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HEPATIC PARASYMPATHETIC NERVE DYSFUNCTION INVOLVED IN THE DEREGULATION OF POSTPRANDIAL WHOLE-BODY INSULIN SENSITIVITY: A ROAD TO DIABETES AND ASSOCIATED PATHOLOGIES

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Aos meus avós Vitória e Eduardo

“The most exciting phrase to hear in Science, the one that heralds new discoveries, is not ‘Eureka!’ but ‘That’s funny…’.”

Isaac Asimov (1920-1992)

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ABBREVIATIONS

A160: Akt substrate of 160 kDa

Ach: acetylcholine

ACTH: adrenocorticotropic hormone

ADP: adenosine monophosphate

Akt: protein kinase B (also PKB)

APS: associated protein substrate

AMPK: 5'adenosine monophosphate-activated protein kinase

ANOVA: analysis of variance

aPKC: atypical protein kinase C

ATP: adenosine triphosphate

BMP-9: bone morphogenetic protein 9

CAMK: Ca2+/calmodulin protein kinase

cAMP: cyclic adenosine monophosphate

CAP: Cbl-associated protein

Cbl: Casitas b-lineage lymphoma protooncogene

CHB: vagus nerve common artery branch

C-peptide: connecting peptide

DNFB: 2,4-dinitro-1-fluorobenzene

ED_{50}: effective dose

eNOS: endothelial nitric oxide synthase

ERK: extracellular signal-regulated kinase

GDP: guanosine diphosphate

GSH: reduced glutathione

GSH-Px: glutathione peroxidase

GSSG: oxidised glutathione

GSSG-R: oxidised glutathione redutase

GTP: guanosine triphosphate

GIP: glucose-dependent insulinotropic peptide

GKRP: glucokinase regulatory protein

GLP-1: glucagon-like peptide 1

GLUT: glucose transporter

GSNO: S-nitrosoglutathione

H_2O_2: hydrogen peroxide

HbA1c: glycosylated haemoglobin

HCl: hydrochloric acid

HIEC: hyperinsulinemic euglycaemic clamp

HISS: hepatic insulin sensitising substance

HNO: hepatic nitric oxide

HPLC: high-pressure chromatography

HPN: hepatic parasympathetic nerves

IDE: insulin degrading enzyme

IFG: impaired fasting glucose

IGF1: insulin-like growth factor 1

IGT: impaired glucose tolerance

iNOS: inducible nitric oxide synthase

ipv: intraportal

IR: insulin receptor

IRS: insulin receptor substrate

ITT: insulin tolerance test

iv: intravenous

KO: knock-out

L-NMMA: N^g-methyl-arginine

MAPK: p38 mitogen-activated protein kinase

mTOR: mammalian target of rapamycin protein

MTT: meal tolerance test

NEM: N-etilmaleimide

NO: nitric oxide

NOS: nitric oxide synthase

OGTT: oral glucose tolerance test

PDI: protein disulfide isomerase

PDK: 3-phosphoinositide-dependent kinase

$PI(3,4,5)P_3$: phosphatidylinositol-3,4,5-triphosphate

$PI(4,5)P_2$: phosphatidylinositol-4,5-biphosphate

PI3K: phosphoinositide-3-kinase

PKA: protein kinase A

PKC: protein kinase C

RIST: rapid insulin sensitivity test

RSNO: S-nitrosothiols

SD: Sprague-Daley rat strain

SEM: standard error of the mean

SGLT: sodium-dependent glucose co-transporter

SH: thiol group

SHR: spontaneously hypertensive rat

SIN-1: 3-morpholinosydnonimine hydrochloride

SNARE: soluble SNF attachment protein

SNP: sodium nitroprusside

TCA: trichloroacetic acid

TRIS-HCl: Tris(hydroxymethyl)aminomethane hydrochloride

VAMP2: vesicle-associated membrane protein 2

WIS: Wistar rat strain

WKY: Wistar Kyoto rat strain

ABSTRACT

The postprandial state is characterised by an increment in whole-body insulin sensitivity. This increased insulin-stimulated glucose disposal seen after a meal has been proposed to be regulated by a mechanism to which the integrity of the hepatic parasympathetic nerves (HPN) is crucial. The present thesis focus on the hypothesis that a dysfunction of the HPN-dependent component of insulin sensitivity is involved in the insulin resistance seen in various conditions related to the aetiology of type 2 diabetes.

The physiological impacts of age and gender were here studied in Wistar rats. In males, age was correlated with a gradual decrease of total postprandial insulin sensitivity. However, while the HPN-independent component of insulin action decreased early, between 6 and 9 weeks of age, and remained unchanged thereafter, the HPN-dependent component decreased from 9 weeks of age throughout ageing. Females showed similar developmental changes, although at different rates in some stages.

The repercussion of lifestyle on postprandial insulin sensitivity was evaluated by providing Wistar and Sprague-Dawley rats with a high-sucrose supplement. The high-sucrose diet induced a decrease in insulin sensitivity by affecting solely the HPN-dependent component. Additionally, this development of postprandial insulin resistance was evident after only 2 weeks of diet manipulation; prior to inducing weight gain and hyperglycaemia.

Studies in healthy animals argued in favour of the integration of the HPN on the hepatic insulin sensitising substance (HISS) pathway. Blockade of the HPN through physical sectioning or pharmacological muscarinic antagonism, or inhibition of the synthesis of hepatic nitric oxide, another proposed step of the HISS pathway, were shown to similarly abrogate the postprandial increment of insulin sensitivity; while having no effect on fasting insulin sensitivity.

The analysis of a rat model of essential hypertension (SHR) has shown an impairment of the HISS-dependent component of insulin action, partially compensated by an increase of the HISS-independent component; in relation to normotensive Wistar rats. That another normotensive control strain for the SHR, the Wistar Kyoto, showed already a decreased in the HISS-dependent component hints to a possible genetic interference on the HISS pathway; particularly on parasympathetic function. Ageing studies further support these conclusions.

In summary, dysfunction of the HPN-determined branch of the HISS mechanism was shown here to be involved in the development of insulin resistance related with ageing, diet-induced metabolic deregulation, and hypertension. This was also shown to predate other risk factors for the development of type 2 diabetes and related pathologies; and seems to constitute an attractive target for behavioural and pharmacological interventions, especially those able to ameliorate parasympathetic function.

RESUMO

O estado pós-prandial é caracterizado por um incremento da sensibilidade à insulina. Este aumento da captação celular da glucose por acção da insulina, observado após a ingestão de uma refeição, parece estar relacionado com um mecanismo dependente da integridade dos nervos parassimpáticos hepáticos (HPN). A presente dissertação teve como base a hipótese de que a disfunção desta componente dependente dos HPN está relacionada com a insulino-resistência observada em várias condições envolvidas na etiologia da diabetes tipo 2.

O impacto da idade e do género foram aqui estudados em ratos Wistar. Em machos, observou-se que a idade está relacionada com um decréscimo gradual da sensibilidade total à insulina. No entanto, enquanto a componente independente dos HPN decresceu entre as 6 e 9 semanas de idade, mantendo-se depois inalterada, a componente dependente dos HPN decresceu com o envelhecimento a partir das 9 semanas de idade. As fêmeas demonstraram alterações de desenvolvimento semelhantes aos machos, apesar de algumas diferenças na taxa de decréscimo.

A influência do estilo de vida sobre a sensibilidade pós-prandial à insulina foi avaliada fornecendo um suplemento de sacarose a ratos Wistar e Sprague-Dawley. A dieta rica em sacarose induziu um decréscimo na sensibilidade à insulina, afectando apenas a componente dependente dos HPN. Mais, o desenvolvimento de insulino-resistência pós-prandial foi obtido após 2 semanas de manipulação; antes do surgimento de obesidade e hiperglicémia.

Estudos em animais saudáveis apoiam a integração dos HPN na via da substância hepática sensibilizadora da insulina (HISS). O bloqueio dos HPN por intervenção cirúrgica ou antagonismo muscarínico químico, assim como a inibição da síntese de óxido nítrico hepático, outro passo proposto da via da HISS, provocaram a anulação total do incremento pós-prandial de sensibilidade à insulina; não tendo tido, no entanto, qualquer efeito no estado de jejum.

A análise de um modelo animal de hipertensão essencial (SHR) revelou um decréscimo da acção da insulina dependente da HISS, parcialmente compensada por um aumento da acção da insulina independente da HISS; em relação aos ratos normotensos Wistar. O facto de outro controlo normotenso dos SHR, o Wistar Kyoto, apresentar já uma diminuição da componente dependente da HISS sugere uma possível determinação genética da via da HISS, provavelmente actuando sobre a função parassimpática. Estudos com a idade fortalecem estas conclusões.

Em conclusão, a disfunção da componente dependente dos HPN na via da HISS foi aqui demonstrada estar envolvida no desenvolvimento da insulino-resistência relacionada com o envelhecimento, a desregulação induzida por factores nutricionais, e a hipertensão. Foi também observado que essa disfunção antecipa outros factores de risco para o desenvolvimento de diabetes tipo 2 e patologias relacionadas; o que parece constituir um alvo promissor para intervenções comportamentais e farmacológicas capazes de melhorar a função parassimpática.

1. INTRODUCTION

1.1. Thesis rationale

The basis of this doctoral thesis is related to the involvement of the hepatic parasympathetic nerves (HPN) on the augmented whole-body insulin sensitivity seen in the postprandial state; and the possible contribution of the disruption of HPN function to the genesis of insulin resistance and type 2 diabetes.

Ingestion of a meal is the greatest challenge faced by glucose homeostasis. The surge of nutrients has to be disposed off quickly, as their high concentrations in the bloodstream may have pathophysiological effects; and properly, as misplaced reserves may induce problems on the affected tissues. Thus, loss of the ability to adequately dispose of ingested nutrients can be expected to lead to a deterioration of glucose metabolism control, and favour the development of the aforementioned pathologies.

This dissertation regards studies designed to uncover the behaviour of metabolic pathways determined by HPN function status, both in physiology and pathophysiology. Likewise, the inquiry spread to address the impact of modern lifestyle, especially feeding habits, on the HPN control of glucose metabolism. This was done bearing in mind that the HPN may have a particular relevance during the postprandial state as a determinant in the action of a hormone, the hepatic insulin sensitising substance (HISS), which has been implicated in the peripheral allocation of the ingested glucose.

The term “HISS” is not yet a scientific household name. With it, as befits the scope of a doctoral thesis, there are certainly many biological whys and hows to be addressed in this “fairy tale”, as Marie Curie would call it[1]. For that, a case will be presented based both on the performed experiments and on the evidence found in other works, as to the validity of this novel mechanism. Surely, when that is done, the scientific notions and data provided throughout this work will have earned due consideration.

To begin with, the current dimension of diabetes will be alluded to, and the concepts of diabetes and prediabetes will be clarified.

[1] “I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.” Marie Curie (1867-1934).

1.2. Diabetes as a modern epidemic

The Western World has seen in the last decades a sharp increase in the general incidence of diabetes mellitus (Zimmet *et al.*, 2001). More, the Developing Countries have been witnessing ever more rapid rates of incidence and, similarly, an increasingly lower age of disease onset (Tong and Cockram, 2003). This has made diabetes into one of the most alarming worldwide health problems facing us presently, to the point of being recently recognised by the United Nations as a full epidemic (United Nations Resolution 61/225, 2007).

As if this scenario was not bad enough, it is expected to deteriorate further in the near future. From the estimated 150 million people affected worldwide in 2000, the incidence of diabetes is foreseen to rise to a total of 366 million people by 2030 (King et al., 1998; Wild et al., 2004). Even worse if taken into account that these are conservative estimates (Wild et al., 2004); the higher incidence of obesity and diabetes in children, the rise in life expectancy enlarging the elderly strata of the population, compound sedentary and stressful lifestyles, and urbanisation of developing countries, can further exacerbate these extrapolations.

Diabetes mellitus has a considerable premature morbidity and mortality, especially due to the accompanying panoply of complications and associated pathologies (Zimmet et al., 2001). It is now considered to be globally the fifth leading cause of death (Roglic et al., 2005). Additionally, it has a radical impact upon a growing portion of the world population, in terms of life quality, and to society in a whole, in terms of an increased expenditure of either private or public national systems of Health (Bruno et al., 2008; Dall et al., 2009).

This is especially true for Portugal. With a number of documented cases of diabetes, between the ages of 20 and 80, reaching around 0.5 million in 2003, predictions were that the number of cases would rise by 2025 to 0.7 million (IDF, 2003; King et al., 1998). Unfortunately, the recent disclosure of the preliminary report for the first Portuguese epidemiologic study solely dedicated to diabetes (PREVADIAB-2009) has surpassed the worst expectations (Pina e Brito, 2009). In it, it was found that more than 10% of individuals between ages 20 and 80 are already diabetic, which represents an astounding 0.9 million people. To make matters worse, an additional 1.8 million

Portuguese (23%) were found to suffer from at least one kind of prediabetes[2]. Summarising, more than a third of the Portuguese population is either already diabetic, or possesses a significant impairment of glucose metabolism that increases the risk of developing diabetes in the near future. Considering that the rate of mortality from diabetes in Portugal was more than double the mean in the European Union, and was rising (WHO, 2003), this poses a dire prospect in terms of national Health.

Beyond finding ways to treat this hailing and financially cumbersome disease, it becomes essential to understand the physiological mechanisms that are related to the development of insulin resistance, the step that seems to predate and link diabetes and its cluster of commonly associated pathologies (Gerich, 1999; Reaven, 1988).

To act in an early phase of this process would consequently provide the best approach to a line of research capable of bearing answers into postponing the metabolic degradation that eventually leads to type 2 diabetes. To contribute to this approach was the main thought at the basis of this dissertation.

1.3. Concept, classification and diagnosis of diabetes and prediabetes

One of the oldest surviving accounts of the existence of diabetes is a basic description recorded by Aretaus of Cappadocia in the 2nd century AD. Aretaus used the Greek word for 'siphon' to first coin the term 'diabetes', 'because the fluid does not remain in the body, but uses the man's body as a channel whereby to leave it' (Tattersall, 2003)[3].

Diabetes mellitus is in our days more than simply a disease; it is now consensually described as 'a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycaemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels.' (ADA, 1997).

[2] The definition and characteristics of the prediabetes stage(s) will be further discussed in the next item.
[3] This description is a clear allusion to the state of polyuria that sets later in the condition, often the tell-tale sign that alerts people to the onset of the disease; ultimately leading to death, if untreated. The term 'mellitus' was coined well after that, in the 19th century, based on the Greek and Latin word for 'honey'.

In our times, classification criteria aim mainly to reflect the aetiology and pathogenesis of diabetes (ADA, 1997; Genuth et al., 2003). There are two main forms of diabetes. Type 1 diabetes involves the autoimmune-mediated failure or destruction of pancreatic β–cell islets, eventually leading to absolute insulin deficiency (Dotta and Eisenbarth, 1989; Muir et al., 1992). These patients, generally young people and children (Taplin and Barker, 2008), rely on exogenous insulin for survival, and are prone to ketoacidosis, coma and death. Type 2 diabetes is by far the most prevalent form, representing 90% of diabetes cases. It is an heterogeneous and multifactorial condition, probably determined by the crosstalk of several pathophysiological mechanisms. Of both genetic and environmental origin (Beck-Nielsen and Groop, 1994), type 2 diabetes is characterized by defects in insulin action (insulin resistance) and/or in insulin secretion (β-cell dysfunction) (Kashyap and Defronzo, 2007), either of which may predominate (Gerich, 1999). Other forms of diabetes include gestational diabetes mellitus, which is defined as any degree of inability to deal with a glucose load (i.e. glucose intolerance) during pregnancy (independently of the onset of diabetes being during or prior to that state), and several rarer forms induced by endocrinological, drug or chemical, infectious, autoimmune, or genetic factors (ADA, 1997).

The epidemic of diabetes is thus more correctly referred to as an epidemic of type 2 diabetes. Indeed, even among young adults and children, where type 1 diabetes was the most common chronic disease (Rosenbloom et al., 1999), the rising incidence of physical inactivity, bad food habits, and obesity, is allowing a higher prevalence of type 2 diabetes (Cali and Caprio, 2008; Ten and Maclaren, 2004).

Diabetes is diagnosed when exaggerated blood glucose levels and/or abnormal handling of a glucose load are detected (ADA, 2009). This diagnosis is based on glycaemia magnitude in relation to established reference, or “cut-off”, values (Figure 1). Hyperglycaemia is an important risk factor (Stratton et al., 2000); and the cut-off values are chosen taking into account the prevalence thresholds for several diabetic complications, especially retinopathy. From this stems that diagnosis can be made already with or without the presence of known symptoms of diabetes[4]. With new developments in the clinical and research fields, the cut-off values have been

[4] Common symptoms of already established diabetes include polyuria (excessive excretion of urine), polydipsia (excessive or abnormal thirst), ketonuria (presence of excessive ketone bodies in the urine) and rapid weight loss.

progressively adapted following current knowledge. It is presently considered that diabetes is established if blood glucose level after an overnight fast is equal or over 126 mg/dl, and/or the blood glucose level obtained two-hours after an oral glucose tolerance test (OGTT)[5] is equal or over 200 mg/dl. Although the debate raged from early on about the best method for evaluating diabetes, fasting or 2h post-challenge plasma glucose, mainly an effort was made to try to find cut-off points that represent similar biological risk factors in the two approaches.

<table>
<tr><td colspan="2" rowspan="2"></td><td colspan="3">2h-post challenge blood glucose level (mg/dl)</td></tr>
<tr><td>< 140</td><td>140-199</td><td>≥ 200</td></tr>
<tr><td rowspan="3">Fasting glucose level (mg/dl)</td><td>< 100</td><td>Normal</td><td>IGT</td><td>Diabetes (isolated post-challenge hyperglycaemia)</td></tr>
<tr><td>100-125</td><td>IFG</td><td>IFG/IGT</td><td></td></tr>
<tr><td>≥ 126</td><td colspan="2">Diabetes (isolated fasting hyperglycaemia)</td><td>Diabetes</td></tr>
</table>

Figure 1- Current criteria to diagnose diabetes and prediabetes. Glycaemic control evolves from normal homeostasis to diabetes, with visible degradation either in the fasted state or in response to a glucose challenge, or both. Impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) represent stages of prediabetes (adapted from (Tong and Cockram, 2003)).

A further distinction has been created, to describe an intermediate stage between normal glucose homeostasis and diabetes; which has become known as 'prediabetes'. This stage can be characterized either by impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) (Figure 1). IFG is considered when the fasting glucose level is higher than 100 mg/dl but lower than 126 mg/dl, and IGT is considered when blood glucose assessed 2h after an OGTT is higher than 140 mg/dl but lower than 200 mg/dl (Ryden et al., 2007). Less commonly, patients show both IFG and IGT. This last condition presents a more extreme stage of prediabetes, sometimes able to develop independently (Perreault et al., 2008), which usually progresses more rapidly towards type 2 diabetes (de Vegt et al., 2001; Meigs et al., 2003; O'Rahilly et al., 1994).

[5] Usually by ingestion of a glucose load containing 75g anhydrous glucose in water after an overnight fast.

Prediabetes is estimated to affect presently almost 350 million people worldwide (Garber et al., 2008). Its importance is highlighted by the increased medical costs associated (Zhang et al., 2009), since a considerable proportion of prediabetics already present microvascular and cardiovascular complications (Garber et al., 2008).

Some authors argue that, more than a risk factor for diabetes, prediabetes is in itself a disease state that must be clinically addressed (Eldin et al., 2008). Others see the constant lowering of cut-off points, especially those related to IFG, as a risk of losing biological relevance and a danger of generalising treatments with possible side-effects over groups that would never evolve type 2 diabetes or suffer from cardiovascular complications (Rosenstock, 2007).

Anyway, IFG and IGT seem to be both related to the development of type 2 diabetes through the presence of insulin resistance; even if possibly under slightly different pathophysiological mechanisms (Meyer et al., 2006; Nathan et al., 2007; Perreault et al., 2008; Weyer et al., 1999). IFG seems to be connected mainly to early defects in the pancreas and liver; a loss of the first-phase of insulin secretion and a decreased capacity of insulin to inhibit hepatic glucose output. Paradoxically, this seems to be accompanied by normal skeletal muscle insulin sensitivity (Nathan et al., 2007). On the contrary, IGT seems to only present hepatic insulin resistance at a later stage, or not at all, but develops extensive peripheral insulin resistance by lack of ability to sensitise the skeletal muscle[6] (Crandall et al., 2008; Garber et al., 2008; Guerrero-Romero and Rodriguez-Moran, 2006; Stancakova et al., 2009). Eventually IGT may also show alterations in both early- and late-stage insulin secretion (O'Rahilly et al., 1994). Nonetheless, notwithstanding dynamic changes in insulin secretion, hyperinsulinemia is usually a sustained feature in prediabetes (Weyer et al., 1999)[7].

In real life these expressions of prediabetes show a considerable heterogeneity, especially in individuals with isolated IFG (Kim and Reaven, 2008). This is not surprising in terms of a natural history of development of type 2 diabetes. Traditionally,

[6] Peripheral insulin resistance that leads to IGT development has been retraced even to the stage where individuals are still within normal 2h-post challenge blood glucose levels (Stancakova et al, 2009).

[7] The eventual failure of the ability of β-cells to compensate for the rise on blood glycaemia by producing more insulin may well be the hallmark of the final progression of prediabetes to type 2 diabetes (Guerrero-Romero and Rodriguez-Moran, 2006; Crandall et al, 2008). Strategies to spare and maintain β-cell function as long as possible are nowadays one of the main clinical targets (Crandall et al, 2008; Garber et al, 2008).

type 2 diabetes has been seen as arising from early defects in peripheral insulin sensitivity. The pancreas counterbalances the growing loss of tissue sensitivity by secreting more insulin, leading to a maintained state of hyperinsulinemia. This process continuously degrades, with both glycaemia and insulinemia rising, until β-cell islets reach a critical point and begin to fail. Other sites, as the liver and adipose tissue, begin to also be affected. By then, usually diabetes sets with both full-blown hyperglycaemia and possibly a decrease in insulinemia due to β-cell exhaustion. Intuitive as this order of events may seem, in reality these processes are so interrelated that evolution to type 2 diabetes may begin in any of those steps.

As in other biological processes, nature seldom finds only one way of doing and undoing things, unlike what humans so often find comfortable to conceptualise. However, it is true that some mechanisms may be more relevant than others. And in pathophysiological issues this relevance may lie in those physiological pathways more prone to be disrupted by environmental factors, since those are the ones that will aggravate what can be the unavoidable disadvantages of the genetic background (Buren and Eriksson, 2005). The natural history of progression from normal glucose metabolism to type 2 diabetes, and logically the possibility of successful intervention to delay it, may thus depend heavily on the correct identification of the aetiological defect. It should also be critical to detect it at an early stage of the prediabetic range.

This rationale seems to be supported by epidemiological follow-up studies. In those studies, people diagnosed with IFG and/or IGT are revaluated after several years of the first diagnosis. Even taking into account all the circumstances that may induce variability on the quantification given by the OGTT, which may (Ko et al., 1998; Yudkin et al., 1990) or may not (de Vegt et al., 2001; Ko et al., 1998) make the OGTT a lesser reproducible method than the measurements done on the fasting state (Balion et al., 2007), reversion from IGT to normalcy seems to be more common than from IFG (Lu et al., 2008). Considering that a first diagnosis of IGT or IFG is followed by an increased awareness of each afflicted individual to the potential outcome of a prediabetic condition, and that this may lead to immediate changes in lifestyle, this data may hint to a higher sensitivity of IGT to environmental factors.

As mentioned before, IFG is diagnosed simply by assessing the blood glucose level in the fasted state; and IGT is diagnosed through an OGTT. During an OGTT, after the ingestion of a known solution of glucose, the changes of blood glycaemia, and

insulinaemia, are followed at regular intervals to assess the ability of the organism to deal with the glucose challenge. Additional analytical quantifications and mathematical models are able to provide more information on the dynamics of glucose appearance/disappearance in the blood and the response of the pancreas in secreting insulin and of the liver in insulin removal (Abdul-Ghani et al., 2007; Cobelli et al., 2007).

The OGTT is usually reported as assessing glucose metabolism response in a postprandial state, in opposition to the fasting state evaluation. However, this method may not be the most suited for this purpose (Lefebvre and Luyckx, 1976). Indeed, the comparison of the OGTT with a meal tolerance test (MTT)[8] shows that a challenge of only glucose leads to a higher level of blood glycaemia and triggers insulin secretion in a different dynamic curve than a challenge posed by a mixed meal (Berthiaume and Zinker, 2002; Meier et al., 2008; Rijkelijkhuizen et al., 2009). This is explained by the fact that glucose appearance in the blood is quicker when it comes from a diluted solution than when the same quantity of glucose is part of a mixed meal, and by the additive secretory response of the pancreas to nutrients other than carbohydrates (Bock et al., 2007b), namely protein (Bock et al., 2007b; Krezowski et al., 1986). Additionally, the arrival of solely glucose to the intestine was shown not to be enough to produce some of the feeding signals involved in postprandial glucose homeostasis (Sadri et al., 2006).

It becomes evident that a mixed meal challenge will constitute a more appropriate test to fully assess the postprandial state. It would also be more accurate to evaluate changes in real-life postprandial glucose metabolism, as has been already proposed (Meier et al., 2008). Further, the previous considerations indicate that both clinically advised measurements for evaluating diabetes and prediabetes, fasting and 2h post-OGTT plasma glucose levels (ADA, 2009), may be unable to adequately detect the impairment of real-life glucose metabolism responses in the postprandial state.

Now the question remains if those potential real-life alterations in postprandial nutrient handling are relevant to the natural history of the development of type 2 diabetes; and if the additional effort in detecting them has any real clinical applicability

[8] The MTT is initiated by the ingestion of a standardised mixed meal, but is in all the rest similar to the OGTT; potentially sharing the same methodological approaches to assess both insulin secretion and insulin sensitivity parameters.

(specifically, if they are able to predict development to type 2 diabetes; if they are sensitive to strategies aimed at averting or postponing that development; and, in later stages, if there is a potential for therapeutic pharmacological intervention).

However, before focusing on those points, the relevance of setting the cornerstone of the present doctoral thesis on the study of alterations of insulin sensitivity can only be ascertained after dealing with one other subject, envisioned today as one of the most heated debates in the international field of diabetes clinical research: currently called 'the metabolic syndrome'.

1.4.Clinical relevance of the metabolic syndrome, type 2 diabetes, and insulin resistance

Type 2 diabetes is related to a multitude of complications, especially cardiovascular, undoubtedly due to concomitant dysfunctions throughout several pathways involved in energy management, as for example carbohydrate and lipid metabolism. Even prediabetes can likewise be involved, since the risk of complications can already be evident at this stage[9] (Goldberg et al., 2009; Haffner et al., 1990). For this, prediabetes and type 2 diabetes can also be regarded as part of a broader picture.

In 1988, Gerald Reaven proposed (Reaven, 1988) that insulin resistance and hyperinsulinemia are the main factors involved in the aetiology of conditions like type 2 diabetes, hypertension and coronary artery disease. This was termed "Syndrome X". In its initial proposition, Syndrome X was an informal cluster of risk factors that included decreased sensitivity in insulin-stimulated glucose uptake, glucose intolerance, hyperinsulinemia, dyslipidemia (increased very-low-density lipoprotein triglycerides and decreased high-density lipoprotein cholesterol), and hypertension. Although it was named "syndrome", this was more a recognition of a set of whole-body metabolic and hemodynamic alterations induced by the sustained hyperinsulinemia that compensated for insulin resistance and glucose intolerance than a suggestion for a real new disease (Kim and Reaven, 2004). In a later paper, the same author expanded the number of cardiovascular conditions possibly related to the Syndrome X (Reaven, 1993).

[9] In the same way, the reversion from prediabetes, specifically IGT, to normal glucose tolerance, achieved through intensive alterations in lifestyle (food habits, exercise, etc.), leads to an improvement in risk factors related to cardiovascular diseases (Goldberg et al, 2009).

Soon after, the initial proposal was taken by others; not always with the same focus. From 1988 to 1991, the Syndrome X was given a multitude of other incarnations ('deadly quartet', 'insulin resistance syndrome', 'visceral fat syndrome', 'atherogenic metabolic triad', among others) (Oda, 2008). While some preserved insulin resistance and hyperinsulinemia at the core of this cluster (Balkau and Charles, 1999; DeFronzo and Ferrannini, 1991; Ferrannini, 2006; Ferrannini and Balkau, 2002), others argued that the central aetiological role should be given to obesity (Oda, 2008; Samaras et al., 2006; Yudkin, 2007). From 1991 onwards, several organisations established clinical guidelines for this supposed entity, by then mostly named "metabolic syndrome" (Grundy, 2006a). These guidelines also reflected the variability of aetiological points and diseases considered by previous descriptions of the syndrome (Kim and Reaven, 2004; Oda, 2008); with some of them even excluding insulin resistance as a parameter worth evaluating, either due to technical or conceptual issues (Alberti et al., 2006; Kim and Reaven, 2004)[10]. Not surprisingly, a comparison between the applicability of several of the most used definitions for the metabolic syndrome wielded the result of less than 30% of people surveyed being eligible for treatment simultaneously by all three of them (Day, 2007).

Not surprisingly with a high prevalence worldwide (Grundy, 2008), by now the proper clinical relevance, and even desirability, of the existence of the "metabolic syndrome" category has been put in question (Alberti and Zimmet, 2008; Gale, 2008; Kahn et al., 2005). While supporters of the metabolic syndrome argue that it is not an attempt to label a new disease, but to identify a risk state, like prediabetes (Alberti and Zimmet, 2008; Grundy, 2006b), detractors highlight the confusion arising from considering the same conditions as both risk factors and outcomes, and the redundancy of the diagnosis of the metabolic syndrome in comparison to the diagnosis and treatment of each of its components (Gale, 2008; Kim and Reaven, 2008; Sundstrom et al., 2006).

It has been shown that the prevalence of the individual components of the metabolic syndrome increases with decreasing insulin sensitivity (Lee et al., 2007). However, that a common aetiological feature has yet to be established, even among well known

[10] It is also relevant to point out that even those criteria guidelines that took into account measurements of insulin resistance were generally inconsistent in regards to the use and significance given to IFG and IGT (Kim and Reaven, 2004).

associations of included pathologies, further complicates the analysis of these matters (Gale, 2008).

Besides those objections, the simultaneous occurrence of obesity may be more a complication than an aetiological factor for this cluster. There is no doubt that obesity is related to insulin resistance. Indeed, obesity may aggravate insulin resistance and other features of the metabolic profile by fat mass deposition (Alberti et al., 2006) and an higher release of free fatty acids (Opie, 2007), or/and a rise in secretion of inflammatory cytokines from the adipose tissue (Oda, 2008; Yudkin, 2007). However, the other features of the metabolic syndrome seem to develop independently of these obesity-induced factors (Petersen et al., 2007; Reaven, 1993), when already in the presence of peripheral insulin resistance (Petersen et al., 2007).

To highlight this fact, it has been observed that a minority of primarily obese people develop diabetes (Perreault et al., 2008); while lean prediabetics generally progress to diabetes and obesity (Perreault et al., 2008). Consistent with this, obesity has been described has presenting, in the absence of the other defects, a minor risk factor (Kaplan, 1998). Thus, obesity may be related to the metabolic syndrome not by a direct effect but by increasing the chance of an individual to become less sensitive to insulin-stimulated glucose uptake (Matthews, 1999; Reaven, 2006) and/or by accelerating the metabolic degradation that follows insulin resistance.

Elevated blood pressure is another common feature of the metabolic syndrome with ties with insulin resistance, obesity, and cardiovascular complications (Antic et al., 2003; Reaven, 2006). And although only half of patients with essential hypertension show insulin resistance and hyperinsulinemia (Reaven et al., 1996; Zavaroni et al., 1992), those are precisely the ones that develop the other features of the metabolic syndrome (Reaven, 2006; Zavaroni et al., 1999).

In healthy individuals, insulin has a net vasodilator effect (Anderson et al., 1991); which reflects the balance between the vasoconstrictor and vasodilator effects of insulin. These effects are originated both through the central nervous system and directly at the peripheral vasculature (Cabou et al., 2007; Sartori et al., 2005); and are integrated in the overall control of blood pressure. Several mechanisms have been proposed to contribute to the link between insulin resistance and essential hypertension (Antic et al., 2003; Ginsberg, 2000): a defect in the pathway by which insulin stimulates the release of

nitric oxide, a potent vasodilator[11], from endothelial cells (Claxton and Brands, 2003; Cook et al., 2003; Macedo and Lautt, 1996; Mather et al., 2001; Montagnani et al., 2002; Sartori et al., 2005; Steinberg et al., 1994; Turini et al., 2007), the increased reabsorption of sodium and water due to hyperinsulinemia (DeFronzo et al., 1975), and/or an exaggerated overactivity of the sympathetic nervous system in response to insulin (Kopp, 2005; Lembo et al., 1992). Again, insulin resistance seems to lie at the genesis of a pathology included in the metabolic syndrome.

Ageing has also been related with features of the metabolic syndrome; such as obesity, hypertension, and diabetes (Denys et al., 2009). The normal process of ageing is known to gradually present insulin resistance (Scheen, 2005), which has been proposed as a possible common origin to this cluster (Muller et al., 1996). The development of these other would also determine the acceleration of the age-determined defects (Morley, 2008).

If the criteria for metabolic syndrome diagnosis was created to find individuals who have an increased risk of developing diabetes and cardiovascular disease (Oda, 2008), then it seems more profitable to centre this question in insulin resistance instead; as was initially proposed (Reaven, 1988). Furthermore, excessive dependence upon the diagnosis of the metabolic syndrome may provide a sense of false security for those with abnormalities not yet serious enough to be classified as suffering from the metabolic syndrome (Kim and Reaven, 2004); which falls paradoxically short from the declared objective of creating a social weapon for public awareness about the epidemic of type 2 diabetes and associated pathologies, like obesity and hypertension.

Even considering the aforementioned relevance, insulin resistance estimates are badly regarded in the clinical field; either when derived from fasting or post-challenge insulin blood level measurements (Chevenne et al., 1999; Kahn et al., 2005; Samaras et al., 2006). Also, prediabetes, with no organ damage *per se* yet established, is regarded solely as an indicator of potential future complications (Genuth et al., 2003). In spite of this, the alternative (Samaras et al., 2006), nearly comes up to waiting for the 'metabolic

[11] This dysfunction can be due either to an inability of insulin action or to a defect on nitric oxide synthesis/bioavailability (Sartori et al, 2005). Furthermore, the vasodilator effect of nitric oxide is felt peripherally, by relaxation of the vascular smooth muscle (Steinberg et al, 1994; Mather et al, 2001), and centrally, by suppressing the sympathetic outflow (Turini et al, 2007) that locally induces peripheral vasoconstriction (Macedo and Lautt, 1996; Claxton and Brands, 2003). This has prompted that defective nitric oxide availability was proposed as a common point to the development of several abnormalities included in the metabolic syndrome (Cook et al, 2003).

syndrome' to become visible. This may sum to an aggravation of metabolic and hemodynamic complications, leading, in the near future, to a harder-to-maintain quality of life.

On the other hand, the cautions of clinical intervention seem well founded. Mainly, it is ruled by finding the best way to foresee and prevent life-threatening conditions or those that lead to gross deterioration of general health; making a diagnosis through the most practical and cost-effective method of analysis. This implies a difficult balance between the ability of a risk factor to predict a real future progression of a disease and the possible negative side-effects that a pharmacological intervention may carry.

The present situation seems to hinge in a dispute between the relevance of insulin resistance to the aetiology and early prediction of future metabolic degradation and the necessity of recognising an immediate negative impact upon a patient to make a medical intervention worthwhile in terms of cost effectiveness and patient safety. Browsing through the literature, one could become convinced that a stalemate about this question has been reached. If so, perhaps exploring the possibly distinct defects inherent to each type of prediabetes (i.e., determined either in fasting or post-ingestion) may shed more light into the relevance of the loss of insulin sensitivity to the development of prediabetes and diabetes; and to the features associated with the metabolic syndrome.

1.5.Tracking the influence of the prandial state to prediabetes and diabetes

Circulating glucose is able to glycosylate valine residues of erythrocyte haemoglobin, in a process that evolves linearly with blood glucose concentration (Little and Sacks, 2009). Due to the long half-life of erythrocytes, glycosylated haemoglobin (HbA1c) is used to estimate the degree of glycemic control in the last 2 to 3 months. A higher HbA1c is indicative of exposure to longer or more extreme hyperglycaemia[12] (ADA, 2010; Fonseca et al., 2009; Genuth et al., 2003; Kilpatrick et al., 2009; TIEC,

[12] Glycosylated haemoglobin (HbA1c) has been pondered as a potential tool in the diagnosis of diabetes. Its use in that capacity was considered up until now premature (Genuth et al, 2003); while still maintaining its role as an evaluator of glycemic control effectiveness. Indeed, a recent report (TIEC, 2009) spurred the adoption of HbA1c $\geq$6.5% as criteria for the diagnosis of diabetes by the American Diabetes Association (ADA, 2010). However, this is still under considerable dispute (Fonseca et al, 2009; Kilpatrick et al, 2009).

2009). The current recommendation is that HbA1c should be lower than 6.5%, to reduce the risk of microvascular complications (ADA, 2010; Ceriello and Colagiuri, 2008).

Prospective studies have shown clearly that intensive glycemic control can lead to a lowering of HbA1c, and a decrease of the risk of microvascular complications with type 2 diabetes (Matthews, 1999). This was achieved solely by intervening on fasting glucose levels; which correlate well with HbA1c (Wettre et al., 1993), but produced a much smaller impact on cardiovascular disease risk reduction than expected and, on follow-up studies, soon shown an inability to staunch the progression of glycemic deregulation (Del Prato, 2002). Indeed, the relative importance of either augmented fasting glucose levels or postprandial glucose excursions to the formation of HbA1c has been subject to debate (Avignon et al., 1997; Bonora et al., 2001). Nonetheless, it now seems that, in individuals with declared diabetes, postprandial hyperglycaemia is the primer determinant factor in subjects with moderate glycemic control (ie, with moderate values of HbA1c) (Monnier et al., 2003), while fasting hyperglycaemia is more determinant in stages of higher values of HbA1c (Bonora et al., 2001; Monnier et al., 2003). This raises the hypothesis that reaching fasting hyperglycaemia may represent a state of worsened metabolic profile; and that at this stage the importance of fasting hyperglycaemia masks the greater relevance of postprandial glucose excursions to HbA1c on earlier glucose metabolism deregulation (Monnier et al., 2003). The rise of HbA1c with worsening diabetes would thus indicate a transition between an earlier phase of metabolic compromise dictated by postprandial hyperglycaemia and a later phase of diabetes deterioration determined by the deleterious effect of fasting hyperglycaemia (Monnier et al., 2003). This has been shown in type 2 diabetics, where the primary loss was of postprandial glycemic control, mainly at breakfast, followed by deterioration of the pre-meal periods, and only after that was possible to observe a maintained hyperglycaemia during the night period where fasting is more pronounced (Monnier et al., 2007).

The above argument also hints to the hypothesis that the distinct prediabetic states mentioned earlier, IFG and IGT, influence differently the formation of HbA1c, the development towards diabetes, and cardiovascular complications.

IGT and IFG are both predictors for type 2 diabetes, showing a higher risk than normal subjects in prospective or retrospective studies; but IGT seems to provide a more wide recognition of individuals that progress to this disease (DECODE, 1999; Ferrannini et al., 2009; Qiao et al., 2003b). Likewise, IGT is a better predictor than IFG

of future cardiovascular complications, morbidity and mortality (Heine et al., 2004); independently of further development of diabetes (Qiao et al., 2003a). Several studies showed that the risk of cardiovascular disease and mortality of people with IGT was almost as high as on subjects already diabetic, and that IFG showed a similarly lower risk as individuals with normal fasting glycaemia (DECODE, 2001; IDF, 2003; Qiao et al., 2002; Tominaga et al., 1999). Furthermore, most individuals with HbA1c between 6.0 and 7.0% have normal fasting glucose but already abnormal 2h post-OGTT glucose levels (Woerle et al., 2004).

This makes the isolated IGT prediabetics the most promising group for intervention to prevent the development of type 2 diabetes and metabolic complications (DECODE, 1999). Interestingly, controlling postprandial glycaemia in patients that have already IFG was shown not to prevent the development of type 2 diabetes, probably because by then they had developed β-cell dysfunction (Kirkman et al., 2006). Furthermore, HbA1c may not reflect all the relevant influence of hyperglycaemia. Indeed, the same HbA1c level may be related to different glycemic control profiles, depending on glucose level fluctuations (Del Prato, 2002). Since fluctuations are expected to be of a higher magnitude precisely during postprandial excursions, this provides an additional contribution of postprandial glucose levels to the development of type 2 diabetes and other complications, even if these hyperglycaemias are sporadic and otherwise glycemic control is still maintained (Bonora, 2002; Wettre et al., 1993).

The prior mentioned results where obtained by using the OGTT, but the same is expected to happen in response to a meal (Del Prato, 2002). Certainly, glucose excursions measured by the OGTT are more pronounced than when the MTT is used (Berthiaume and Zinker, 2002; Meier et al., 2008); and individuals characterised as IGT by an OGTT can show a normal glucose level in response to a MTT, due to an adequate hyperinsulinemia brought by the sum of glucidic and non-glucidic driven insulin secretion (Bock et al., 2006), and to other factors that influence insulin sensitivity and are only present in response to a mixed meal (Sadri et al., 2006). But this does not necessarily indicate that the MTT is a less adequate test in detecting defects in glucose metabolism. Indeed, the MTT is a more physiological test, as people usually consume mixed meals and not pure glucose loads. Furthermore, after the MTT, the rise in other nutrients, as lipids and aminoacids[13], may independently uncover further risks for

[13] These non-glucidic nutrients can influence glucose metabolism through indirect metabolic pathways.

complications (Del Prato, 2002; Heine et al., 2004; Henkel et al., 2002; Kim et al., 2000; Rebolledo and Actis Dato, 2005)[14].

Since subjects with postprandial glucose intolerance have been described to present similar endogenous glucose production after feeding as normal individuals, the site of defect of postprandial glucose disposal in IGT must lie at the peripheral tissues (Weyer et al., 1999). This stage corresponds to an hypersecretion of insulin; which may partially offset peripheral insulin resistance, but is not enough to avoid higher-than-normal postprandial glycemia. Later, with β-cell exhaustion, IGT is expected to finally evolve to type 2 diabetes.

Interestingly, a lower insulin-stimulated glucose uptake at the peripheral tissues may also be happening in the early stages of IFG. In the fasted state, these individuals present an higher hepatic glucose output, and lower skeletal muscle glucose disposal, than normal subjects; leading to fasting hyperglycemia. On the other hand, in the postprandial state, the rise in circulating insulin is able to induce an adequate shutdown of the hepatic glucose output. However, not even this insulin level seems able to compensate for the skeletal muscle insulin resistance (Bock et al., 2007a); albeit this defect is not yet severe enough to provoke postprandial hyperglycemia.

Skeletal muscle, the major peripheral insulin-sensitive tissue, seems to be affected by insulin resistance before the liver or the adipose tissue (Petersen et al., 2007). As reported, peripheral insulin resistance is the underlying defect for IGT subjects, and may be even present in early IFG ones. In fact, it may even be acquired as a hereditary trait, since normoglycemic first-degree relatives of type 2 diabetic patients can already show similar skeletal muscle insulin resistance to IGT subjects (Abdul-Ghani et al., 2006). Furthermore, the mentioned studies support the notion that these defects are expressed earlier, and in a more insidious way, in the postprandial state. This, taken together with the fact that microvascular and macrovascular complications begin several years before overt diabetes, provides the clinical relevance desired to warrant a closer look into the contribution of postprandial insulin resistance in the development of type 2 diabetes and other related pathologies.

[14] However, it must be referred that glucose intolerance was shown to predate lipid intolerance (Henkel et al, 2002; Heine et al, 2004), which may show at a later stage, specifically when the excess of nutrients unable to be stored in skeletal muscle overburdens the adipose tissue or when glucose metabolism at the liver is already compromised (Kim et al, 2000).

The next sections will provide an overview into the mechanistic physiology of whole-body glucose metabolism and insulin action, giving a particular attention to the prandial status. After that, a novel mechanism will be presented that seems to contribute specifically to postprandial insulin-stimulated glucose uptake at certain peripheral tissues, as skeletal muscle. In that respect, the importance of the autonomic nervous system to postprandial glucose metabolism will also be highlighted; and the liver will rise as more than a target for insulin action, but also as a promoter of insulin sensitivity in the extrahepatic tissues.

1.6.The need for glucose homeostasis

The energy required for both resting and active functions of the body is obtained by the oxidation of ingested nutrients. Thus, each gram of carbohydrates provides 4 kcal, of fat 9 kcal, and of proteins 4 kcal. A monosaccharide (glucose) and lipids (fatty acids and ketone bodies) are the major oxidative fuels; and, in line with a rationale of energy management, glucose and fatty acids are physiological competitors (Frayn, 2003).

The challenges posed presently by nutrition are quite different from those that shaped our inherited ability to deal with nutrient disposal. This impacts our metabolism in two major ways: frequency and composition.

Nowadays, we are exposed to an almost ubiquitous access to food, at least in considerable parts of the globe, which is much unlike what happened during our evolutionary early times. As hunter-gatherers, human beings would best prosper if able to store enough energy during the feast periods to maintain activity during the famine periods (Neel, 1962). This ability ultimately determined the capacity of procuring for the next period of nourishment. Since then, this "thrifty genotype"[15] has seemed to lose its evolutionary advantage and became a ground for possible metabolic deregulation of

[15] Regarded as "thrifty genotype" or "thrifty phenotype", depending on the relevance given to the actual influence of type 2 diabetes on the pre- and post-reproductive periods (Ozanne and Hales, 1998). The latter would make evolution "blind" to the detrimental effect of diabetes through reproduction. However, it must not be forgotten that metabolic deregulation related to the eventual development of diabetes may predate the onset of disease by several decades. Nonetheless, the influence of the phenotype seems beyond debate, independently of the real relevance of the genotype.

energy fluxes, with ramifications into general physiological control (Ozanne and Hales, 1998).

The economy of energy is self-evident in terms of evolutionary value; independently of the evolutionary advantage being determined either by a sharp action of insulin in inducing nutrient storage during feasting times (Neel, 1962), or by the imposement of insulin resistance in peripheral tissues during famine periods (Reaven, 1998). The former would prevent wasting nutrients that later could be much needed, while the latter guarantied the unfaltering provision of glucose to the central nervous system while still preventing the necessity to degrade muscle protein (Wang and Mariman, 2008).

More importantly, diet composition has changed dramatically (Eaton, 2006; Mann, 2000); as well as the balance between energy intake and energy expenditure[16]. This can lead to early detrimental effects on insulin sensitivity, either by exposure to a high nutrient uterine environment (Mingrone et al., 2008; Shankar et al., 2008), or to a mismatch between the prenatal and postnatal energy balance (Hanson and Gluckman, 2008; Thompson et al., 2007).

The new challenge presented by overnutrition, changes in food composition, and lowered physical activity, likely involves the subversion of both mechanisms related to insulin sensitivity mentioned above; leading simultaneously to maintained hyperinsulinemia and insulin resistance (Wang and Mariman, 2008). Not only meals are supersized, but foodstuff has become calorie dense, low on fibres, and of a high-glycemic index; at the same time, less physical work is required of us[17] (Lieberman, 2003).

Furthermore, the pathways related to the metabolism of carbohydrates, lipids and proteins are interconnected at several steps; which enables the organism to shift energy production and storage depending on the prandial state, and on energy requirements and availability. If there is a problem of inadequate energy fluxes within normal

[16] Perhaps against popular belief, ancestral humans seem to have had an even higher energy intake than in present times; which was, of course, met by also a much higher energy expenditure than ours (Eaton, 2006).

[17] And even the mental state of the modern way of life may be of relevance: traditional physiological stress responses that mobilise glucose to the bloodstream, like adrenalin secretion, are no longer met with an increase in physical activity as in the primordial fight-or-flee dilemma; originating a link between the so-called “stress diseases” and type 2 diabetes (Neel, 1962).

metabolism, it might lead to the development of insulin resistance and type 2 diabetes. A closer look (Figure 2) shows that the key molecules that mark the critical points between these pathways are glucose-6-phosphate, pyruvic acid, and acetyl coenzyme A.

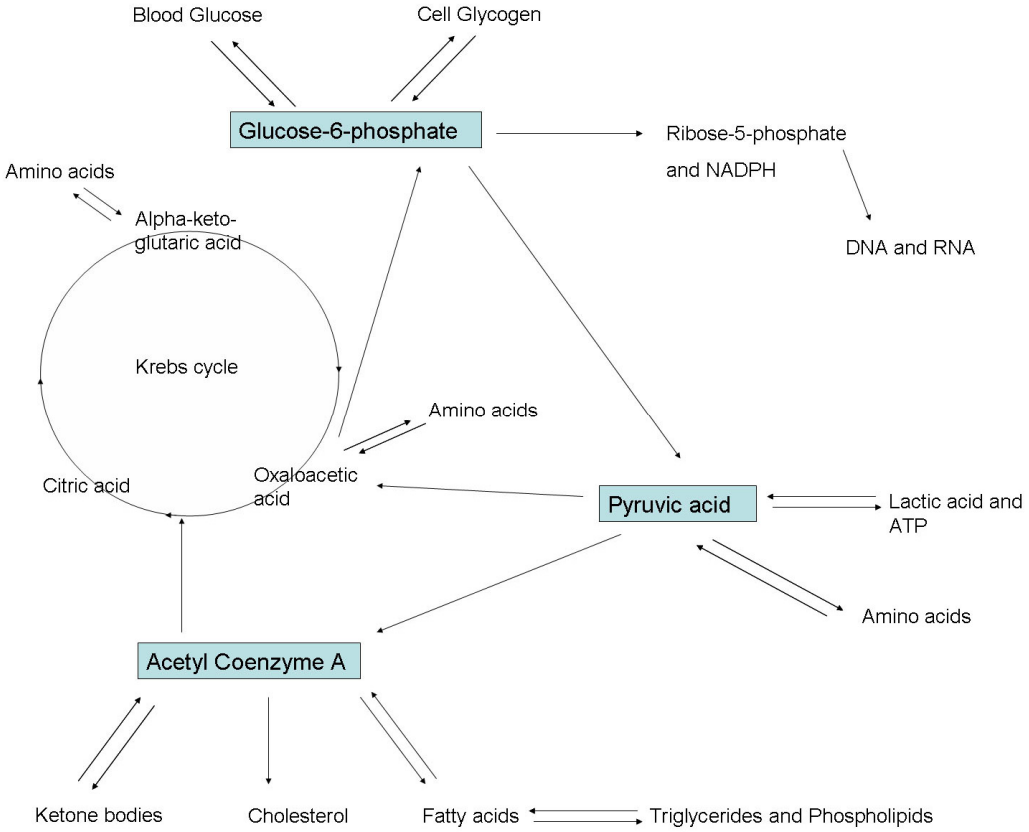

Figure 2 - Pathways of nutrient metabolism crosstalk. Key molecules and pathways linking carbohydrate, lipid, and protein metabolism (Adapted from (Tortora and Grabowski, 1996)).

As mentioned above, glucose and fatty acids are the main oxidative fuels. This fact is not without control, and the interdependent relation between glucose and fatty acids seems to lead to the preferential usage of glucose, when available, sparing fat depots as reservoirs to be tapped during long-term fasting or exercise periods. This highlighted importance of glucose is also seen as to its consumption as fuel for the central nervous system.

During fasting, the brain is responsible for almost 60% of whole-body glucose uptake from blood circulation; being it almost all spent in oxidative metabolism (Amiel,

1995). The rate limiting step in this process is glucose transport. Indeed, unlike other tissues, brain glucose uptake is not dependent on insulin or blood flow but only determined by the gradient difference through the blood-brain barrier (Dienel and Hertz, 2001), mediated by glucose facilitative transporters. For this, glucose is the main substrate in the blood that is able to supply the energy demands of the brain (Dienel and Hertz, 2001). After a meal, the brain is responsible for disposing of ~20% of the ingested glucose (Kelley et al., 1988; Meyer et al., 2002). This new–arrived glucose is oxidised in place of blood glucose originated by the breakdown of reserves stored in other organs, which is the way the level of blood glucose is maintained during a short-term fasting.

Additionally, under conditions of glucose shortage, the brain is able to consume other substrates. With increased time of fasting, or during exercise, it becomes capable of uptaking lactate with adequate effectiveness (Dienel and Hertz, 2001). Lactate also seems to be shuttled between different neural cells, to provide for local increases in energetic necessities (Pellerin, 2008)[18], or to be eliminated to the bloodstream, as a result of the excess of glucose taken by the brain (Dienel and Hertz, 2001). If fasting is prolonged into starvation, the brain may start to consume ketone bodies, β-hydroxybutyrate and acetoacetate (Amiel, 1995). By this time, the other body tissues are consuming mainly fatty acids and ketone bodies.

In times of nutrient shortage, the other body tissues are coerced to give priority to the brain (Peters et al., 2004). The first physiological response is the secretion of adrenaline and an activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (Amiel, 1995; Wang and Mariman, 2008). Then, glucose output is promoted from reserves at the liver, and in less magnitude in the kidneys, while glucose uptake is inhibited in skeletal muscle and adipose tissue. Similarly, fatty acids are launched into the blood, by increased lipolysis in adipose tissue. This helps the peripheral tissues to change from glucose to fatty acid oxidation as an energy source (Frayn, 2003), leaving more glucose available for brain uptake. The brain also acts to promote food intake, by stimulating feeding behaviour through neuroendocrine factors (Burcelin, 2005).

[18] This is related to the increased neural activity in selective parts of the brain, due to the quick summon of cognitive functions, and the necessity to promptly allocate energy to those specific points (Pellerin, 2008).

If even this is not enough to prevent hypoglycaemia, body proteins, especially from the skeletal muscle, may be degraded to provide fuel for *de novo* glucose synthesis in the liver. Eventually, this necessity to compensate for hypoglycaemia can lead to tissue wasting, coma and eventually death. Likewise, an excess of glucose availability in the blood can be harmful to tissues throughout the whole body. Circulating hyperglycaemia can become toxic, mainly leading to damage by oxidation and free radical formation (Brownlee, 2001), and even inflammatory reactions (Ceriello, 2005; Evans et al., 2002).

Due to the risks derived from these two situations – hypoglycaemia and hyperglycaemia - the level of glucose in the blood has to be actively clamped by the organism within a tight range (in humans, roughly around 90 mg/dl). For this, energy must be adequately stored and utilised.

Although three forms of carbohydrates are absorbed into the blood, fructose and galactose are quickly converted to glucose in the liver. Since glucose is a preferential source of energy, its fate is mainly ATP production (glycolisis) or storage as either glycogen (glycogenesis) or eventually triglycerides (lipogenesis). Glycogen can later be converted back to glucose (glycogenolysis) in several tissues, although only the liver and the kidneys have the ability to release it as glucose back into the blood circulation; thus providing glucose for other tissues. To complement this, the liver is also able to uptake certain intermediaries of lipid and protein metabolisms, and produce new glucose (gluconeogenesis). Glucose can likewise be used to provide the backbone for amino acid synthesis, later incorporated into new proteins. If glucose is excreted in the urine, it marks one of the main symptoms that diabetes is already established.

Lipids are also oxidised to provide energy; it should not be forgotten that one lipid molecule yields more than twice the energy of a glucose molecule. If in excess, lipids are stored as triglycerides in adipose tissue; making in fact the largest reserve of energy in the organism. However, triglycerides are continuously degraded and resynthesised; which means that they are turned over between the blood and adipose tissue. This favours the availability of lipids to be included in the formation of several substances needed for structural and regulatory functions.

In contrast, digested proteins and amino acids are not stored. But they are likewise turned over. Body protein is constantly degraded, and the resulting amino acids are used either in new protein synthesis or oxidised for very specific functions. If amino acids are in excess, they are not excreted but rather are converted into glucose or triglycerides.

If glucose homeostasis is thus affected by the metabolism of non-carbohydrate nutrients, then a proper control to maintain it requires the interrelation of the whole organism. A tight account must be kept to optimise glucose availability, but likewise to prevent the adverse effects of too much circulatory glucose. The correct relation to lipid and protein metabolism has also to be maintained, as gluconeogenesis may provide an unwelcome increase in circulating glucose. Several pathways of information and intervention, both neural and humoral routes of signalling, have to be simultaneously managed to provide an optimal level of control (Wasserman, 2009). And to be able to withstand all the energy requirements during the periods of wanting, the first critical constraint of the organism is to be capable of properly storing energy when nutrients are available.

1.7.Hormonal and nervous control in the fed/fasting/fed transitions

Digested carbohydrates and lipids, if not directly oxidised to provide energy in the form of ATP, are dispatched to storage. Carbohydrates are stored as glycogen, and lipids are stored in fat as triglycerides. Amino acids are integrated in the whole-body course of protein turnover. Within a few hours, body reserves are summoned to help maintain glucose homeostasis. But reserves are not indefinite, and eventually food ingestion is again wanted.

Metabolic reactions in the immediate postprandial state are under strong hormonal regulation, with insulin as the major coordinator of several processes.

Insulin secretion in response to feeding happens in three distinct phases. Upon feeding or even by the presentation of a meal, which can be referred broadly to as a preabsorptive phase[19], the parasympathetic autonomic nervous system mediates the secretion of a first peak of insulin from the pancreas, called the cephalic phase (Ahren and Holst, 2001). This first burst of insulin[20] is thought to be important in the measure

[19] This phase is restricted to the first 10-15 min after meal ingestion. It can be referred to as preabsorptive, since plasma glycemia only starts to rise from 15 min after meal ingestion onwards (Ahren and Holst, 2001).

[20] Since insulin is secreted continuously in a pulsatile manner (Porksen et al, 2002), the term “burst” is used here to express the increase in mean insulin secretion. Postprandial changes in insulin secretion seem

that it may prepare the liver for the impending influx of nutrients, shifting it from glucose output to uptake; and is dependent on insulin already synthesised and stored in vesicles docked to the plasma membrane in pancreatic β-cells[21] (Bratanova-Tochkova et al., 2002). Lack of this response influences negatively the glucose disposal through the entire postabsorptive period (Ahren, 2000). This is followed by an early and later phases in insulin secretion, dependent on subsequent insulin synthesis, transport, vesicle docking and exocytosis (Duckworth et al., 1998), which vary in kinetics dependent upon the type of meal stimulus provided[22] (Caumo and Luzi, 2004).

The initial cephalic phase is complemented by responses induced by the arrival of nutrients to the stomach, their absorption in the intestine and delivery to the blood circulation, and subsequently to the local effects of nutrients at the pancreas. Hence, gastrointestinal peptides (specifically, GLP-1 and GIP; also known as incretins) are released from the stomach upon food arrival. This, plus the rise in blood glucose concentration in the hepatoportal circulation (phase 2 of insulin secretion) and eventually in the pancreatic circulation (phase 3 of insulin secretion), further stimulates insulin release from pancreatic β-cells.

The effect of glucose-sensing at the hepatic portal circulation on pancreatic insulin secretion (phase 2) is mediated by the parasympathetic nervous system; first by an afferent, from liver to brain, and then by an efferent pathway, from brain to pancreas (Lee and Miller, 1985).

In relation to the direct pancreatic effect of glucose on insulin release (phase 3), the contribution of glucokinase must be pointed out. This enzyme functions, in the pancreas, as a glucose sensor (Matschinsky et al., 1998). Additionally, other nutrients besides glucose, mainly amino acids, also have a direct effect on modulating pancreatic insulin secretion (Bock et al., 2007b).

Glucokinase is the enzyme responsible for the intracellular phosphorilation of glucose into glucose-6-phosphate; the first step to several metabolic pathways, as seen

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to be translated in higher insulin burst mass magnitude, rather than in changes of burst frequency. Also, this pulsatile behaviour is shown even after hepatic insulin extraction (Porksen et al, 2002b).

[21] The state of maturation of the insulin granules seems to configure at least three pools: a reserve pool, a physically docked pool, and a readily releasable pool (Bratanova-Tochkova et al, 2002).

[22] Glucose ingestion produces a well-defined early peak of insulin, followed by a steep descent and eventually a more delayed rise. In a mixed-meal ingestion, this is substituted by an early fast-rising insulin secretion and a later maintenance of the level achieved (Caumo and Luzi, 2004).

before. It plays a vital role in glucose homeostasis; with different, although complementary, roles in the pancreas and liver[23] (Al-Hasani et al., 2003; Levin et al., 2004; Matschinsky et al., 1998). Due to the equilibrium in glucose concentration between the blood and both hepatocytes and pancreatic β-cells, achieved through facilitative transport provided by glucose transporter 2 (GLUT2), the glucose sensor needed by these cells is able to rest intracellularly, rather than on the surface of cell membranes. This, together with a cooperative relation between glucose and glucokinase (Iynedjian, 2009), contributes to the close maintenance of a glucose set point. Any deviation from this set point, either positive or negative, makes the body respond in a coordinated effort to maintain euglycemia (Zelent et al., 2005).

Glucokinase expression and activity is highly tissue-specific and dependent on prandial status. In hepatocytes, during fasting, glucokinase is inactivated by binding to the glucokinase regulatory protein (GKRP). This complex is stored in the hepatocyte nuclei. After a meal containing carbohydrates, blood glucose rises, as well as liver fructose-1-phosphate[24]. Entering through nuclear pores, fructose-1-phosphate breaks the sequestered glucokinase-GKRP complex, and allows glucose to bind to glucokinase. The new complex is shuttled to the hepatocyte cytoplasm, where it catalyses glucose-6-phosphate generation. Fructose-6-phosphate, which is in equilibrium with the concentration of glucose-6-phosphate by isomerisation, provides a negative feedback to control glucokinase activity; by competing with glucose and inactivating the enzyme when able to bind to it (Iynedjian, 2009). Further, while hepatic glucokinase mRNA is almost absent during fasting and rises sharply with feeding, pancreatic glucokinase mRNA and protein levels are constitutive, independently of prandial status (Iynedjian et al., 1989).

However, the later is still activated by feeding, but by increased catalytic activity (Iynedjian et al., 1989). Feeding also potentiates this mechanism through the vagus nerve and incretins, bypassing glucokinase. Acetylcholine released by increased activity of the pancreatic parasympathetic vagus innervation, and incretins, bind to receptors in the β-cell membrane, and promote insulin exocytosis by enhancing the effect of intracellular calcium (Matschinsky et al., 1998) in specific pools of insulin-containing granules (Bratanova-Tochkova et al., 2002). In relation to acetylcholine, this action has

[23] Glucokinase also has a role as the main brain glucose sensor (Levin et al, 2004).
[24] Fructose-1-phosphate is generated by phosphorilation of ingested fructose. It is further metabolised into glyceraldehyde-3-phosphate and dihydroxyacetone, which then enter glycolysis.

been shown to be mediated by muscarinic M3 receptors, in the pre-meal and postprandial stages (Gautam et al., 2007; Gilon and Henquin, 2001).

In β-cells, GKRP is essentially absent (Iynedjian, 2009). This makes pancreatic glucokinase activity more strictly dependable on glucose concentration. Thus, it facilitates the coupling of glucose metabolism, signalled by the metabolic products arising from the pathways to which glucose-6-phosphate is the precursor, to other pancreatic functions essential for whole-body glucose homeostasis, as insulin secretion (Matschinsky et al., 1998). The increased rate of glycolysis and oxidative metabolism, induced by a rise of available β-cell pooled glucose-6-phosphate, leads to the production of ATP. The rise in intracellular ATP/ADP ratio causes the inactivation of ATP-sensitive Kir6.2 potassium channels, inducing depolarization of the cell membrane (Miki et al., 1998). This leads to the activation of voltage-sensitive calcium channels, with the subsequent influx of calcium to the cytoplasm (Ashcroft and Gribble, 1999). Cell membrane depolarization and calcium influx seem to be related to the inducement of exocytosis of different pools of insulin granules (Bratanova-Tochkova et al., 2002; Straub and Sharp, 2002); hence the ability of the pancreas to coordinate various phases of insulin secretion[25] (Straub and Sharp, 2002; Zawalich et al., 1989).

Insulin is a major effector in the anabolic metabolism that prevails in the immediate postprandial state. It facilitates tissue uptake of glucose and enhances active uptake of amino acids. It also stimulates phosphorylation of glucose and conversion to glycogen in hepatocytes and skeletal muscle (Al-Hasani et al., 2003). Further, in hepatocytes and adipocytes, insulin enhances triglyceride synthesis. Protein synthesis is likewise stimulated by insulin.

All these actions fit together, whole-body wise, as for the direct and indirect insulin action on the control of hepatic glucose metabolism. Insulin acts directly on the liver to enhance glucose uptake and glycogen formation, and indirectly by favouring the release of gluconeogenic substrates from the periphery to the liver (Edgerton et al., 2006).

Further, insulin has a role in the "turn off" of responses that are related to the fasting state. In fact, the relation in concentrations of insulin and glucagon, released respectively by β- and α-cells of the pancreas, is highly dependent, and at the same time

[25] This is further modulated by cephalic and incretin factors (Zawalich et al, 1989). These signals promote the readiness of releasability of the insulin granules already docked to the plasma membrane (Straub and Sharp, 2002).

influential, on the prandial status. Thus, postprandial increased insulin release blocks the output of glucose from the liver; since at this stage the available ingested glucose can be used to fuel the tissues that, during fasting, where dependent upon hepatic-generated glucose.

The regulation of the aforementioned processes in the fasted and fed states is further conditioned by a more wide control of food intake and disposal. This mechanism has both short and long-term control loops. The first, as seen before, answers rapidly to dietary challenges and is involved in meal initiation and termination. This short-term response depends on blood concentrations of nutrients and metabolites, and on signals from the gastrointestinal tract (Havel, 2001). The second is responsible for alterations in feeding patterns, and is modulated by the status of the stocks of energy. This long-term control is mainly influenced by signals that are proportional to body adipose tissue, as insulin and leptin (Havel, 2001).

Consistent with this, acute hepatic denervation does not alter feeding patterns (Martin et al., 1990). Thus, although the central nervous system is able to manage alterations of hepatic glucose metabolism during fast/fed/fast transitions through the hepatic autonomic nervous system (Edgerton et al., 2006), it seems that the long-term control of energy balance and feeding behaviour is able to function properly in the absence of this signal. Hepatic parasympathetic afferents seem however to be related to fat intake (Warne et al., 2007), and to the regulation of plasma metabolite levels dependent on the insulin/corticosterone balance (Cailotto et al., 2005). This hints to a different regulation of the mechanism of choice of caloric source, modulated by insulin action.

Body energy balance is monitored mainly by brain centres located in the hypothalamus. While the feeding centre in the lateral hypothalamus is related to the necessity of food ingestion, the satiety centre in the ventromedial nuclei of the hypothalamus is responsible for inducing the sense of fullness. Apparently, the instinct to eat is always present, which makes the feeding centre always active, and it is the satiety centre that modulates it. The hypothalamus integrates information from all over the body, either through nervous or hormonal pathways. Changes in neuron activity within each centre leads to changes in feeding behaviour.

Hypothalamic control of feeding behaviour can be perceived as a response either to a positive or negative energy balance. A decrease of blood glucose concentration may signal the depletion of energy reserves. This leads to a decrease in the firing frequency of neurons in the satiety centre, with a subsequent decrease in the inhibition maintained upon the feeding centre, and resulting stimulation of food intake. On the other hand, an increase in the amount of adipose tissue and circulating lipids leads to the activation of neurons in the satiety centre, thus increasing inhibition on the feeding centre and decreasing food intake.

The central nervous system is also responsible for a circadian rhythmic control in the daily variation of glucose metabolism (Cailotto et al., 2005; Kalsbeek et al., 2004). This mechanism allows, even prior to food ingestion, blood glycemia to rise at dawn, in anticipation of the beginning of higher body activity. It also influences blood glycemia in the remaining parts of day.

This circadian signal seems to be mostly modulated by the sympathetic connection between brain and liver (Cailotto et al., 2005). Since the hepatic parasympathetic nerves are related to the afferent and efferent central responses necessary to regulate the disposal of a meal, this hints to a possible critical role of the autonomic sympathovagal balance in the global regulation of glucose metabolism.

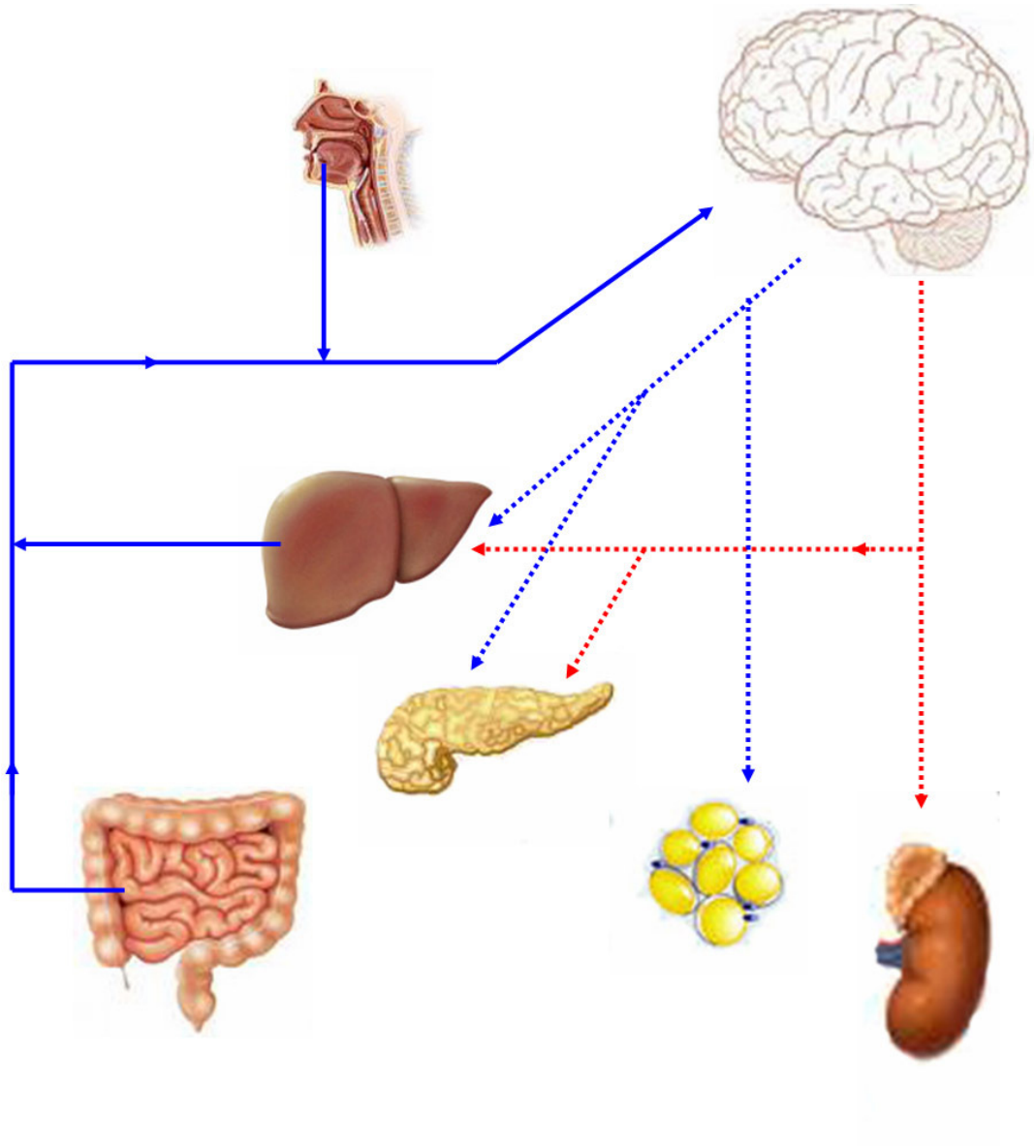


Figure 3 - **Autonomic nervous control of glucose homeostasis through central integration** (Adapted from Niijima, 1989). This is a general representation of parasympathetic (blue) and sympathetic (red) autonomic nervous signals that influence glucose homeostasis management. Afferent (continuous lines) parasympathetic innervation provides the brain with data from nutrient mechanical and chemo sensors. This is integrated, mainly in the hypothalamus, with information from nutrient sensors within the brain. Parasympathetic and sympathetic efferent signals (dotted lines) participate in the regulation ascertained by the brain on several organs.

Brain inputs are provided by humoral factors and neural pathways (mainly from gut, adipose tissue, and liver) (Yamada and Katagiri, 2007).

Signals received by the hypothalamus are several, as well as the organs they control (Figure 3); allowing the hypothalamus to function as the brain main integrator and effector in terms of energy homeostasis (Yamada and Katagiri, 2007). After receiving

information both from local sensors and from afferent nerves, the hypothalamus exerts control over several organs either by stimulating efferent nerves or by hormones secreted from the pituitary gland.

The liver is chief among the organs in which the hypothalamus interferes on metabolic homeostasis (Kiba, 2002; Uyama et al., 2004). Afferent signals from the hepatic region[26] are believed to be related to cytokines and nutrient metabolites sensors (Berthoud, 2004; Berthoud, 2008; Uyama et al., 2004). Additionally, intrinsic hepatic nerves may also have sensory functions[27] (Forssmann and Ito, 1977). The hypothalamus has direct efferent innervation to the liver, but also influences it by stimulating three endocrine axis; modulating actively the pancreas, adrenal glands, and the pituitary gland.

As mentioned, the pancreas secretes both insulin and glucagon, which have antagonic effects upon glycogenesis, glycogenolysis, and gluconeogenesis. Insulin favours anabolic processes, while glucagon favours catabolic reactions. Catecholamines are released from the adrenal glands, and mainly facilitate glycogen degradation and gluconeogenesis. The hypothalamus also regulates adrenal glands indirectly, by leading to adrenocorticotropic hormone (ACTH) release from the pituitary gland, which stimulates the production of cortisol and corticosterone from the adrenal glands. These act on peripheral tissues to facilitate gluconeogenesis at the liver. Likewise, growth hormone, released from the pituitary gland, increases hepatic glucose production indirectly, by stimulating lipolysis in adipose tissue. Thyroid hormones have a direct effect on the liver promoting gluconeogenesis, besides enhancing the action of catecholamines and glucagon (Uyama et al., 2004).

In this intraorgan communication, the liver may also be able to influence β–cell mass through the existing neural relay mediated by the central nervous system. This mechanism is stimulated by hepatic extracellular signal-regulated kinase (ERK) signalling, through the parasympathetic nervous system (Imai et al., 2008; Uno et al.,

[26] These nerves are originated at the hepatoportal region and not from the liver proper (Berthoud, 2004; Uyama et al, 2004).

[27] The distribution of these intrahepatic nerves is highly species-dependant; and may be inversely related to the degree of direct parasympathetic innervation of hepatic lobules (Forssmann and Ito, 1977). Alternatively, hepatic parasympathetic innervation feeds into the hepatocyte triads.

2006); and may be involved in the adaptative behaviour to an increase in fat accumulation within the liver[28] (Eaton et al., 2009), the so-called "fatty liver".

Adipose tissue has direct parasympathetic innervation (Kreier et al., 2006). It also has sympathetic innervation, which acts by inducing lipolysis (Bartness and Song, 2007). Again, energy-related information derived from the liver can influence the adipose tissue, through a neuronal pathway (Uno et al., 2006). Additionally, the adipose tissue releases several adipokines able to interfere on skeletal muscle glucose uptake (Sell et al., 2006b). This relation between adipose tissue and skeletal muscle is at the basis of the detrimental effects of inflammation on whole-body glucose uptake.

This is even more relevant as an impairment of parasympathetic function has been related to an overexpression of the inflammation response (Czura and Tracey, 2005; Sloan et al., 2007). Indeed, the parasympathetic autonomic nervous system seems to be involved in the regulation of cytokines release (Sloan et al., 2007). Thus, a decrease in parasympathetic function would, besides from determining possible alterations in glucose metabolism by interfering in glucose absorption and insulin secretion, further hinders insulin-stimulated glucose uptake by inducing the interference of major pathways of inflammation, cellular stress, and mitogenesis (Sell et al., 2006a).

To conclude, changes in the ability of any tissue to adapt to the transition between the fasting and fed states, may hamper the regulation of glucose fluxes body-wise; by disrupting the aforementioned hormonal and nervous crosstalk systems (Ukropec et al., 2008).

1.8.Glucose transport and intracellular signalling

Glucose entry on cells is generally achieved by facilitated diffusion[29] (Wood and Trayhurn, 2003). Circulating glucose attaches to the extracellular face of a carrier protein that spans the entire plasma membrane. These glucose transporters (GLUT)

[28] It has been proposed that those intrahepatic fat deposits are a critical determinant of metabolic deregulation, rather than visceral or subcutaneous body fat deposition (Eaton et al, 2009).

[29] Apart from the facilitative transporters, the GLUT family, glucose can also be transported by another class, the sodium-dependent glucose co-transporters (SGLT). These are present mainly in cells lining the intestinal lumen and the proximal tubules of the kidneys, to ferry glucose against the concentration gradient. This transport is powered by a sodium gradient provided by the sodium-potassium ATPase pump (Wood and Trayhurn, 2003).

undergo conformational changes, ferrying the glucose molecules to (and, in some cases, from) the cell cytosol; this depending upon the existence of a concentration difference between the two media. As already mentioned, this allows glucose to keep moving into cells to produce energy or be stored, and out to provide energy for other tissues.

There are more than a dozen isoforms of the GLUT family; slightly different in tissue distribution, specificity and affinity for sugars, and in additional stimuli able to change these characteristics (Mueckler, 1994; Wood and Trayhurn, 2003).

The GLUT1 is present in all tissues, being related to basal glucose uptake. Albeit its ubiquitous function, it is especially critical in providing glucose to the brain, through the blood-brain barrier (Leybaert, 2005). GLUT2 is expressed mainly in pancreatic β-cells, hepatocytes, enterocytes, and renal tubules. It is responsible for the ability of β-cells to act as glucose sensors for insulin secretion, and of hepatocytes to shift between output and uptake of glucose. Likewise, it is involved in the intestinal absorption and renal reabsorption of glucose (Wood and Trayhurn, 2003). The GLUT3 has the highest affinity to glucose. For this, it is expressed in situations of high demand of this fuel, as during fetal development and in the brain (Simpson et al., 2008). In some types of glucose transporters, especially GLUT4, which can be found mainly in skeletal muscle and adipose tissue, the rate of facilitated diffusion is able to be accelerated by insulin and other stimuli (Huang and Czech, 2007; Khan and Pessin, 2002). These mechanism arise from the necessity of either quickly disposing of glucose from the blood, as in immediately after a meal, or providing glucose for energy production, as in exercise, cold exposure, or hypoxia (Dohm, 2002; Huang and Czech, 2007). Some transporters ferry other hexoses than glucose; like fructose, in the case of GLUT5 (Douard and Ferraris, 2008).

The characteristics of these and other facilitative hexose transporters can be found in Table 1.

Table I - Tissue distribution and function of facilitative glucose transporters. Adapted from (Doblado and Moley, 2009; Mueckler, 1994; Rogers et al., 2002; Stuart et al., 2009; Wood and Trayhurn, 2003)**.**

Name	Tissue expression	Proposed function
GLUT1	Ubiquitous; adipose, muscle, liver, especially brain and erythrocytes	Basal glucose metabolism; transport across blood-brain barrier
GLUT 2	Hepatocytes, pancreatic β-cells, intestine, kidneys	Glucose sensing; high-capacity low-affinity transport; transepithelial transport
GLUT 3	Especially important in brain	High affinity for glucose; basal transport; uptake from cerebral fluid into brain parenchimal cells
GLUT 4	Skeletal muscle, heart, adipocytes	Insulin-stimulated glucose uptake; especially important in the postprandial state
GLUT 5	Small intestine, testes, adipose, muscle, brain, and renal tissues	Intestinal absorption of fructose
GLUT 6	Brain, spleen, leucocytes	Glucose transport
GLUT 7	Hepatocytes and gluconeogenic tissues	Mediates flux across endoplasmic reticulum membrane
GLUT 8	Testes, brain, and other tissues	Glucose transport
GLUT 9	Liver, kidneys	Hexose and uric acid transporter
GLUT 10	Liver, pancreas	n.d.
GLUT 11	Heart, skeletal muscle, liver, lung	Fructose and glucose transport
GLUT 12	Heart, prostate, muscle, small intestine, adipose tissue	Provides redundancy for the insulin-stimulated GLUT4

As stated before, GLUT4 is especially relevant for insulin-stimulated glucose uptake. In the basal state, GLUT4 cycles slowly between being inserted at the plasma membrane and being stored at intracellular sites. These sites, where GLUT4 molecules are sequestered within vesicles, include the *trans*-Golgi network, recycling endossomes, and diverse tubulo-vesicular bodies (Ishiki and Klip, 2005). At this stage, only a small fraction of GLUT4 is integrated in the cell membrane at a given time (Kanzaki, 2006). The 90% that are sequestered may be so in two "pools", corresponding to different intracellular structures (Ishiki and Klip, 2005; Thong et al., 2005).

Insulin action triggers a series of signalling pathways that change the traffic of the GLUT4-containing vesicles among diverse intracellular depots, accelerates their translocation to the plasma membrane, as well as sometimes decreases GLUT4 endocytosis from the membrane back into vesicles (Ishiki and Klip, 2005). The activation provided by insulin may also lead to differences in the GLUT4-containing vesicles that are translocated to the cell membrane; specifically, in the content of

proteins involved in vesicular docking/fusion to the plasma membrane[30] (Pessin et al., 1999; Thong et al., 2005). Furthermore, the action of insulin on GLUT4 trafficking is likely due to changes in actin and microtubule cytoskeleton (Chang et al., 2004; He et al., 2007).

GLUT4 endocytosis is a subject less well known than exocytosis. However, the effect of insulin on reducing the rate of internalisation of GLUT4 seems to be relevant in adipocytes but not in myocytes (Antonescu et al., 2009). In adipocytes, insulin seems to prevent the endocytosis of GLUT4 in caveolae[31] (Khan and Pessin, 2002), rather redirecting them to be internalised through slower clathrin-coated vesicles. In skeletal muscle cells, insulin seems to have no effect on endocytosis rate, in opposition to its drastic increase in GLUT4 exocytosis. Nonetheless, the same decrease in endocytosis seen in adipocytes can be elicited in myocytes, but rather by influence of factors related to muscle contraction (Wijesekara et al., 2006).

[30] This is related mainly to the vesicular content of SNARE protein VAMP2, which interacts with its SNARE protein counterpart on the plasma membrane to induce docking and fusion of the GLUT4-containing vesicle (Pessin et al, 1999).

[31] Caveolae are small (50-80 nm) invaginations in the lipid rafts of plasma membrane, maintained by the protein caveolin (Kahn and Pessin, 2002).

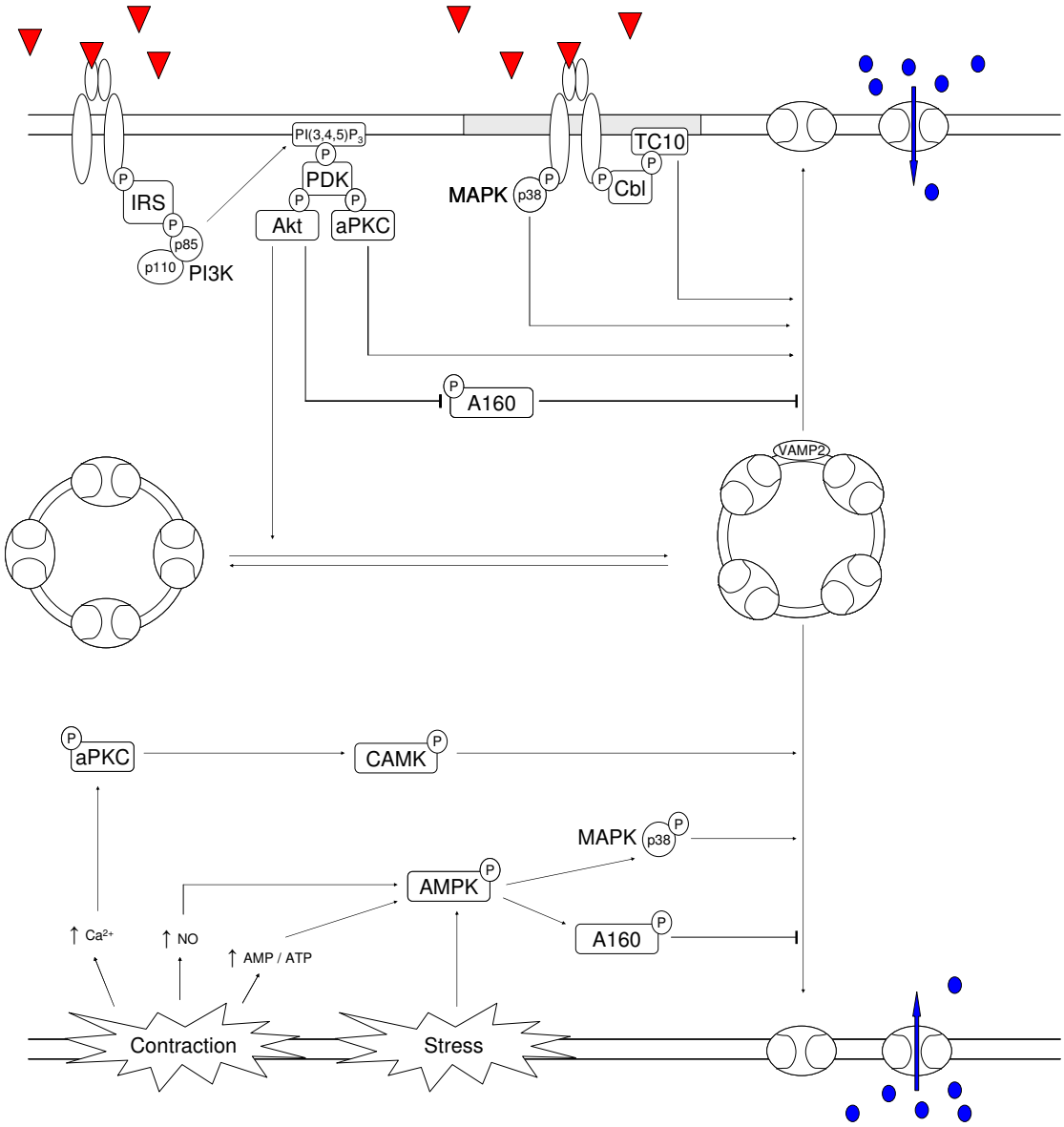


Figure 4 - **Induction of GLUT4 translocation.** Representation of stimuli and intracellular pathways that lead to GLUT4 translocation and activation (adapted from (Higaki et al., 2001; Jessen and Goodyear, 2005; Long and Zierath, 2005; Rockl et al., 2008; Thong et al., 2005)). Insulin is represented by red triangles and glucose is represented by blue circles. A160: Akt substrate of 160 kDa, Akt: protein kinase B, AMP: adenosine monophosphate, AMPK: 5'adenosine monophosphate-activated protein kinase, aPKC: atypical protein kinase C, ATP: adenosine triphosphate, CAMK: Ca^{2+}/calmodulin protein kinase, Cbl: Casitas b-lineage lymphoma protooncogene, IRS: insulin receptor substrate, MAPK: mitogen-activated protein kinase, NO: nitric oxide, P: phosphate group, PDK: 3-phosphoinositide-dependent kinase, PI3K: phosphoinositide-3-kinase, $PI(3,4,5)P_3$: phosphatidylinositol-3,4,5-triphosphate, TC10: GTPase protein, VAMP2: vesicle-associated membrane protein.

The transmembrane insulin receptor has two α extracellular subunits, where insulin binding occurs, and two β intracellular subunits, with various sites[32] of autophosphorylation with tyrosine kinase activity (Chang et al., 2004). After insulin binding is effective, the activated receptor induces the phosphorylation of tyrosine residues on intracellular substrate proteins.

The intracellular substrate proteins bind to the activated insulin receptor and are responsible for signal transmission to the diverse metabolic, mitogenic, and anti-apoptic signalling pathways that are modulated by insulin[33]. These proteins are named insulin receptor substrates (IRS) and possess several isoforms, with tissue-specific functions (Thirone et al., 2006). Among them, IRS1 and IRS2 seem to play the most important part in glucose homeostasis (Fritsche et al., 2008; Thirone et al., 2006); with complementary actions among them[34] (Chang et al., 2004). The phosphotyrosine residues in these IRS provide docking sites for other proteins, belonging to different signalling pathways (Khan and Pessin, 2002).

Although insulin actions, relayed by activated IRS proteins, are widely diverse, its effect on GLUT4 translocation is mainly achieved through the phosphatidylinositol-3-kinase (PI3K) pathway (Khan and Pessin, 2002). Additionally, apparently on adipose tissue but not on skeletal muscle (JeBailey et al., 2004), that signal has to be complemented with the contribution of a second signalling pathway, the Cbl-TC10, to allow for correct GLUT4 translocation and induction of glucose uptake (Khan and Pessin, 2002). Both pathways will be briefly described below.

PI3K is essential to the metabolic and mitogenic actions of both insulin and insulin-like growth factor 1 (IGF1) (Saltiel and Kahn, 2001). In the case of insulin action on glucose metabolism, the activated IRS interacts with the p85 regulatory subunit of

[32] The intracellular portion of the insulin receptor has sites for phosphorylation at juxtamembrane, kinase, and C-tail regions (Chang et al, 2004).

[33] The insulin receptor substrates (IRS) are also responsible for relaying the signal from insulin-like growth factor I (IGF1) binding. IGF1 can bind either to specific receptors, or hybrid receptors that bind both IGF1 and insulin. This versatility explains how the same signal can be diverted into different pathways.

[34] This has been observed in studies regarding specific IRS knock-out (KO) mices. IRS1KO are growth retarded, have peripheral insulin resistance, and show glucose intolerance, albeit not becoming diabetics. IRS2KO present both peripheral and liver insulin resistance, and develop type 2 diabetes (Chang et al, 2004).

PI3K. This activates the other half of the PI3K dimmer, the p110 catalytic subunit. The enzyme then catalyses the phosphorilation of the lipid substrate phosphatidylinositol-4,5-biphosphate ($PI(4,5)P_2$). The resultant phosphatidylinositol-4,5-triphosphate ($PI(3,4,5)P_3$) migrates but remains bound to the plasma membrane, from where it regulates the activity of several effector proteins in the insulin signalling pathways (Thong et al., 2005). $PI(3,4,5)P_3$ recruits these effectors to the plasma membrane and activates them. These include PDK1, which then phosphorilates other downstream effectors, as protein kinase B (known as Akt or PKB) and atypical protein kinase C (PKC ζ/λ) (Khan and Pessin, 2002). Akt further acts on the substrate AS160, inactivating its GTPase-activating domain by phosphorylation, and removing the block that AS160 imposes on GLUT4 translocation when able to interact with Rab proteins pertaining to GLUT4-containing vesicles. In turn, PKC ζ/λ may induce actin remodelling essential for GLUT4 translocation. These effectors seem to produce a general signal for the trafficking of GLUT4-containing vesicles among intracellular pools, and translocation to the cell surface.

As mentioned, this general signal may not be enough to promote glucose transport into the cell; even when it leads to GLUT4 translocation to the plasma membrane (Sweeney et al., 2004). A second signal has been identified as being generated from lipid raft microdomains (Chang et al., 2004). These plasma membrane regions are highly enriched in cholesterol, sphingolipids, and assorted anchored proteins. Likewise, insulin receptors reside in these lipid rafts, by interaction with the protein caveolin (Kanzaki, 2006). The activation of insulin receptors in these caveolae leads to the tyrosine phosphorylation of proto-oncogenes Cbl (Kanzaki, 2006; Thong et al., 2005), by formation of a complex with the associated protein substrate, APS. Further, the Cbl-associated protein, CAP, is also recruited to the complex. Upon phosphorylation, Cbl interacts with the protein CrkII, which is constitutively associated with the nucleotide exchange factor C3G. The C3G stimulates the activation of a small G-protein, TC10, by substituting GDP with GTP. The activated TC10 is a potent regulator of actin remodelling (Kanzaki, 2006), through pathways that likely intersect the PI3K pathway by also recruiting PKC ζ/λ (Chang et al., 2004). The crosstalk between these two pathways seems thus to govern the translocation, tethering and fusion of GLUT4-containing vesicles, and the activation of glucose transport proper.

Although the previous mechanism for GLUT4 activation seem to be present in adipocytes but absent in skeletal muscle cells (JeBailey et al., 2004), several studies have raised the hypothesis that nonetheless the myocytes present a similar signalling differentiation between translocation and activation (Michelle Furtado et al., 2003; Somwar et al., 2001). According to this, insulin also activates the p38 mitogen-activated protein kinase (MAPK). Interestingly, p38 MAPK is recruited to the plasma membrane, where it may be involved in activating the GLUT4, either by promoting their dephosphorylation[35] or by removal of another inhibitory protein (Michelle Furtado et al., 2003). This is consistent with the specificity of insulin action; although the PI3K pathway is activated by other hormones and growth factors, only insulin induces this effect on GLUT4 translocation and activation (Isakoff et al., 1995).

Besides the previously mentioned sites for tyrosine phosphorylation on the insulin receptor and on the IRS, that induce an increase in insulin-stimulated glucose uptake, additional sites of phosphorylation, but with serine kinase activity, are further involved in the regulation of the insulin receptor activation (Saltiel and Kahn, 2001); but providing a negative feedback for insulin signalling. Thus, several protein serine/threonine phosphatases have regulatory functions of insulin signalling, either with stimulatory or inhibitory effects (He et al., 2007). Likewise, nitric oxide is reported to potentiate insulin signalling at low doses, but to attenuate that signal when in high doses, by S-nitrosation of the insulin receptor, IRS1, and Akt[36] (Badal et al., 2006; Carvalho-Filho et al., 2005; Fritsche et al., 2008; Wu, 2009; Wu et al., 2009). Pathophysiologically, a wide range of factors (hyperglycemia and hyperinsulinemia, fatty acids, cytokines, glycated proteins, and others) have the ability of interfering with at least one of the multiple steps of insulin signalling (Pirola et al., 2004; Yu et al., 2002).

Beyond the action of insulin in stimulating glucose removal from the blood to the cell, thus crucially helping to manage postprandial blood glucose levels, intracellular

[35] GLUT4 are more phosphorylated when fused to the myocyte plasma membrane than when sequestered on intracellular compartments. However, paradoxically, surface GLUT4 loose more than half their phosphorylation when insulin is present, while intracellular GLUT4 phosphorylation is unaffected by insulin administration (Michelle Furtado et al, 2003).

[36] This may be specifically related to the expression of the inducible nitric oxide synthase (iNOS), and hence with inflammatory stimuli that induce insulin resistance at the skeletal muscle (Carvalho-Filho et al, 2005; Badal et al, 2006). The amount of nitric oxide production originated by iNOS when activated far surpasses that of the constitutive isoforms.

insulin signalling is responsible for its role in regulating overall glucose and lipid metabolism, and in blocking catabolic reactions.

Especially in the liver, increased insulin signalling and glucose concentration induce the transcription of glycolytic and fatty-acid synthetic genes in the nucleus and the activation of cytosol enzymes (Saltiel and Kahn, 2001). Similarly, genes related to gluconeogenesis and lipolysis are down-regulated; by Akt/PKB or atypical PKC phosphorylation of several transcription factors, as, for example, fork-head box proteins (Fox0). Since this insulin action does not affect gluconeogenic enzymes already present in the cytosol, this is not a rapid inhibition (Fritsche et al., 2008). Insulin signalling has also a role in protein metabolism, increasing synthesis and blocking degradation of proteins through activation of the mTOR pathway (Saltiel and Kahn, 2001), which is also related to the mitogenic and growth effects of insulin.

It remains to be referred the question of insulin signalling initiation/termination. It seems consensual that insulin stimulation of glucose uptake is initiated when insulin binds to its receptor on the cell surface. However, from then on, insulin has several possible fates, which surely impinge on signal termination.

The insulin bound to the receptor may be released or be caught by internalisation; considering that, even when it is released without internalisation, it may have been partially degraded by enzymes present in the cell surface (Duckworth et al., 1998). The clearance of insulin from the blood happens primarily in the liver and kidneys, and in a lesser degree in the skeletal muscle and other insulin sensitive tissues. When the complex insulin/insulin receptor is internalised into endosomes, insulin may suffer differential degrees of degradation, mainly induced by the insulin degrading enzyme (IDE)[37] (Camberos et al., 2001; Hersh, 2006; Valera Mora et al., 2003). These sequential steps induce first cleavages of the insulin B-chain at several points, and only then the cleavage of disulfide bonds, by the protein disulfide isomerase (PDI). The results are multiple; mainly yielding intact A-chains and several B-chain fragments. By this time, the fragments may be further degraded inside the endosomes, freely or still bound to the receptor. Potentially at any of these steps, intact or fragment insulin may

[37] Insulin degrading enzyme (IDE) activity is inhibited by ATP (Camberos et al, 2001). This may allow the regulation of insulin degradation by the current cell energy status; linking the need/surplus of energy signalled by the availability of ATP to the processing of insulin, both by regulating the extracellular concentration of insulin by modifying the rate of insulin clearance and/or modulating the intracellular signalling by insulin and its degradation products.

be able to stimulate or inhibit intracellular processes; expectedly those related to lipid or protein metabolism, cell growth or mitogenesis (Duckworth et al., 1998).

If intended for disposal, insulin or its fragments are delivered to lysosomes. The endosomes can eventually fuse again with the plasma membrane, releasing to the exterior the intact insulin or its fragments. The insulin receptor is reinstated to its transmembrane position (Duckworth et al., 1998; Zhang and Radziuk, 2001).

Insulin was thus shown to be the main coordinator of the metabolic responses after a meal, in tissues as liver and skeletal muscle. On the other hand, during fasting, when circulating insulin levels decrease, this predominant role is taken by other hormones, as glucagon, catecholamines, and glucocorticoids.

Glucagon binds to its respective plasma membrane receptor, inducing the activation of adenylate cyclase, which catalyses the formation of cyclic adenosine monophosphate (cAMP). The increased cAMP binds to protein kinase A (PKA), resulting in the blockade of glycogen synthesis and glycolysis, and the activation of glycogenolysis (Jiang et al., 2001). Catecholamines act through α-adrenergic receptors in a cAMP-independent manner, but with the same results of increased glycogenolysis and gluconeogenesis (Clark et al., 1983). Glucocorticoids have to reach the nucleus to bind to their receptor, where they increase the transcription of genes related to gluconeogenesis, lipid β-oxidation, and ketogenesis (Fritsche et al., 2008). This favours the release of glucose from the liver during short-term fasting or exercise[38] (Farghali et al., 2008), as well as the production of gluconeogenic substrates and ketone bodies for longer fasting periods. As expected, the hormones involved here act at quite the same points as insulin does, but in an inverse manner.

Still, other stimuli are able to lead to cell glucose uptake, independently from insulin.

With exercise, contraction increases glucose uptake by skeletal muscle cells, but in an insulin-independent manner (Wasserman, 2009). This pathway is not yet well

[38] The same mechanism seems to be triggered in response to inflammation (Farghali et al, 2008). This opens the hypothesis of a dose-dependent mediation of glycogenolysis by hepatic nitric oxide; where high concentrations of nitric oxide, achieved through activation of inducible nitric oxide synthase, would favour hepatic glycogen degradation.

established[39] (Clark et al., 1983), but AMPK, calcium, and nitric oxide seem to play an important role on the signalling that leads to contraction-induced glucose uptake (Dohm, 2002; Jessen and Goodyear, 2005; McConell and Wadley, 2008; Zierath, 2002). AMPK is a metabolic sensor, activated when the cell is deficient of energy; this feature may enable it to fine tune the crosstalk between the contraction- and insulin-stimulated pathways of glucose uptake (Long and Zierath, 2005).

Hypoxia seems to constitute the stimulus for a third pathway that is able to stimulate skeletal muscle glucose uptake, which may or may not overlap certain steps of the previous two pathways (Brahimi-Horn et al., 2007; Mu et al., 2001). It has been recently proposed that all these pathways converge on a single nexus within the skeletal muscle, by activating the AS160 (Kramer et al., 2006) through phosphorylation of the TBC1D1 protein (Taylor et al., 2008).

Other peptides, derived from the biosynthesis of insulin, as connecting peptide (C-peptide)[40], may also have an effect on glucose uptake (Forst and Wahren, 2008). C-peptide binds to the plasma membrane through a G-protein coupled receptor, and increases glucose uptake at the skeletal muscle by activation of MAPK and endothelial nitric oxide synthase (Hills and Brunskill, 2008; Li et al., 1999). This effect of C-peptide on glucose utilisation seems to overlap insulin intracellular signalling, although bypassing the insulin receptor and early stages of insulin signalling (Wilhelm et al., 2008). Additionally, considering the unknown areas regarding the hepatic endossomal metabolism of the insulin/insulin-receptor complex, products of the degradation/alteration of the insulin molecule proper may yet likewise come to be found to have a biological action on glucose metabolism.

This interplay of intracellular processes provides a combined metabolic regulation of the alternation between fasting and fed periods; explaining how the phenomena described on topic 1.7 are induced. As described, almost each step in these processes is subjected to diverse activating and inhibitory influences; with the main objective of

[39] Contraction-stimulated glucose uptake seems to be modulated by the effect of an adrenergic balance. The α- and β-receptors mediate responses on liver, pancreas, heart, and skeletal muscle, which lead to lower insulin release, tissue glycolysis, glycogenolysis, and gluconeogenesis (Clark et al, 1983). This explains how, outside exercise, catecholamines induce insulin resistance; by decreasing pancreatic insulin release, and increasing the pool of available intracellular glucose-6-phosphate.

[40] The connecting peptide (C-peptide) favours the structural assembly of the A- and B-chains of insulin (forming proinsulin). The cleavage of C-peptide happens within β-cell granules, prior to exocytosis. Until recently, C-peptide was thought to have no biological effect once released concomitantly with insulin into the bloodstream (Forst and Wahren, 2008).

integrating information from parallel pathways. This is yet a burgeoning field of study, with novel mechanisms being investigated; as is the case of the suggested prandial-dependent modulation of peripheral insulin sensitivity by an hepatic signal that is presented below.

1.9.Hepatic modulation of postprandial peripheral insulin sensitivity

It is well known that insulin-stimulated whole-body glucose uptake assessed after a meal doubles that observed in the fasted state. This has been mainly attributed to the increase in insulin secretion in response to a meal, with a subsequent rise in circulating insulin bathing the peripheral tissues. The greater insulin availability would explain the increase in insulin-stimulated glucose uptake. Thus, the classic role of the liver in influencing peripheral insulin action would be derived from how hepatic insulin extraction modulates the concentration reaching the peripheral tissues (Balks and Jungermann, 1984; Jaspan and Polonsky, 1982)[41].

However, it was observed that this postprandial increase in insulin sensitivity can be blocked without changing postprandial peripheral insulin levels (Lautt, 1999). This has led to the proposition of a new hypothesis, one that involves the liver as a key effector in the modulation of peripheral tissue insulin sensitivity. This hypothesis relates the hepatic parasympathetic nerve tonus to the insulin sensitivity at the skeletal muscle through the action of a hormone tentatively called “HISS”, which stands for hepatic insulin sensitising substance (Figure 5).

[41] Hepatic insulin extraction has been shown to also reflect insulin action at the liver. After a meal, when insulin secretion is augmented, insulin extraction is both fractionally and quantitatively larger. This has been related to the action of insulin in suppressing glucose output and promoting glucose uptake in the liver (Jaspan and Polonsky, 1982), but can be expected to favour also other pathways associated with insulin-processing in the liver.

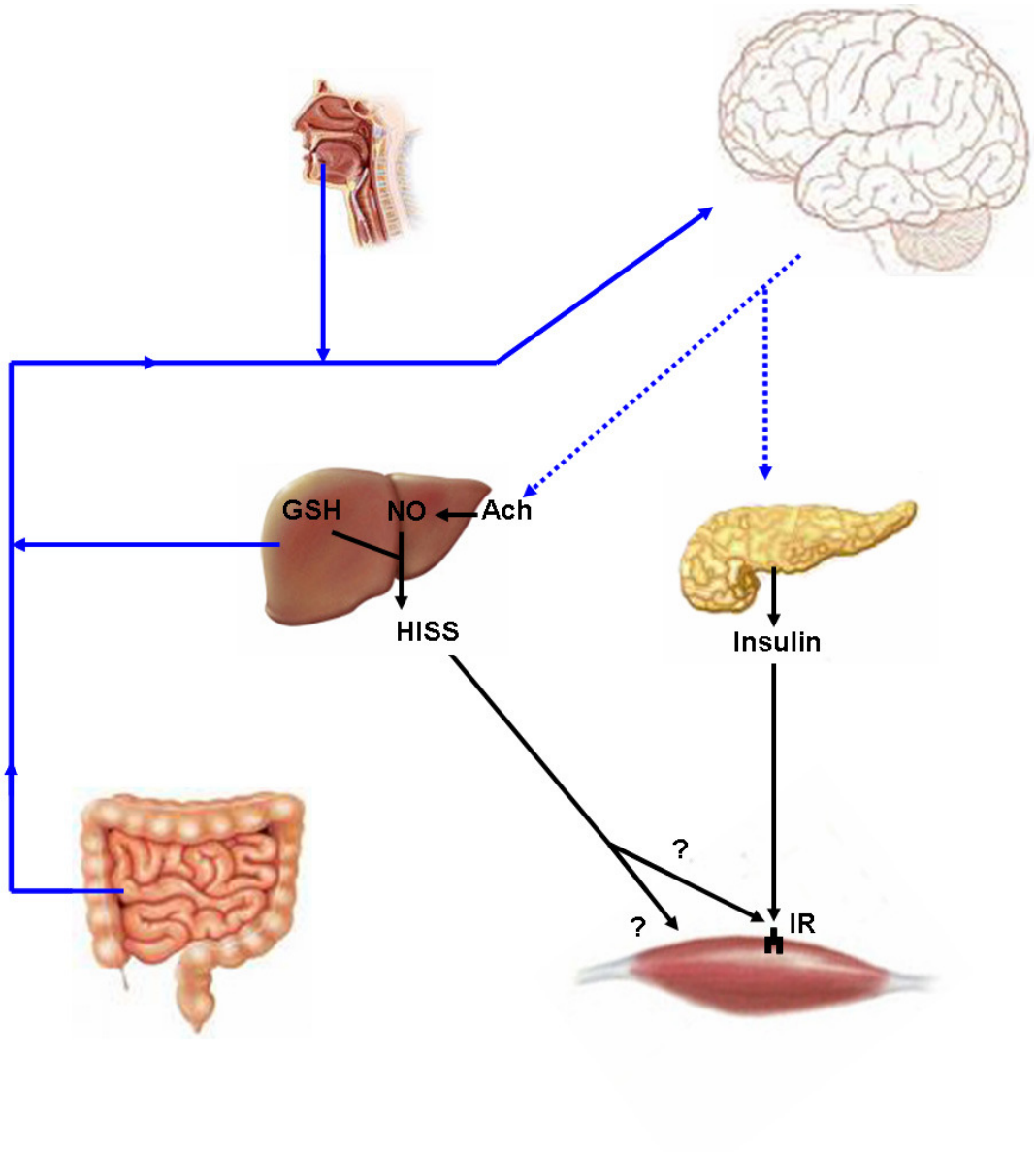


Figure 5 - Proposed hypothesis for the postprandial regulation of peripheral glucose uptake. Several feeding signals trigger the release of the Hepatic Insulin Sensitising Substance (HISS) from the liver. This will act at the skeletal muscle, either through a direct interaction with the insulin receptor or through a direct action on other component of the plasma membrane, to potentiate intracellular insulin signalling. The effect will be an increase in GLUT4-mediated glucose uptake. Ach: acetylcholine, GSH: reduced glutathione, HISS: hepatic insulin sensitising substance, IR: insulin receptor, NO: nitric oxide.

In the 1980s, the observation that the vagal parasympathetic innervation to the liver was essential to allow for the increase in insulin-stimulated glucose uptake seen after oral ingestion of a glucose load led to the hypothesis that hepatic autonomic dysfunction could lead to a decrease in hepatic glucose uptake and glycogen synthesis. This was

thus posited as a link between liver disease and type 2 diabetes; with autonomic neuropathy being proposed as an aetiological factor to diabetes development (Lautt, 1980). Later studies were to dispel the former assumption, although still preserving the notion of liver-related dysfunctions as determinants of glucose metabolism deregulation, and diabetes.

More than ten years passed until the study of this pathway could be properly addressed. First, it was shown that indeed it was the parasympathetic hepatic innervation that, if ablated, led to a decrease in the hypoglycaemic effect of insulin (Xie et al., 1993). The neutralisation of the hepatic sympathetic innervation had no additional detrimental effect (Xie et al., 1993). Then, it was shown that the administration of a cholinergic muscarinic antagonist, atropine, was able to produce the same degree of impairment of the hypoglycaemic action of insulin as the hepatic parasympathetic denervation (Xie and Lautt, 1994). Subsequently, more detailed studies identified the hepatic muscarinic M1 receptor as the one mediating this parasympathetic modulation of insulin action (Xie and Lautt, 1995b). Again, even considering that pharmacological parasympathetic blockade could influence other sites, atropine was shown not to have any additional detrimental effect over insulin-stimulated glucose uptake in relation to hepatic denervation (Xie and Lautt, 1995a).

The influence of hepatic parasympathetic innervation was definitely established by showing that acetylcholine administered directly in the liver by the portal vein was able to regenerate the insulin-stimulated glucose uptake abolished after denervation (Xie and Lautt, 1996a). As expected, peripherally administered acetylcholine was unable to restore insulin-stimulated glucose uptake after denervation. Similarly, atropine was shown to induce the same degree of insulin resistance as hepatic denervation at a lower dose when given intraportally than intravenously (Takayama et al., 2000).

Until this time, the studies performed analysed whole-body measures of insulin sensitivity. When arteriovenous glucose gradients were measured, surgical hepatic denervation was shown not to impair insulin-stimulated glucose uptake in the liver. Surprisingly, it was the skeletal muscle that revealed an impaired in insulin-stimulated glucose uptake after either hepatic denervation or atropine administration (Xie and Lautt, 1996b). This observation disproved the original hypothesis that hepatic parasympathetic dysfunction produced an impairment in liver glucose uptake, but, on the other hand, showed how those autonomic dysfunctions could be related to whole-body insulin resistance, through decrease of skeletal muscle glucose uptake. This led to

the hypothesis that the liver, after stimulation by insulin and acetylcholine, secretes an hormone, tentatively called hepatic insulin sensitising substance, or HISS, which travels through the bloodstream to act selectively at the skeletal muscle (Lautt, 1999).

Since many physiological actions of acetylcholine are mediated by nitric oxide, it was studied as a possible next step in the intrahepatic metabolic pathway that leads to HISS secretion. Indeed, the administration of a nitric oxide synthase inhibitor was shown to produce insulin resistance similar to atropine administration or hepatic denervation (Sadri and Lautt, 1999). Again, an intraportally dose capable of producing this magnitude of insulin resistance was not able to produce it when given intravenously. Also, the administration of a nitric oxide donor was able to reinstate normal insulin sensitivity after hepatic denervation or nitric oxide synthase inhibition when given intraportally, but not intravenously (Sadri and Lautt, 1999).

By this time, the hypothesis was raised that some high variability seen in previous studies could be related to the lack of knowledge regarding the prandial status of the animal experiment subjects at the time of analysis. This prompted the study of the relative importance of the HISS pathway on fasted and fed insulin sensitivity. This showed clearly that insulin sensitivity was higher immediately after feeding, and decreased gradually with time of fasting. Furthermore, it was shown that the component of insulin sensitivity preserved after atropine administration did not change, in rats, from the fed to, at least, the 24h-fasted state (Lautt et al., 2001). So, the HISS pathway was uncovered as being the responsible for the incremental insulin sensitivity seen after feeding.

Since insulin sensitivity assessed in the fasted state or in the fed state after atropine were similar (Latour and Lautt, 2002b; Lautt et al., 2001), the HISS pathway is not only the responsible for the postprandial increment, but is also only activated after the ingestion of a meal. It decreases gradually with fasting duration (Latour and Lautt, 2002b) and, given enough time, becomes in practicality non-existent. Similarly, hepatic parasympathetic denervation, atropine, or nitric oxide synthase inhibition, abolished entirely the effect of food to increase peripheral insulin sensitivity.

The dependence of the activation of the HISS pathway on the postprandial state raised the concept that a “feeding” signal would also be mandatory for this mechanism

to be properly expressed. Among the compounds that show an increase in their hepatic concentration after a meal ingestion, glutathione was uncovered to be of particular relevance to the postprandial increase in glucose tolerance.

Reduced glutathione (GSH) was a prime candidate since it rises to unparalleled high hepatic concentrations in relation to all other coenzymes after a meal (Tateishi et al., 1977). Furthermore, GSH impairment had been already linked to the development of glucose intolerance. Decreased levels of GSH were described to be present in diabetic patients, which was interpreted as either a consequence or a contributor to increased oxidative stress and subsequent defects on insulin secretion or action. However, surprisingly, GSH cellular depletion by synthesis inhibition was shown to be able to directly impair insulin action, without inducing changes in oxidative stress or insulin secretion (Khamaisi et al., 2000).

This led to studies that showed that hepatic GSH was likewise essential for the optimal function of the HISS pathway (Guarino et al., 2003). The postprandial whole-body insulin sensitivity was lower in rats subjected to chronic GSH depletion than in control animals. Furthermore, while in control animals the decrease in insulin sensitivity seen after inhibition of hepatic nitric oxide synthase was fully reinstated by the portal administration of the nitric oxide donor 3-morpholinosydnonimine hydrochloride (SIN-1), in GSH-depleted animals SIN-1 had no effect on the observed insulin resistance (Guarino et al., 2003). This seemed to indicate that, although equally essential, GSH acted on the HISS pathway in a parallel stage to nitric oxide.

Another piece of the puzzle was further glimpsed in the previous study (Guarino et al., 2003). Besides the positive results obtained with SIN-1, another nitric oxide donor, sodium nitroprusside (SNP), was used. However, SNP was not capable, under the same conditions, to restore the postprandial insulin sensitivity. Thus, the insulin resistance obtained after hepatic nitric oxide synthase inhibition remained unchanged with SNP. The main difference between these two nitric oxide donors is that SIN-1 acts similarly to the physiological effect of the enzyme nitric oxide synthase, by leading to the release of both nitric oxide and superoxide (O_2^-), while SNP releases solely nitric oxide. Since nitric oxide and superoxide can react rapidly with GSH, to form biologically active intermediates, S-nitrosothiols (Schrammel et al., 1998), this was proposed to be the common point in the parallel signalling pathways provided by nitric oxide and GSH.

To ascertain the relevant pathways, the order of the factors had first to be checked; mainly to determine the relation between acetylcholine and nitric oxide. Intraportal

acetylcholine administration was unable to revert insulin resistance induced by hepatic nitric oxide synthase inhibition. On the other hand, the intraportal administration of the nitric oxide donor SIN-1 was able to ameliorate insulin sensitivity after parasympathetic blockade by atropine (Guarino et al., 2004). In conclusion, it is indeed the release of acetylcholine by the hepatic parasympathetic nerves that leads to the formation of nitric oxide, and the subsequent activation of HISS action. It was also postulated that the effect of hepatic nitric oxide on whole-body insulin sensitivity is further regulated by guanilate cyclase (Guarino et al., 2004).

The necessity of the presence of the two signals was elegantly shown in fasted animals, where HISS is not present. Here, 24h-fasted rats were able to show normal postprandial insulin sensitivity only when both GSH and nitric oxide were administered to the liver (Guarino and Macedo, 2006). The prior administration of any one of them produced no effect on insulin sensitivity; the combined hepatic administration was indispensable to mimic normal postprandial HISS action (Guarino and Macedo, 2006). Again, the HISS mechanism was ascertained as the signalling pathway responsible for the increment in insulin sensitivity seen in the postprandial state.

More recently, the identification of the trigger that induces this feeding response has been pursued. Intragastric administration of nutrients on fasted animals was used to assess the nature of the feeding signal that leads to HISS action. Administration of a liquid mixed meal in this way was able to produce normal postprandial insulin sensitivity (Sadri et al., 2006). It was concluded that it must have a chemical nature, rather than a physical one, since the administration of the same volume of water was unable to instigate any rise in insulin sensitivity. Likewise, the administration of a solution of glucose or sucrose was unable to properly generate the feeding signal that leads to insulin sensitivity potentiation. Hepatic denervation prior to the intragastric administration of the mixed meal prevented in absolute the triggering of the mechanism (Sadri et al., 2006). Again, the relevance of the hepatic parasympathetic nerves to this reflex was shown.

The exact nutrient or combination of nutrients that induce this increased postprandial response in the skeletal muscle are not yet know; but is expectable that it might include proteins, since the consumption of a lipid enriched diet was shown to quickly induce the abrogation of HISS action (Afonso et al., 2007a).

Although the genesis of the autonomic nervous system feeding signal is still poorly understood, recent studies have uncovered that it is dependable of nutrient arrival at the intestine. Indeed, if the intragastrically administered mixed meal is prevented from progressing to the intestine, by a rubber band located at the pylorum, the postprandial response is blunted. On the other hand, if, also with the applied band, the mixed meal is given directly on the intestine, the increment in insulin sensitivity is optimal (Afonso and Macedo, unpublished results).

Other recent advancements have reaffirmed that this increment in whole-body insulin-stimulated glucose uptake seen after feeding a mixed meal happens mainly at the skeletal muscle. In fed animals, hepatic parasympathetic nerve ablation has been shown to induce insulin resistance at soleus and extensor digitorum longus (EDL) skeletal muscles; and not at liver or adipose tissue (Fernandes and Macedo, unpublished results).

The simultaneous dependence upon hepatic GSH and nitric oxide raised the hypothesis that they could be directly involved in the synthesis of HISS (Guarino et al., 2003). Indeed, GSH and nitric oxide/superoxide react together to form S-nitrosothiols (RSNO). This has been since corroborated by the observation that intravenous S-nitrosoglutathione (GSNO) administration is able to mimic postprandial insulin sensitivity in fasted animals (Fernandes and Macedo, unpublished results). Interestingly, if GSNO is perfused directly in the portal vein, it induces insulin resistance rather than augmenting insulin sensitivity. This suggests that RSNO may be related to the HISS concept, having a role in promoting peripheral insulin-stimulated glucose uptake; and that a negative feedback may be present if the RSNO concentration reaching the portal vein is too high. If the elusive HISS factor is indeed a RSNO, or another nitrosylated molecule for that matter[42], remains to be shown (Ramachandran et al., 2001; Santos et al., 2006). However, the fact that a surgical or pharmacological intervention on fed animals is able to quickly abrogate the postprandial increment in insulin sensitivity argues for a short half-life of the HISS factor in circulation; which is consistent with these compounds. Furthermore, similar mechanisms involving

[42] Both the A and B chain of insulin have been shown in vitro to be able to bind one nitric oxide molecule (Santos et al, 2006). This raises the hypothesis that, in hepatocytes, insulin may be degraded and form adducts of each chain with nitric oxide. These would be able to be transported through the bloodstream and reach the skeletal muscle, where the cell surface-bound enzyme PDI seems to be able to promote intracellular s-nitrosilation by NO transfer from extracellular s-nitrosothiols (Ramachandran et al, 2001).

nitrosylation/denitrosylation have been proposed as simple, but highly selective, forms of nitric oxide-dependent physiological signalling (Tannenbaum and White, 2006).

In summary, the HISS pathway seems to provide a way to optimise the removal of circulatory glucose to the skeletal muscle after the ingestion of a meal. This postulates a potentiating effect of HISS on insulin-stimulated glucose uptake, either directly at the insulin receptor or by synergistically activating the intracellular pathways that lead to GLUT4-mediated glucose uptake. This HISS mechanism is triggered by two feeding signals reaching the liver: GSH and the parasympathetic tonus, besides the presence of insulin. This provides a facilitator signal that, upon the increase in insulin concentration, potentiates insulin-stimulated glucose uptake. Absence of HISS is expected to favour that glucose remain more time in circulation, and eventually end up by being stored as fat.

After the review of the basics of glucose metabolism, it remains to be addressed exactly what is the fate of the glucose ingested during a meal.

1.10. Disposition of a meal and glucose metabolism

Of the glucose ingested during a meal, the greater percentage is taken up by the liver and the skeletal muscle. Besides the 20% already mentioned that are taken up by the brain, around 30% is taken up by the splanchnic tissues, mainly the liver, and almost another 30-40%[43] goes into the skeletal muscle (Foss, 1994; Holtz et al., 2008; Kelley et al., 1988; Meyer et al., 2002). The remaining 20% is taken by the kidneys (10%), heart (4%), fat (3%), and other tissues (Kelley et al., 1988; Meyer et al., 2002).

The liver, for its anatomical location (receiving blood from the portal vein, directly from the gut outgoing circulation; which means that it is the most nutrient rich right after a meal), is in a prime position to act as a buffer for blood glucose. Indeed, one third of the hepatic glucose disposal of a meal is done on the first passage (Selz et al.,

[43] Since skeletal muscle glycogen is spared when at rest, the ingested glucose directed to this tissue will depend upon physical activity intensity and glycogenolysis in the period between meals (Holtz et al, 2008).

2003); this lowers the magnitude of the glucose excursion, thus greatly diminishing the degree of postprandial hyperglycemia that peripheral tissues are exposed to. Also, being the liver the main organ responsible for the maintenance of glucose homeostasis during fasting, and, together with the kidneys, unique on the capacity to both uptake and output substantial amounts of glucose (Moore et al., 2003), the repletion of hepatic glycogen reserves is a priority upon feeding[44] (Capaldo et al., 1999; Greenberg et al., 2006).

On the other hand, the skeletal muscle, although representing the greatest depot of glycogen, is unable to release it into the bloodstream as glucose. Furthermore, muscle glycogen is spared during fasting, since it must be instead available to be spent during physical exercise (Wasserman, 2009); so, the need to replenish it with ingested glucose is lower than that of the liver.

The liver is also located immediately following the pancreas in terms of circulation, which is pertinent here mainly for the secretion of insulin[45] (Bollyky and Greenbaum, 2007). This allows the liver to modulate of the quantity of insulin being distributed systemically (Jaspan and Polonsky, 1982; Rabinowitz and Liljenquist, 1978); likewise contributing to delay peripheral glucose uptake and allow hepatic reserves to be firstly replenished[46] (Bjorntorp and Sjostrom, 1978).

Delay in insulin secretion produces changes in both carbohydrate and lipid metabolisms. The loss or delay of early insulin secretion allows higher postprandial glucose excursions to occur, and lowers inhibition of lipolysis. This is evident in type 2 diabetics (Dimitriadis et al., 2004), and has been reversed by the pharmacological mimetisation of the normal insulin profile[47]. However, as stated before, defects in insulin secretion appear more generally at later stages of glucose intolerance (Saad et al., 2005), by effect of glucotoxicity.

More, the hepatoportal region is rich in glucose sensors. This produces the so-called "portal signal" (Gardemann et al., 1986; Moore et al., 2003), which represents the

[44] Surprisingly, feeding also increases hepatic glucose cycling, with simultaneous degradation and synthesis (Capaldo et al, 1999; Greenberg et al, 2006).
[45] The pancreatic secretion of glucagon may also be relevant, but its exact action in the metabolic balance needs yet to be clarified (Bollyky and Greenbaum, 2007).
[46] Differences in glucose uptake determined by other routes of delivery further show the importance of the liver. Indeed, if glucose is administered intravenously, it leads to lower insulin secretion and consequently higher systemic hyperglycaemia, and a greater disposal of glucose into adipose tissue (Bjorntorp and Sjostrom, 1978). The response to an intravenous glucose load lacks the effect of several physiological triggers; as, for example, the cephalic phase of insulin secretion, the afferent neural signalling derived from the hepatoportal glucose sensors, or the presence of other nutrients besides glucose, like amino acids and fat.
[47] This is much better achieved with an insulin secretagogue than by administration of exogenous insulin prior to a meal (Dimitriadis et al, 2004). This has also an ameliorating long-term effect on HbA1c.

glucose gradient between the arterial and portal circulations. Instead of depending solely on absolute glycaemia, this gradient permits to quickly stimulate hepatic glucose uptake even with a modest rise in blood glucose and insulin (Capaldo et al., 1999); thus also enhancing hepatic glycogen synthesis (Bollen et al., 1998).

The liver as a buffer for energy storage is even more relevant when there is the need to handle greater glucose loads. In cases of the disposal of meals with complex carbohydrates, for example starch, which delay gastric emptying and thus prolong glucose absorption, hepatic glucose disposal may even reach 40% of the ingested glucose (Capaldo et al., 1999). As before, here the correspondent glucose disposal by skeletal muscle is estimated to be still around 30% (Capaldo et al., 1999).

Nonetheless, in cases of exacerbated positive energy balance between intake and consumption, even the capacity of the liver to accommodate energy reserves may be overwhelmed. In this scenario, adipose tissue becomes the final destination for excessive carbohydrates (Bjorntorp and Sjostrom, 1978)[48]. This may lead to increased triglyceride accumulation in fat pads, liver, and, eventually, even in skeletal muscle.

As stated before, insulin dominates the postprandial state as the main controller for glucose disposal fate; both by inhibiting endogenous glucose production, and free fatty acids release, and by promoting glucose disposal into tissues.

Immediately after a meal, circulating insulinaemia is increased even more than what is accounted by the increase in pancreatic insulin secretion (Gibby and Hales, 1983). This is achieved first by a decrease in hepatic and skeletal muscle insulin fractional extraction (0-15 min from meal ingestion) and only after by an increase in pancreatic insulin secretion (from 10 min after meal ingestion onwards) (Brundin, 1999). This timing discrepancy may hint to a temporal uncoupling of the hepatic and extrahepatic effects of insulin. First by an action on the liver to inhibit endogenous glucose release and maximise glucose uptake (Brundin, 1999), then by sensitising peripheral tissues to glucose uptake[49] (Gibby and Hales, 1983; Samnegard and Brundin, 2001). A small increment of circulating insulin suppresses hepatic glucose output and inhibits free fatty

[48] This ability of fat tissue to hold the excessive carbohydrate seems more efficient in other species, like rat, than in man (Bjorntorp and Sjostrom, 1978). This hints to an even lower tolerance of humans to overnutrition, leading to a more rapid and maintained circulating hyperglycaemia; even if thermogenesis is increased to compensate for the overwhelming energy intake.

[49] On the other hand, the kidney seems to have a role in attenuating the postprandial accumulation of insulin in the systemic circulation (Samnegard and Brundin, 2001), by doubling renal insulin fractional extraction after a meal. Paradoxically, the renal fractional extraction of C-peptide (which is mainly cleared here, and not in the liver as insulin), is not altered by meal ingestion.

acids oxidation at the skeletal muscle (Groop et al., 1989). The latter helps peripheral tissues to switch from fat to carbohydrate consumption, an hallmark of the transition from the fasted to the fed state. Insulin has also effects on adipose tissue, suppressing lipolysis and free fatty acids release. The insulin-stimulated glucose uptake at the peripheral tissues, mainly the skeletal muscle, happens only at higher insulin concentrations.

Besides the amount of glucose disposed of into each tissue, the metabolic fate of that glucose is also relevant.

Almost 40% of the absorbed glucose is immediately oxidised (Fery et al., 1998). More than half of this is oxidised in the brain; corresponding to almost all the uptake of ingested glucose by this organ. Another 35% is oxidised in skeletal muscle. The heart (2%)[50] and other tissues are responsible for the rest (Berger et al., 1976; Kelley et al., 1988).

Of the remaining 60% of ingested glucose, disposed by non-oxidative means (Fery et al., 1998), more than half is disposed of at the splanchnic tissues, mainly the liver (Kelley et al., 1988). This accounts for both glucose stored as glycogen by the direct pathway or indirectly by hepatic uptake of gluconeogenic substrates (Edgerton et al., 2006), which have been proposed to contribute equally to postprandial hepatic glycogen formation (Huang and Veech, 1988; Kurland and Pilkis, 1989; Lefebvre and Scheen, 1999)[51]. Only 15% of stored glucose was placed on skeletal muscle, and the kidneys stored 11% (Kelley et al., 1988); which is not surprising, since skeletal muscle is spared unless physical activity is required during fasting (Wasserman, 2009), and renal glycogen is turned over in response to a meal ingestion (Meyer et al., 2002).

Indeed, to further facilitate glucose uptake by the liver, the hepatic endogenous release of glucose to the bloodstream, essential to maintain glucose homeostasis during fasting, is quickly inhibited after a meal (Moore et al., 2003). On the other hand, while hepatic glucose release is almost entirely suppressed by feeding, postprandial renal

[50] The myocardium has been described as resembling the contracting skeletal muscle in many ways (Berger et al, 1975). However, in terms of energetic fuel, it shows a more marked, and maintained, preference for fatty acids oxidation than the skeletal muscle.

[51] The postprandial synthesis of hepatic glycogen is variably attributed to these two pathways; ranging from predominance of one, another, or an equal importance of both (Huang and Veech, 1988; Kurland and Pilkis, 1989; Lefebvre and Scheen, 1999).

glucose release increases twofold[52] (Meyer et al., 2002). This transient postprandial renal mechanism is believed to augment the efficiency of hepatic glycogen repletion.

The fact that hepatic glycogen synthesis is not exclusively derived directly from ingested glucose should be underlined. After an overnight fast, around half of the remaining hepatic glycogen has been synthesised from glucose that was first converted to lactate and other gluconeogenic intermediaries (Bollen et al., 1998). This may seem a futile cycle; but may rather be an hepatic mechanism to prevent excess glycogen stores, and to provide a combined control over glycogenolysis and gluconeogenesis (Greenberg et al., 2006; Wise et al., 1997)[53].

Maintenance of glucose homeostasis in the postprandial state is dependent upon the suppression of glucose hepatic output, a shift from fat to glucose oxidation, and storage of glucose as glycogen at the liver and the skeletal muscle (Firth et al., 1986; Groop et al., 1989). So, in terms of postprandial usage of glucose in the skeletal muscle, much depends upon the metabolic flexibility of alternating between glucose and fatty acid catabolism (Corpeleijn et al., 2009; Storlien et al., 2004). However, since glucose oxidation in skeletal muscle is more rapidly saturated than nonoxidative metabolism (Foss et al., 1992), it can reveal to provide no great alternative if glycogen synthesis becomes impaired.

It has been shown that glucose transport is the rate limiting step affected by the glucose metabolism deregulation involved in the progression to diabetes (Cline et al., 1999). Indeed, the aetiology of the problems with postprandial handling seems generally to be restricted to glucose uptake. Impaired insulin-stimulated uptake is apparently already present when there is still a normal suppression of hepatic glucose output (Lefebvre and Scheen, 1999; Nielsen et al., 2005). Likewise, glucose intestinal absorption seems to remain normal, even with diabetes (Basu et al., 2001).

The inability to properly dispose of ingested glucose at the skeletal muscle, a considerable portion of the total, even more if taken into account the glucose shuffled indirectly to the liver through the peripheral tissues, must lead to metabolic changes.

[52] The underestimation or disregard of renal changes may explain why the postprandial suppression of hepatic endogenous glucose production varies greatly among studies, between around 40 and 85% inhibition.

[53] This feedback mechanism is further hinted by the influence of hepatic glycogen concentration on the balance between glycogenolysis and gluconeogenesis contribution to hepatic glucose endogenous production (Wise et al, 1997).

Among them is the increased postprandial accumulation of triglycerides in skeletal muscle and liver (Ravikumar et al., 2005).

In this introduction, the hypothesis has been raised that the liver is able to influence extrahepatic glucose uptake during the postprandial state both by modulating insulin delivery to the peripheral tissues and producing an hormonal signal that potentiates insulin action at the skeletal muscle. This turns the spotlight into those pathologies that affect the health condition of the liver or the integrity of parasympathetic autonomic nervous system function, two essential points in the proposed mechanism. Not surprisingly, both conditions are known to present commonly glucose intolerance and a greater prevalence of diabetes.

Patients with chronic liver disease present frequently disturbances of glucose homeostasis. However, they do not present insulin resistance at the liver, but do so at the skeletal muscle (Kruszynska et al., 1993; Selberg et al., 1993). Cirrhotic patients also present hyperinsulinemia in fasting and after oral glucose (Kruszynska et al., 1993; Letiexhe et al., 1993), due to increased insulin secretion and lower hepatic insulin extraction (Letiexhe et al., 1993). Even so, as in IGT and diabetics (Thorburn et al., 1991), cirrhotic patients seem to hinge glucose intolerance upon the ability of hyperinsulinemia to compensate for the lower insulin action (Nielsen et al., 2005), the excess insulin levels are not always able to achieve that.

Cirrhotic individuals present normal postprandial glucose oxidation, but lower nonoxidative glucose disposal, which accounts for the decreased glucose uptake (Riggio et al., 1992; Selberg et al., 1993). Thus, paradoxically to the affected organ, liver disease patients show decreased glycogen deposition at the skeletal muscle.

This should not be derived from a defect in the molecular machinery needed for postprandial insulin-stimulated glucose uptake, since GLUT4 protein content and activity (Holland-Fischer et al., 2007; Jessen et al., 2006) and the initial steps of intracellular insulin signalling (Jessen et al., 2006) seem to be similar to normal subjects[54]. Again, it argues for the impairment of a factor that potentiates insulin-stimulated glucose uptake in the postprandial state.

[54] Even though these patients seem to loose the redundancy in GLUT4 expression, indicated by a decrease in GLUT4 mRNA (Holland-Fischer et al, 2007), the ability of the cell to function at an optimal level of

Also pathologies related to an impairment in parasympathetic nerve function, or an increase in sympathovagal balance, have been related to glucose intolerance and the development of diabetes. Not just neuropathies, but even more subtle conditions as depression (Das, 2007).

Paradoxically, isolated skeletal muscle shows no difference in insulin-stimulated glucose uptake coming from short-term fasted or refed states (Berger et al., 1976). This discrepancy with the higher muscle insulin-stimulated glucose uptake and glycogen deposition seen in the postprandial state is explained by the presence of a circulating factor in response to a meal ingestion that potentiates insulin action; which is consistent with the presented HISS hypothesis.

Animal models have been used widely to study insulin resistance, the development to type 2 diabetes, and the metabolic syndrome (Oron-Herman et al., 2008). On this basis, the present research was directed into what are considered to be the risk groups according to studies based on the OGTT analysis: history of diabetes, age, hypertension, and obesity (IDF, 2003). The uncovering of problems in glucose uptake related to the HISS mechanism would provide further avenues for prevention and treatment of glucose intolerance and type 2 diabetes, since glucose transport is seen as the more effective step in which agents will be able to improve insulin sensitivity (Shulman, 2000).

glucose uptake with a radical loss of this redundancy at either the insulin receptor or glucose transporter argues against a limitation in glucose uptake by this observation.

2. OBJECTIVES

The present work is based on the **hypothesis** that postprandial whole-body insulin sensitivity is regulated by a mechanism to which the integrity of the hepatic parasympathetic nerves (HPN) is crucial. In accordance with this, the **overall objective** of this doctoral thesis was to determine if a dysfunction of the HPN-dependent component of insulin sensitivity is involved in the insulin resistance seen in several situations related to the aetiology of type 2 diabetes.

It is known that with age animals commonly become glucose intolerant and that the prevalence of type 2 diabetes is higher in old individuals. The **first objective** of this thesis was to assess what happens to the HPN-dependent component of insulin sensitivity in otherwise healthy male and female animals during those developmental changes.

This thesis **second objective** was to evaluate the impact of environmental factors, such as diet manipulation, on postprandial insulin sensitivity. To this end, it was studied the exposure to several ways to administer a diet with a high content of the disaccharide sucrose, drawing a parallel to the increased diet ingestion of refined sugars seen today all over the world, and that undoubtedly contributes to the rising of type 2 diabetes seen among children.

Being type 2 diabetes a complex, multifactorial disease, the **third objective** was to study the postprandial increment of insulin sensitivity, and its possible dysfunction, in the presence of hypertension – a pathology usually related to insulin resistance and type 2 diabetes. The impact of the sustained presence of hypertension was also assessed. The **fourth objective** was related to the hepatic insulin sensitising substance (HISS) hypothesis, a proposed pathway that influences glucose uptake and to which the HPN seems to pertain. The status of other steps of the HISS pathway besides the hepatic parasympathetic nerves, specifically hepatic nitric oxide and hepatic glutathione, was also studied in the presence of hypertension.

3. MATERIAL AND METHODS

3.1. Animals

Several strains of rats were used in our studies, specifically Wistar, Sprague Dawley (SD), Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR). The animals were obtained from Charles River Laboratories (Spain) and from the Animal House at the Faculty of Medical Sciences (FCM) of the New University of Lisbon (Portugal). When coming from Spain, animals were kept, after arriving at FCM, on a quarantine period of at least one week.

The animals were housed in cages at the FCM Animal House, following the suggestions of the manufacturer for number of individuals per cage. This also depended at each time on the age of the animals. As an exception, the animal groups used for a special diet protocol were housed one per cage during the time of diet manipulation, to allow for the monitoring of food consumption.

Conditions of controlled temperature and humidity were maintained (21.0 ± 5.0º C and 55.0 ± 5.0 %), as well as a 12h light-dark cycle (lights on from 8:00 to 20:00). Until the chosen age of study, more precisely until the day before the evaluation of insulin sensitivity, all animals had *ad libitum* access to water and solid pellets of standard chow (Panlab A04, Charles River) - composition: 15.5% protein, 58.5% carbohydrates and 2.7% fat - , with the exception of the groups of animals pertaining to the series of studies regarding the high-sucrose diet (detailed on item 3.1.2.3).

Animals were cared for according to the current European and Portuguese legislation related to the protection and welfare of vertebrates used to experimental ends, namely the Directive 86/609/CEE of the European Council (24 of November, 1986), and the National Decree 129/92 (6 of July, 1992).

In these sets of experiments, all animals were maintained under anaesthesia from the time of preparation for the surgical protocol until the end of the experimental protocol. After, they were euthanized with a lethal dose of sodium pentobarbital.

3.2. Experimental design

3.2.1. Studies related to aging

To study the evolution of insulin sensitivity with age we used male Wistar rats. The animals were analysed, on the immediate postprandial state, at 6, 9, 16 and 52 weeks, which translates, in human terms, to two phases of maturation (6 and 9 weeks of age), the end of puberty (16 weeks of age) and middle-age (52 weeks of age). The Wistar rat strain has a mean life expectancy of two years, and we have not recorded any spontaneous mortality in any of the age groups that we maintained at the Animal House.

Additionally, we analysed male Wistar rats on the fasted state, at 9 and 52 weeks of age, to provide us with a comparing template to study the influence of age on the increment of insulin sensitivity observed after meal ingestion.

3.2.2. Studies related to gender

To clarify the possibility of differences in insulin sensitivity with age between genders, we used male and female Wistar rats, analysed in the postprandial state at 9 and 52 weeks of age. After the first experiments, Wistar rats of both genders were also analysed in the postprandial state at old-age (78 weeks of age).

3.2.3. Studies related to high-sugar content diets

Wistar and SD rats were randomly divided into several groups and were given free access to water and standard solid food pellets (Panlab A04; Charles River) or to a liquid or solid supplement of sucrose on their diet, as detailed below per feeding protocol.

The liquid diet was obtained by mixing sucrose in tap water in a 35% solution (w/v). Each bottle was filled with 200 ml of solution, changed each two days. Alternatively, the high-sucrose solid diet D11725 and corresponding control diet D11724 were used, both from Research Diets. These solid diets were formulated to differ only in the source of carbohydrates, that being sucrose in the first diet and corn starch in the control diet. Both have 20.0% protein, 66.0% carbohydrates and 5.0% fat.

The sucrose diet was coloured red and the control diet was coloured blue to avoid mix up when refilling the pellets partition in each cage.

3.2.3.1. Effect of a liquid high-sucrose diet in Wistar rats

Wistar rats were randomly divided into two groups. Both standard-fed and sucrose-fed groups had free access, for 6 weeks, to a standard solid diet (Panlab A04; Charles River) and water, but while the standard-fed group had only a bottle of drinking water, the sucrose-fed group had an additional bottle of a 35% sucrose solution.

3.2.3.2. Effect of the duration of exposure to a liquid high-sucrose diet in SD rats

SD rats were randomly divided into four groups. Each sucrose-fed group was given a standard solid diet (D11725, Research Diets) and a 35% sucrose solution for the last 2, 6 or 9 weeks. The standard-fed group was given the standard solid diet (D11724, Research Diets) and drinking water for the entire time period of the experiment. Thus, all animals were analysed with 12 weeks of age.

3.2.3.3. Effect of a liquid vs solid high-sucrose diet in SD rats

SD rats were randomly divided into three groups. The standard-fed group was given a standard solid diet (D11724, Research Diets) and drinking water for 9 weeks. Sucrose-fed groups were given either a 35% sucrose solid diet (D11725, Research Diets) and drinking water or a standard solid diet (D11724, Research Diets) and a 35% sucrose solution for the same period. Thus, all animals were analysed with 12 weeks of age.

3.2.4. Studies related to hypertension

For this we used the strain of Spontaneously Hypertensive Rats (SHR), as well as their normotensive control strains Wistar and Wistar Kyoto, analysed in the postprandial state at 9 weeks of age. To further evaluate the impact on the metabolic profile produced by the sustained high arterial pressure, these strains were also analysed at 16 weeks of age.

We also determined insulin sensitivity in the fasted state at 9 weeks of age in a group of SHR animals, using a group of 9 weeks old fasted Wistar rats as their normotensive control.

3.3. Pre-surgical protocol

All animals were fasted overnight for 16 hours (from 16:00 of the day prior to experiment to 8:00 of the day of experiment), but with free access to a bottle of drinking water. Animals to undergo insulin sensitivity testing in the fasted state were maintained fasting for an additional hour. Animals to undergo insulin sensitivity testing in the postprandial state were given access to a standard solid meal for an hour (from 8:00 to 9:00).

After fasting or after we assured that the animals had indeed eaten, and were in the immediate postprandial state, all animals were anesthetised with a solution of sodium pentobarbital (65 mg/kg bw) (Eutasil, Sanofi). This procedure was done with the help of a plexiglass restriction cage. The anaesthesia syringe was introduced 2 cm above the sexual organ, at the midline of the abdomen.

The animal was left unmolested until fully anesthetised, which was checked by the pain reflex on the tip of the tail or the eye reflex. The animal was then removed from the cage and placed on a surgical table, over a heating pad (Homeothermic Blanket Control Unit 50-706, Harvard Apparatus) to maintain body temperature at 37.0 ± 0.5ºC, monitored with a rectal probe thermometer.

3.4. Surgical protocol

A small incision was made centrally from under the chin to the base of the neck of the animal. A tracheotomy was performed and a polyethylene tube (PE 240, Becton Dickinson) was inserted and secured to allow for spontaneous respiration. The left internal jugular vein was isolated from conjunctive tissue. Another small incision was made in the depression at the base of the left leg. The femoral artery was also isolated.
Previously, an external arterial-venous shunt had been made with silicone and plastic connections (Figure 6). Three pieces of silicone tube (Silicone tubing L/S 14, Masterflex) were attached to a plastic T-junction. The end arm of the junction was closed with a three-way plastic valve. To the other arms were connected two long

pieces of polyethylene tubing (PE 50, Becton Dickinson), with a wedge-cut tip. The shunt was primed with a saline-heparin solution (200 U/ml).

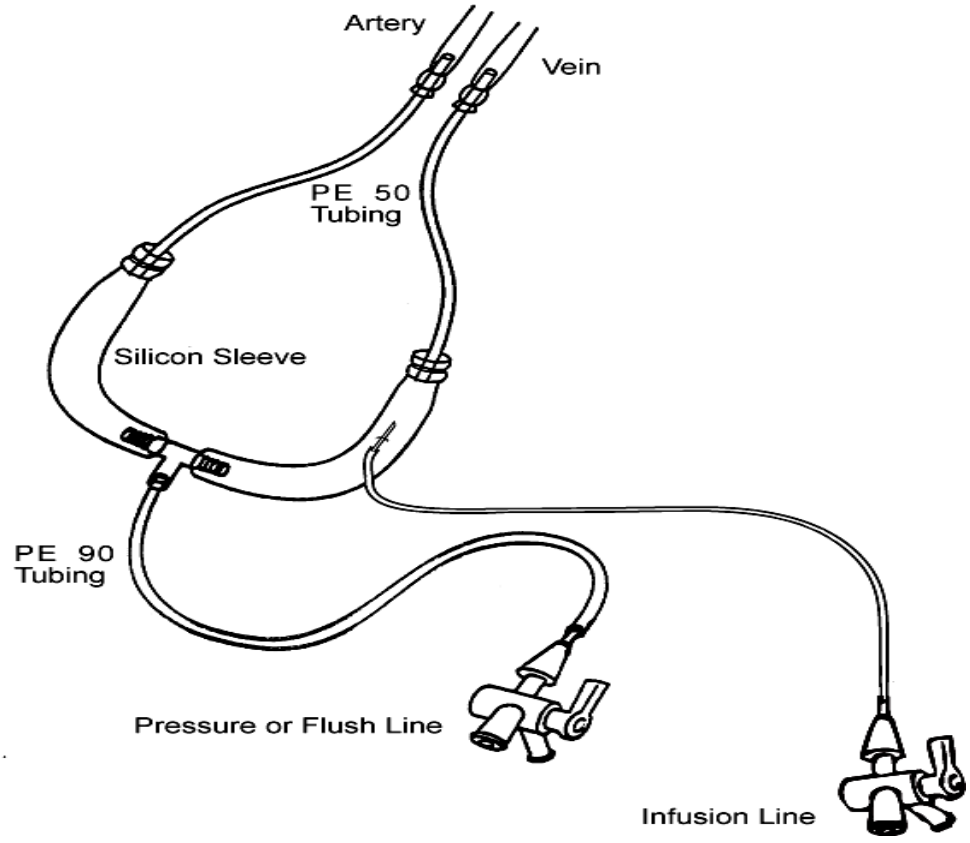


Figure 6 - Extracorporal arterial-venous circuit. Inserted to permit external blood circulation from the femoral artery to the internal jugular vein. The arterial branch is used to collect blood samples. The venous branch is used to perfuse solutions through infusion lines. A third branch can be used to assess blood pressure, or to flush any blood clot that may be formed during experiment. (Adapted from (Lautt, 1999)).

Each end of the arterial-venous shunt was used to catheterise one blood vessel, the internal jugular vein and the femoral artery, with the assistance of a magnifying lens apparatus (SMZ-2B, Nikon). After the loop was secured, and blood was flowing through by difference in pressure between the two ends, the remaining arm was used for periodic measurements of the arterial and venous pressures (Powerlab 8/s, ADInstruments; recorded, through a pressure transducer, by MacLab software); which was done by briefly clamping the opposite end with a surgical clamp. This setup also

enabled the quick detection and removal of any blood clot formed inside the system during the course of the experiment.

For the experiments requiring surgical ablation of the hepatic parasympathetic nerves, a laparotomy was performed prior to the placing of the external shunt. Briefly, a large incision was made vertically on the abdomen, from 1 cm above the genitalia to the xiphoid cartilage. The viscera were gently moved upward (to the right side of the abdomen), taken especial care for the integrity of the pancreas which is diffuse in the rat, and the stomach was exposed. A metal manipulator was inserted above the stomach, pulling down and out the oesophagus, and the liver was carefully pushed. Running parallel to the esophagus, we identified the vagal nerve and, branching from it to the liver, running parallel to the hepatic common artery, we identified the hepatic branch, which possesses the majority of efferent hepatic parasympathetic nerves. The bundle of nerves was isolated and a thread passed behind it. All organs were repositioned in their anatomical place, and the thread extremities were externalised. To perform the selective hepatic vagotomy, the thread was pulled and the bundle of fibres was burned through with a cauteriser (High Temperature Cautery with 500394 tip, World Precision Instruments).

For the experiments requiring the direct delivery of drugs to the liver, a laparotomy was likewise performed. This time, viscera were carefully put aside with the help of moist cotton swabs and covered with moist gauze. After, a catheter was inserted on the portal vein (Abbocath 24G Optiva IV 19 mm, Johnson & Johnson), connected to a syringe by PE50 tubing. The portal vein was punctured 2 cm upstream from the liver and the tip of the catheter was located 1 cm inward. The apparatus was stabilised by points of biological glue (Histoacryl, BBraun) carefully placed on the catheter and line. The laparotomy was finally covered with moist gaze and parafilm.

In all protocols, anaesthesia was maintained throughout the experiments by continuous infusion of a sodium pentobarbital solution (1.0 mg/ml saline given at 1.0 ml/100 g bw) through a cannula (infusion line PE 50 with a cut 23-g needle at the delivery end) inserted into the venous side of the shunt. All drugs and saline solutions were administered intravenously (i.v.) through this shunt or intraportaly (i.p.) through the portal vein catheter.

Operated animals were allowed to stabilise after surgery for at least 30 min before any tests were carried out.

3.5. Insulin sensitivity evaluation

To determine whole body insulin sensitivity, either on the fasted or postprandial state, we used the Rapid Insulin Sensitivity Test (RIST), a modified hyperinsulinemic euglycaemic clamp, as previously described in 1999 by Prof. Wayne Lautt (Lautt, 1999). This test relies in an equipment, the glucose analyser, able to do separate measurements of blood glucose content in less than 2 min.

3.1.5.1. Glucose Analyser

The YSI 1500 Sidekick (Yellow Springs Instruments) is able to determine the glucose content in a relatively small blood sample. The sample is collected directly by puncturing the silicone arterial branch of the external shunt with a microsyringe calibrated for 25 µl. The sample is then injected in the reaction chamber of the analyser.

A magnetic stirrer mixes the blood sample with the analyser buffer (YSI 2357 Buffer, Yellow Springs Instruments). This chamber opens to a biological probe that is covered by a three-layer membrane. Here, a membrane impregnated with immobilised glucose oxidase is separated from the probe by a cellulose acetate membrane and from the sample by a polycarbonate membrane. When some of the substrate diffuses through the membrane, comes into contact with the oxidase and is promptly oxidised, producing hydrogen peroxide (H_2O_2). The H_2O_2 diffuses to the probe and is then oxidised by the platinum anode, producing electrons. When the rate of H_2O_2 production and the rate of diffusion become constant, a dynamic equilibrium is achieved. That steady state means that the electron flow is then linearly proportional to the steady state H_2O_2 concentration and, thus, to the concentration of blood glucose.

Prior to the experiment, the glucose analyser is calibrated with a 180 mg/dl glucose reference solution. This reference solution is also used during experiments to verify the analyser calibration. Sporadically, we also used a 50 mg/dl concentration solution to verify the linearity of the machine.

3.5.2. Determining a baseline glycaemia

As the RIST is a euglycaemic clamp, it can only be performed when blood glucose level is stable. For this, blood samples are measured each 5 min, until three consecutive stable readings are obtained. The mean of those values is referred to as the basal glucose level, and is used as the reference value for the carrying out of the RIST.

3.5.3. Rapid Insulin Sensitivity Test (RIST)

Minute 0 was set at the start of a 5 min i.v. insulin bolus (50 mU/kg bw) given using a perfusion pump (Perfusor fm, BBraun Medical). At minute 1, the arterial blood glucose concentration was measured and a glucose infusion (*D*-glucose/saline, 100 mg/ml, i.v.) was started at a rate of 5 mg/kg/min to avoid hypoglicaemia. Arterial blood glucose concentration was measured at 2 min intervals and the rate of the glucose infusion was adjusted whenever necessary to maintain the glycaemia as near as possible to the basal glucose level. The RIST was concluded when no further glucose infusion was required to maintain euglycaemia (Figure 7).

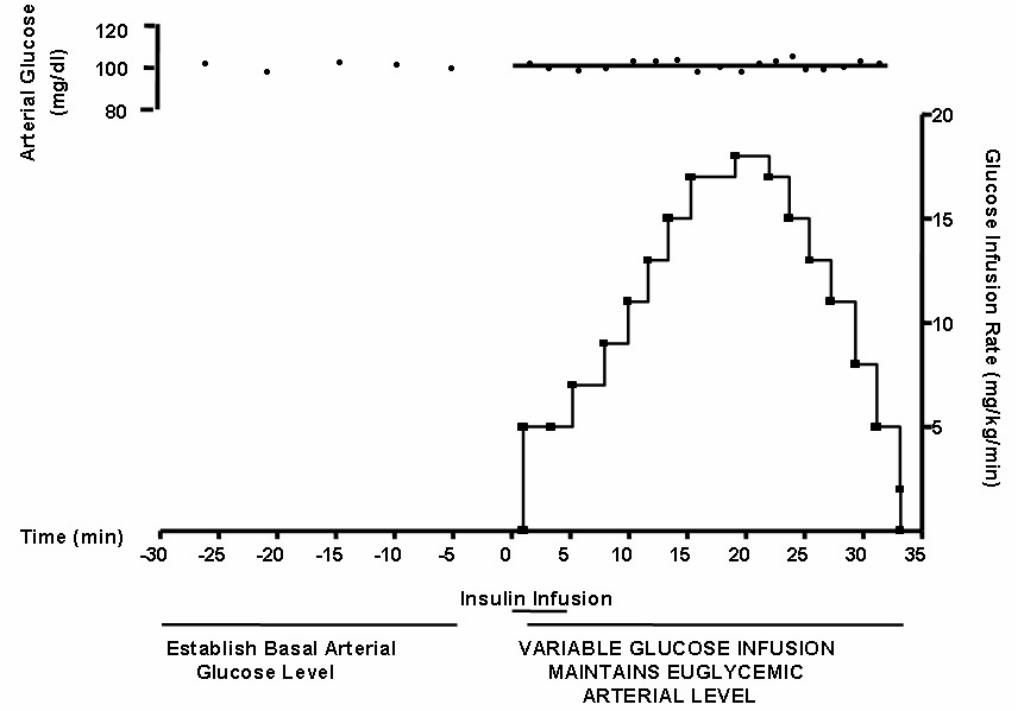


Figure 7 - Example of Rapid Insulin Sensitivity Test (RIST). After the basal glucose level is ascertained, a bolus of insulin is infused. To prevent hypoglycaemia, a perfusion of glucose is started one minute after the beginning of insulin. Glucose perfusion rate is changed in response to changes in glycaemia, so that euglycaemia is maintained throughout the test. The test is completed when no more glucose is needed. The area under the curve is used to quantify the test, and is referred to as the RIST index (Adapted from (Lautt, 1999)).

When the protocol included a following RIST after surgical or pharmacological manipulation, glucose levels were allowed to stabilise for at least 30 min after the end of intervention, after which a new basal glucose level was determined and another RIST performed.

3.5.4. Quantification and graphical representation of the RIST

The total amount of glucose infused quantifies whole-body insulin sensitivity and is referred to as the RIST index (mg glucose/kg bw). The RIST can be performed either in the fasted or postprandial state, providing the control fasted or control fed RIST indexes.

By performing a RIST after surgical ablation of the common hepatic vagal branch, we obtain the HPN-independent component of insulin action. By subtracting the post-denervation RIST from the control fed RIST, we obtain the HPN-dependent component of insulin action, ie, the postprandial increment of insulin sensitivity.

Since the HPN seem to be one of the constituents of the overall HISS pathway, postprandial insulin action reflects the sum of the HISS-dependent and –independent components of insulin action. Thus, pharmacological blockade of the HPN or other step of the HISS pathway allows us to determine the HISS-dependent and –independent components. The RIST index obtained after muscarinic receptor antagonism with atropine (Giachetti et al., 1986) or hepatic nitric oxide inhibition with N^g-methyl-arginine (*L*-NMMA) (Bryk and Wolff, 1999; Moore and Handy, 1997)[55], represents the HISS-independent component of insulin sensitivity, which, subtracted from the control fed RIST index, wields the quantification of the HISS-dependent component of insulin action.

These components can be plotted individually in column bar graphs, or in matched pairs in stacked bars graphs with the HPN-independent component under the HPN-dependent component, thus giving a simultaneous representation of each of the components and of the postprandial control insulin sensitivity.

To obtain the curves representing the time-course of each RIST we plotted the mean values of glucose infusion rate, represented at 0.1 min intervals. The HPN-dependent component action curve was obtained by subtracting the curve values obtained after pharmacological intervention from the corresponding control curve values.

3.6. Glutathione analysis by High-Pressure Liquid Chromatography (HPLC)

The HPLC technique was implemented in collaboration with Prof Jorge Caldeira and Prof Celina Santos, from the Department of Chemistry, Faculty of Sciences and Technology, New University of Lisbon. This technique allows, with great precision, the quantification of glutathione in both the reduced (GSH) and oxidised form (GSSG). This method was adapted from a previously described one (Asensi et al., 1994;

[55] These drugs were used in concentration shown to produce full blockade of the HISS-dependent component of postprandial insulin action (Xie et al, 1995; Sadri et al, 1999). Atropine was given at 3 mg/kg bw and *L*-NMMA was given at 0.73 mg/kg.

Giustarini et al., 2003); which was developed to assess glutathione in the blood. The present technique is based in the quick derivatisation of free thiol (-SH) groups by N-etilmaleimide (NEM). The sample then suffers an acid deproteinisation, and is reacted with 2,4-dinitro-1-fluorobenzene (DNFB). Compounds are separated by chromatography (HPLC) and the glutathione-dinitrobenzene complex is detected by spectrophotometry.

So, after performing a laparotomy in the anesthetised animal, a sample of liver was harvested (0.2-0.3 g), and then stored in liquid nitrogen. In the day of quantification, samples were homogenised separately in NEM 24 $mol.dm^{-3}$ (1 ml) and then left in repose for 1 min. After, trichloroacetic acid (TCA) 50% (m/v) was added, to obtain a final concentration of 20% (m/v) and the samples were placed in 0ºC for 10 min. Then, they were centrifuged at 30000 g and 4ºC, for 20 min, in an Avanti J-25 centrifuge (JA 25-50 rotor, Beckman Coulter Inc., USA). The supernatant was collected (1 ml), washed with dichloromethane (10 ml) to remove NEM traces, and left to repose until the formation of two distinct phases. To the aqueous phase (100 µl) we added Tris(hydroxymethyl)aminomethane hydrochloride (TRIS-HCl) (1 $mol.dm^{-3}$, pH 10, 50 µl) and the dye DNFB (1.5% (v/v), 150 µl). The reactive mix was incubated for 3 hrs, at room temperature and in a dark place. Finally, we added hydrochloric acid (HCl) 37% (10 µl) and eluted the samples through ion exchange HPLC.

The HPLC system used was made up of two solvent injection pumps (LaChrom L-7100, Hitachi, EUA), a spectrophotometric detector (LaChrom L-7400 UV-Detector, Hitachi, EUA) programmed for 355 nm, an interface (LaChrom D-7000 HPLC System, Hitashi, EUA), an injection loop of 20 µl (Rheodyne 7725 Sample Loop, Rheodyne, Germany) and an automatic injector (multi-sampler Marathon-XT v3.1, Pharmacia, Holland). For establishment and analysis of chromatograms we used a LaChrom D-2500/D7500 Data File Conversion Utility software (Hitachi, USA). Additionally, the areas under the curves corresponding to the GSH and GSSG peaks were determined with the software TableCurve 2D v5.01 (Systat Software Inc., USA).

An ionic exchange column (LiChroCART 250-4 HPLC-Cartridge, Purospher Star NH2, 5 µm, Merck, Germany) was used, protected by two pre-columns (LiChroCART 1514960001, Merck, Germany). The mobile phase used in the elution was a mixture of four solvents: A, deionised water; B, acetic acid 3 $mol.dm^{-3}$; C, sodium acetate 3 $mol.dm^{-3}$; and D, methanol (purity > 99.9 %). Solvent D was eluted at a constant concentration. Solvents A, B and C were eluted according to a concentration

gradient (% v/v) throughout the elution cycle, which lasted 45 min[56]. The elution was performed at room temperature, with a constant flow of 1.2 ml/min. The column pressure was maintained between 170-180 bar throughout the whole procedure.

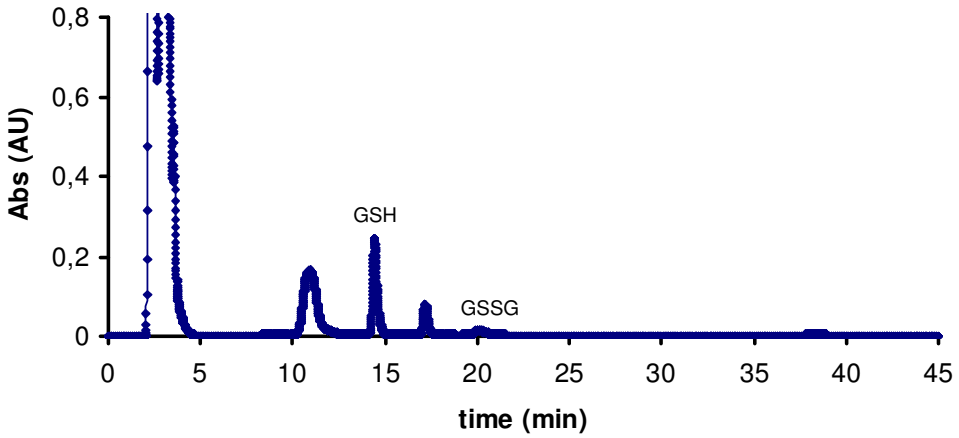


Figure 8 – High-pressure liquid chromatography (HPLC) output example. The areas for the peaks at around 15 and 20 min are calculated to quantify reduced and oxidised glutathione (GSH/GSSG).

The elution of GSH and GSSG happened approximately at 15 min and 23 min, respectively. Peak detection was done by reading absorbency at 355 nm, with the area under the curve being proportional to the glutathione concentration in the sample. To determine the concentration of GSH and GSSG in the sample we performed a daily calibration curve, using standard solutions of GSH (ranging from 1 to 10 mM) and GSSG (ranging from 50 to 300 μM). These standard solutions were prepared in the same conditions as the biological samples.

3.7. Data analysis

Data were expressed as means ± standard error of the mean (SEM) and were analysed, according to the case, by unpaired (ex. fasted/fed) or paired (ex. intact/denervated) Student t-test, one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test or by the two-way ANOVA followed by the

[56] From 0 to 8 min: 14% A, 3 % B, 3% C, 80% D; from 8 to 13 min: linear gradient; from 13 to 44 min: 0% A, 10 % B, 10% C, 80% D; from 44 to 45 min: linear gradient to initial conditions.

Bonferroni post-test. When samples were small (<6 individuals per group), a non-parametric version of these tests was used.

3.8. Drugs and reagents

Atropine, L-NMMA, and D-glucose were purchased from Sigma-Aldrich Chemical. Sucrose was purchased from Panreac Quimica. Sodium pentobarbital (Eutasil) was obtained from Ceva. Heparin was purchased from BBraun Medical. Human insulin (Humulin) was obtained from Lilly. All chemicals were prepared in 0.9% NaCl saline (BBraun). All perfusion were made with the help of Perfusor pumps (BBraun).

TCA, NEM, TRIS-HCl, HCl, methanol, dichloromethane, DNFB, GSH, and GSSG, were purchased from Sigma-Aldrich Chemical Co, Portugal.

4. RESULTS AND DISCUSSION

4.1. Control of postprandial insulin sensitivity by the hepatic parasympathetic nerves in Wistar rats, and integration in the hepatic insulin sensitising substance pathway

4.1.1. Context and work hypothesis

The aim of this study was to assess, in the Wistar rat, if the hepatic parasympathetic nerves (HPN) play a crucial role in the postprandial control of whole-body insulin sensitivity. Furthermore, an intended parallel was drawn by pharmacological intervention, to look at the HPN as a step of the wider pathway that modulates insulin-stimulated glucose uptake.

Previous authors have established the importance of the HPN to the postprandial increment of insulin sensitivity for Sprague-Dawley rats (Latour and Lautt, 2002b; Xie and Lautt, 1996a), cats (Xie and Lautt, 1994; Xie and Lautt, 1995a; Xie and Lautt, 1996b; Xie et al., 1993), and dogs (Moore et al., 2002). Even so, several reasons, stated below, prompted a basic methodological approach to this thesis practical work.

The choice to firstly study the effect of hepatic vagal denervation, and certain pharmacological interventions, on fasted and postprandial insulin sensitivity of Wistar rats was taken considering that:

i) Although the mentioned strain had not been previously studied in this fashion, specific properties of the development of Wistar rats, mentioned later on this dissertation, made it the most appropriate model for a considerable part of this thesis;

ii) No previous published study described the effect of hepatic parasympathetic denervation on the insulin sensitivity of fasted *versus* fed rats, on any strain;

iii) Several authors (Guarino et al., 2003; Sadri and Lautt, 1999; Xie and Lautt, 1994; Xie and Lautt, 1995a; Xie and Lautt, 1996b) reported that certain pharmacological interventions had a similar impact on abolishing the postprandial increment of insulin sensitivity as the physical hepatic parasympathetic nerve ablation. A measure of the decrease of insulin sensitivity produced by denervation was needed here to compare and validate the use on this thesis of the pharmacological agents

described, and allow the conclusion that all interventions affect the same physiological pathway.

At this early stage of research, this permitted to state the role of the HPN on the bigger picture of the hepatic insulin sensitising substance (HISS) pathway hypothesis (see introduction topic 1.9). This fact was pivotal in establishing the working protocols that were to be followed in several animal pathological models throughout the remainder of this doctoral thesis.

The first **working hypothesis** was that HPN denervation would cancel the postprandial increment of insulin sensitivity on Wistar rats. Additionally, in the fasted state, when HISS action is absent, denervation would produce no effect. It was **also hypothesised** that pharmacological interventions reported to interfere with the parasympathetic nervous system or hepatic nitric oxide, known steps of the HISS pathway, would produce a magnitude of postprandial insulin resistance similar to hepatic denervation in Wistar rats.

4.1.2. Experimental protocols

Male 9 weeks old Wistar rats were divided into four groups and fasted for 16 hours. After, one of the groups was subjected to an additional hour of fasting, while the other three groups were given food for 1 hour, to guarantee that they were indeed in the postprandial state. All animals were anesthetised at the end of the fasting or refeeding period.

Each of the four groups was intended for either: i) vagotomy in fasted, ii) vagotomy in fed, iii) parasympathetic pharmacological blockade in fed, or iv) hepatic nitric oxide synthase inhibition in fed animals.

On the fasted group, a thread was surgically placed around the hepatic vagal branch to prepare for the vagotomy. This was also done in one of the fed groups. On the second fed group no additional surgery was needed, since the pharmacological parasympathetic blocker was administered intravenously through the external arterio-venous loop, which was placed in all animals for glucose sampling and continuous anaesthesia perfusion. In the third fed group a catheter was placed in the portal vein to allow for direct administration of drugs to the liver.

In all groups, a rapid insulin sensitivity test (RIST) was firstly performed to assess either postprandial or fasting whole-body insulin sensitivity, depending on the prandial status. Then, the fasted animals and one group of fed animals were subjected to the ablation of the HPN by cauterising the hepatic vagal branch. The second fed group had the administration of the muscarinic antagonist atropine (3 mg/kg). The last fed group had the administration of the nitric oxide synthase inhibitor L-NMMA (0.73 mg/kg). A second RIST was performed in all groups.

4.1.3. Results

4.1.3.1. HISS blockade by surgical manipulation

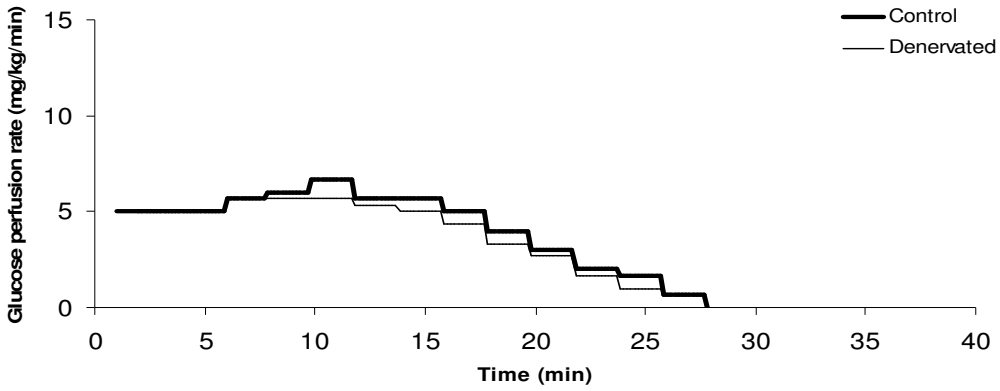
Basal glycaemia, measured as the baseline glycaemia for the RIST, increased with feeding (from 84.3±3.0 after a 17-hour fast, n=3, to 123.8±6.9 mg glucose/dl after refeeding, n=6, $p<0.05$), which is consistent with the recent ingestion and absorption of food. After, it did not change with the ablation of the common hepatic vagus branch, either in fasted or fed animals (fasted: from 84.3±3.0 to 88.0±2.5, fed: from 123.8±6.9 to 145.0±17.9 mg glucose/dl). Both groups showed similar weight (302.0±6.3 for fasted and 313.8±11.8 g for fed animals).

The values of mean arterial pressure were not significantly altered by the hepatic vagotomy, independently of prandial status (fasted: from 116.0±3.1 to 117.3±1.5 mmHg, fed: from 117.5±3.7 to 112.8±3.5 mmHg).

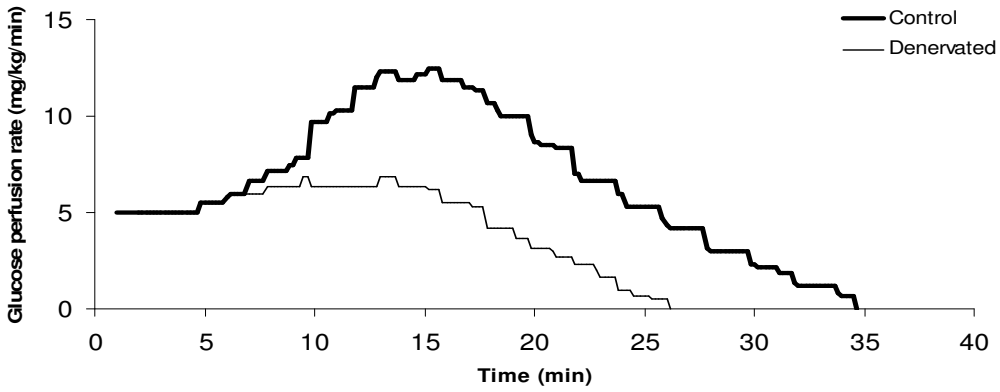
A dynamic curve was obtained at the end of each RIST. Below (Figure 9) are represented the mean dynamic curves calculated for each experimental protocol condition: on fasted or fed animals, before or after hepatic denervation. The mean dynamic curves for the HPN-dependent component are also presented, calculated mathematically by subtracting each post-denervation dynamic curve from the matched control dynamic curve, in fasted and fed animals (Figure 9c).

Additionally, the mean characteristics of the dynamic curves (action peak magnitude, peak time, and action curve duration for control and post-denervation curves; and action peak magnitude, peak time, action curve onset and offset for HPN-dependent component curves) are shown on Table II.

a) Effect of denervation – Fasted state



b) Effect of denervation – Fed state



c) HPN-dependent component in fast and fed

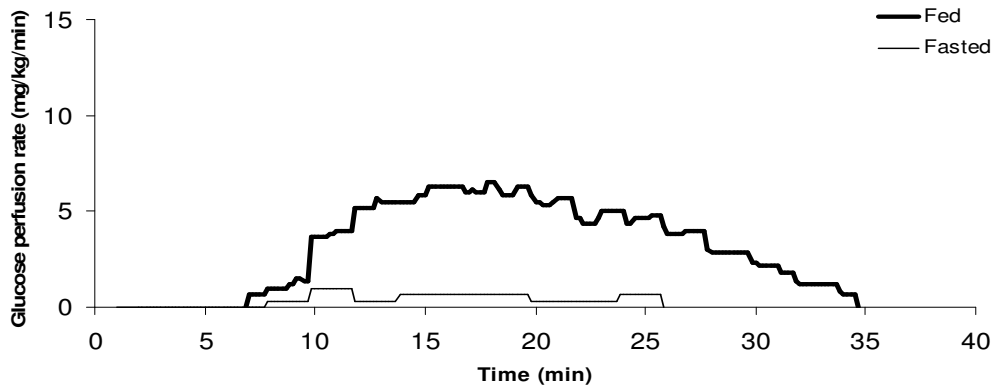


Figure 9 – Effect of denervation on RIST profiles of fasted and fed Wistar rats. Mean dynamic curves for the rapid insulin tests (RIST) performed on control (bold line) and hepatic vagal branch denervation (thin line) conditions in fasted (a) and fed (b) Wistar rats. Mean dynamic profile curve for the HPN-dependent component of insulin action (c), obtained by subtracting the post-denervation curve from the control curve, of fed (bold line) and fasted (thin line) Wistar rats.

In the fasted group, RIST dynamic curves were similar before and after denervation. By contrast, food ingestion led to a greater action curve. This was achieved by an increase of peak magnitude (from 7.0±0.6, fasted control, to 13.2±0.6 $mg.kg^{-1}.min^{-1}$, fed control, $p<0.05$), and duration of the control insulin action curve (from 27.2±0.7 in the fasted, to 33.4±1.2 min in the fed, $p<0.05$).

Further, in fed animals, denervation produced an impairment of the insulin action curve. The surgical ablation of the HPN brought down maximum peak magnitude (from 13.2±1.0, fed control, to 7.2±0.5 $mg.kg^{-1}.min^{-1}$, fed denervated, $p<0.05$) and diminished the duration of insulin action (from 33.4±1.2, fed control, to 23.1±1.4 min, fed denervated, $p<0.01$); reverting both to the same levels seen on the fasted state (Figure 9 and Table II).

Table II – Effect of denervation on dynamic curve main properties of fed and fasted Wistar rats. Dynamic profile characteristics of the control RIST, post-denervation RIST and HPN-component of total insulin action of fasted and fed Wistar rats (* $p<0.05$ vs fasted value, § $p<0.01$ vs fed control duration, # $p<0.05$ vs fed control peak).

		Fasted (n=3)	Fed (n=6)
Control RIST	Peak ($mg.kg^{-1}.min^{-1}$)	7.0±0.6	13.2±1.0*
	Peak time (min)	10.5±1.8	13.2±0.6
	Duration (min)	27.2±0.7	33.4±1.2*
Denervated RIST	Peak ($mg.kg^{-1}.min^{-1}$)	6.3±0.3	7.2±0.5#
	Peak Time (min)	9.2±2.4	8.7±1.0
	Duration (min)	25.8±1.2	23.1±1.4§
HPN-dependent component	Peak ($mg.kg^{-1}.min^{-1}$)	1.7±0.3	7.7±0.5*
	Peak time (min)	17.2±4.1	15.6±1.0
	Onset (min)	8.6±0.3	8.0±0.9
	Offset (min)	20.5±4.7	33.0±1.4*
	Duration (min)	11.9±5.0	21.8±1.2§

The aforementioned results can be further analysed by calculating the area under each dynamic curve. That parameter is called RIST index, and the RIST indexes

obtained from the control and post-denervation dynamic curves, in fasted and fed animals, are presented below, on Figure 10.

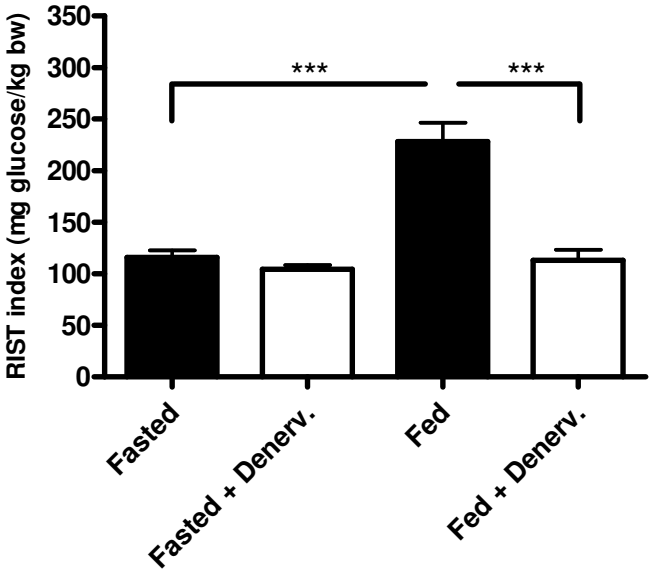


Figure 10 – Effect of denervation on RIST indexes of fasted and fed Wistar rats. Rapid insulin sensitivity test (RIST) indexes for total insulin action in 9 weeks old male Wistar rats in the fasted and fed states, before (filled bars) and after hepatic parasympathetic denervation (open bars). The increment in insulin sensitivity seen with feeding (*** $p<0.001$ vs fasted control Wistar) was eliminated after denervation (*** $p<0.001$ vs fed control Wistar).

In the fasted state, ablation of the HPN did not produce any effect on whole-body insulin sensitivity (from 110.1±5.8 to 102.1±4.1 mg glucose/kg body weight (bw)) (Figure 1) in 9 weeks old male Wistar rats. On the contrary, the increment in insulin sensitivity observed in the immediate postprandial state, i.e., roughly two hours after food ingestion, was significantly impaired by the surgical ablation of the HPN (from 228.1±18.5 to 113.1±10.1 mg glucose/kg bw, $p<0.001$) (Figure 10), lowering insulin sensitivity to the same level seen in the fasted state.

The HPN-dependent component of insulin action (quantified by subtracting the post-vagotomy RIST index from the pre-vagotomy one) had thus an almost neglectable expression when insulin sensitivity was quantified in the fasted state (11.8±5.1 mg glucose/kg bw; corresponding to 9.9±4.0 % of total insulin action). On the other hand,

after food ingestion, it rose almost ten-fold (increasing to 114.9±11.7 mg glucose/kg bw, $p<0.0001$), accounting then for half of total insulin action (50.3±2.2 % of total insulin action, $p<0.001$) and for the entire postprandial increment of insulin sensitivity (Figure 10). The values obtained after hepatic vagotomy, in the fasted and postprandial states, are similar; and alike what was obtained in the fasted animals prior to denervation (Figure 10).

The dynamic response of the HPN-dependent component to feeding could also be uncovered (Figure 1). While food ingestion did not produce changes in the onset of this component, it induced an increase in peak action (from 1.7±0.3 to 7.7±0.5 $mg.kg^{-1}.min^{-1}$, $p<0.05$) and a latter offset (until 20.5±4.7 min in the fasted state and until 33.0±1.4 min in the fed state, $p<0.05$). This resulted in the increase of duration of the HPN-dependent component with feeding (from 11.9±5.0 to 21.8±1.2 min, $p<0.05$) (Table II).

4.1.3.2. HISS blockade by pharmacological manipulation

In regard to the administration of pharmacological substances to mimic the effect of hepatic denervation, the administration of iv atropine or ipv L-NMMA did not change basal glycaemia levels (from 115.7±4.9 to 122.7±4.6 mg glucose/dl after atropine, n=5, and from 134.2±5.8 to 137.0±2.9 mg glucose/dl after L-NMMA, n=5).

The values of mean arterial pressure were not significantly altered by atropine administration (from 120.0±5.8 to 121.7±7.3 mmHg with atropine). In the case of L-NMMA, the transient increase of mean arterial pressure seen during the administration of the drug came back to basal levels, by itself, by the time glucose measurements were restarted to determine the glycaemia baseline for the second RIST (from 120.0±7.0 to 134.4±8.9 during L-NMMA administration, $p<0.01$, back to 118.0±7.2 mmHg by the end of the recovery time, approximately 30 min, $p<0.01$). Both groups showed similar weight as the ones used for the surgical denervation protocol.

Both atropine and L-NMMA had the same effect on the control fed RIST profile (dynamic curves obtained with the same protocol illustrated on following items 4.2.3., 4.4.2 and 4.5.1.; not shown here). Below is represented the mean RIST indexes obtained with both drugs, on fed Wistar rats (Figure 11).

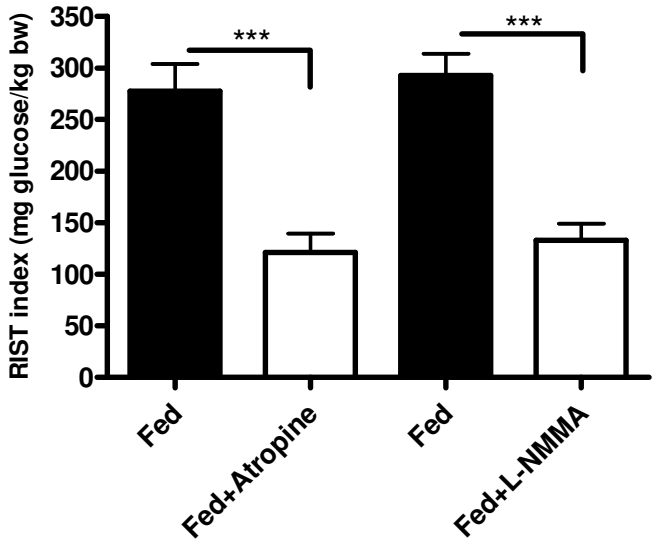


Figure 11 – Effect of pharmacological intervention on fed Wistar rats. Rapid insulin sensitivity test (RIST) indexes for total insulin action in 9 weeks old male Wistar rats in the fed state, before (filled bars) and after (open bars) administration of atropine or L-NMMA (*** $p<0.001$ for both vs fed control Wistar).

As seen with hepatic denervation, parasympathetic blockade by iv administration of atropine or inhibition of hepatic nitric oxide by ipv administration of L-NMMA leads to elimination of the postprandial increment of insulin sensitivity (from 277.5±26.3 to 121.1±18.2 mg glucose/kg bw with atropine, and from 292.6±21.2 to 132.7±16.3 mg glucose/kg bw with L-NMMA, $p<0.001$ for both) (Figure 11); lowering insulin sensitivity to values similar to the control fasted or denervated fed animals (Figure 10).

Considering that the affected pathway is indeed the same, this HISS-dependent component of insulin sensitivity had exactly the same contribution to total insulin sensitivity as seen in the assessment of the HPN-dependent component in the previous protocol. With administration of atropine, the HISS-dependent component found was 156.4±29.5 mg glucose/kg bw, accounting for 55.5±6.8 % of total postprandial insulin action. After L-NMMA, the HISS-dependent component was quantified at 159.8±32.9 mg glucose/kg bw, accounting for 52.7±7.8 % of total postprandial insulin action.

4.1.4. Discussion

At 9 weeks of age, the Wistar rat strain showed a clear increment in insulin sensitivity from the fasted to the postprandial state. Such increment was completely eliminated by the ablation of the HPN at the common hepatic branch level; showing the critical relevance of these nerves for an optimal postprandial insulin action. The same surgical intervention on fasted animals produced no effect on whole-body insulin sensitivity.

The previous statements are clearly shown by the RIST dynamic profiles obtained in fasted and fed animals, before and after denervation. Additionally, the lack of involvement of the HPN on fasting insulin action of rat models is reported here for the first time.

Furthermore, the dynamic curves for the HPN-dependent component of total insulin action were also calculated. The results obtained underline once again that while during fasting the HPN-dependent component has an almost nonexistent, baseline, action, the ingestion of food leads to an activation of the HPN-dependent component.

This supports the concept that the activation of the parasympathetic nervous system, occurring at the beginning and during food ingestion (Teff, 2008; Uijtdehaage et al., 1992), has a role on whole-body glucose metabolism (Latour and Lautt, 2002a; Lautt et al., 2001; Xie and Lautt, 1994; Xie and Lautt, 1996b). More precisely, this HPN-dependent component seems to be wholly responsible for the increment observed in whole-body insulin sensitivity from the fasted to the immediate postprandial state.

It is well known that, in response to a meal, circulating levels of insulin are increased in relation to fasting. However, the same level of hyperinsulinaemia, when produced experimentally in the fasted state, does not seem by itself to be able to induce the increase in insulin sensitivity seen in the postprandial state (Peitl et al., 2009; Xie and Lautt, 1994). So, in accordance with the observations performed in the present work, the postprandial increase in whole-body insulin sensitivity is seen when both signals, HPN activation and insulin, are present. Furthermore, as insulin has been shown to have a dose-dependent influence on the HPN-dependent component of insulin action

(Lautt et al., 2001), this seems to argue for the existence of a modulator effect of both signals on postprandial whole-body sensitivity, expressed by a common pathway.

The parasympathetic nervous system is also known to be involved in the modulation of insulin release from the pancreas. On one hand, it prevents the unwanted release of insulin during fasting (Blat and Malbert, 2001), on the other, it promotes insulin release upon feeding (Benthem et al., 2000; Benthem et al., 2001; D'Alessio et al., 2001)[57]. However, in the present study, by the time the control RIST is performed, glycaemia, and also insulinaemia, are stabilised (Lautt et al., 1998; Xie and Lautt, 1996b); and their levels remain unchanged by hepatic parasympathetic denervation (Lautt et al., 1998; Xie and Lautt, 1996b). Thus, a further alteration in HPN-dependent component from this dynamic steady-state, that would enable us to monitor this component, has to come from an endogenous pulse of insulin brought about by an increase in plasma glucose (Frangioudakis et al., 2008), or, as in the case of the methodology used here, an exogenous administration of a bolus of insulin. That the meal-induced insulin sensitivity increment is abolished after HPN denervation, and that, as reported elsewhere, this can be reverted by continuous intraportal, but not intravenous, administration of acetylcholine (Xie and Lautt, 1996a), further recognises the central role of the liver in processing diverse information and effecting a control on peripheral metabolism.

Morphologically, it has been established that the facilitative parasympathetic vagal signal, shown to be involved in postprandial insulin sensitivity potentiation, follows through the cervical vagus, the hepatic branch, and through the anterior hepatic plexus along the hepatic common artery (Latour and Lautt, 2002a). Ablation of the hepatic common artery branch of the vagus (CHB) has been reported to be enough to promote the total removal of the postprandial increment of insulin sensitivity (Latour and Lautt, 2002a), as seen here. However, the physical interruption of the CHB of the vagus nerve may have other consequences besides to abrogate the effect of the HPN.

Previously considered to feed only into the liver, the CHB has been recently shown to innervate mainly the gastrointestinal tract, and only to a minor extent the

[57] It has been proposed that the promotion of pancreatic insulin release is due to a periprandial parasympathetic suppression of the sympathetic nerve activity to the pancreas. Furthermore, this mechanism has been shown to be non-dependent of muscarinic receptors; so, was not inhibited by atropine (Benthem et al, 2001).

hepatoportal region (Berthoud, 2004). Furthermore, it might contain fibres of nonvagal origin (Prechtl and Powley, 1990). However, against this concern, Xie and colleagues (Xie et al., 1993) have eliminated sympathetic fibres from the hepatic anterior plexus with no further aggravation in insulin sensitivity. Also, since iv atropine, ipv L-NMMA, and selective CHB ablation produced the same effect on insulin sensitivity, it seems unlikely that any element other than the HPN, or any other nerve bundle innervating the liver but that do not pertain to the CHB (Berthoud, 2004), contributes to the postprandial control of glucose metabolism.

The results obtained by muscarinic blockage with atropine, or nitric oxide synthase inhibition with L-NMMA, similar to the surgical ablation of the HPN, also help to integrate the HPN-dependent component on a larger physiological mechanism of postprandial glucose disposal management.

Although the main site of insulin-stimulated glucose uptake in the body is the skeletal muscle, the present study strengthens the notion that there exists a postprandial increment of insulin sensitivity at the periphery which is determined by the action of an hepatic factor (Lang, 1954; Lautt, 1999; Mertz and Schwarzk, 1962; Petersen and Tygstrup, 1994). This data supports the hypothesis presented by the HISS pathway (Lautt, 1999), of a parasympathetic nerve signal delivered to the liver that eventually leads to the increase of whole-body, specifically peripheral, insulin sensitivity in the postprandial state.

Indeed, dose-response curve studies in rats (Takayama et al., 2000) showed that the ED50 of atropine administered intraportally (5.3 ng/kg bw) was lower than that administered intravenously (230 ng/kg bw). Similarly, the nitric oxide synthase inhibitor L-NMMA has been shown, at the given dose of 0.73 mg/kg, to produce a similar decrease of insulin sensitivity to atropine, when administered intraportally, but not intravenously (Sadri and Lautt, 1999).

Thus, the present results support the hypothesis that the referred parasympathetic reflex can be blocked with the same efficacy either by surgical ablation of the HPN or pharmacological intervention.

The efficacy of the pharmacological agents described had also a relevant impact on the experimental methodology followed.

The surgical procedures used, although performed with due caution, were susceptible to hazard. First of all, the access and manipulation of the liver and isolation of the fibre bundle was very sensitive, and led sometimes to tissue damage, haemorrhage, or other event that influenced negatively on glucose metabolic control. Likewise, the catheterisation of the portal vein was prone to surgical problems. Each experiment that showed this was eliminated from final data analysis. However, these complications had sufficient relevance to warrant alternatives.

Considering the present pharmacological results, it became possible to avoid some of the surgical manipulations in subsequent protocols. For the development of the present work, hepatic denervation could thus be forgone in favour of a pharmacological approach to the separation of the HPN-dependent and –independent components of insulin action.

The previous resulted in the choice of the iv administration of atropine as the preferred protocol for assessment of the HPN, or HISS, -dependent component. The iv atropine dose chosen allowed hence to minimise the invasive surgery required; this without showing a statistically different effect on insulin sensitivity from the intraportal administration (Lautt et al., 1998; Takayama et al., 2000), and without exhibiting any undesirable secondary effect.

However, atropine has other described effects that could indirectly induce changes in glucose metabolism. This regards specifically the possible effect of atropine on gastric emptying; delaying the absorption of carbohydrates to the portal circulation (Teff et al., 1999). Nevertheless, in the present study, atropine is given at a late stage of absorption and does not change blood glycaemia, hinting against a role of the parasympathetic fibres innervating the digestive tract on the management of insulin-stimulated glucose disposal.

Direct effects of atropine on hepatic glucose metabolism have also been reported, specifically as hepatic glucose flux alterations (Chap et al., 1985; Tordoff and Friedman, 1994). The fact that atropine was perfused systemically prior to gavage in the mentioned reports (Chap et al., 1985; Tordoff and Friedman, 1994) hints that the altered effect may be due to other parasympathetic responses of feeding[58]. More importantly, arterial-venous gradient studies performed in the postprandial state have shown that

[58] A reported decrease in insulin secretion (Chap et al, 1985) seems a prime candidate.

denervation and/or atropine led to changes in skeletal muscle, but not on liver glucose uptake (Moore et al., 2002; Xie and Lautt, 1996b).

Furthermore, studies that report alterations in hepatic glucose flux by hepatic vagal denervation seem to be related with chronic denervation (Matsuhisa et al., 2000; Xue et al., 2000), in which the liver may be showing an adaptative response. The use of acute hepatic vagal denervation should prevent those confounding factors in the present study.

Still regarding possible alternate interpretations for the presented data, other authors (Baron et al., 1995) have proposed a link between nitric oxide inhibition and a decrease in insulin sensitivity by way of a decrease of blood flow and nutrient availability to the skeletal muscle cells[59].

Besides the fact that the present studies have registered no alteration in blood pressure at the time of the RIST performed after L-NMMA, a case could be presented based on the hypothesis of changes in the microvasculature feeding the myocytes (Baron et al., 2000; Vincent et al., 2006). However, the fact that we obtained the same decrease in whole-body insulin sensitivity with atropine and hepatic vagotomy as with hepatic administration L-NMMA suggests that haemodynamic changes were not responsible for altering glucose uptake.

Furthermore, even if L-NMMA changed blood pressure, alterations in blood flow have been shown not to alter peripheral glucose uptake (Ronnemaa et al., 1999; Scherrer et al., 1994; Utriainen et al., 1996). In spite of that, a recent study (Levesque et al., 2006) reported the effect of atropine on decreasing skeletal muscle insulin-stimulated glucose uptake and related it to a decrease in insulin-stimulated blood flow[60] (Scherrer et al., 1994; Steinberg et al., 1994; Taddei et al., 2001). This seems to be clearly refuted by the present results; and by the fact that either a nitric oxide synthase inhibitor, or a parasympathetic muscarinic antagonist, is more effective in reducing

[59] This hypothesis states that availability of insulin and glucose from the bloodstream to skeletal muscle cells would be the limiting rate step in insulin-stimulated glucose uptake (Baron et al, 1995).

[60] This proposed mechanism sprung from the notion that, in the endothelium, insulin leads to vasodilation, as well as potentiating the vasodilator effect of acetylcholine (Steinberg et al, 1994; Taddei et al, 2000). This mechanism leads to the release of vascular nitric oxide (Steinberg et al, 1994; Scherrer et al, 1994). Through this chain of events, insulin would control blood flow and capillary recruitment (Baron et al, 1995). Atropine administration would lead to decreased blood flow, poorer capillary recruitment, and lower nutrients availability at the peripheral tissues; thus limiting whole-body glucose uptake.

insulin sensitivity through ipv than iv administration (Sadri and Lautt, 1999; Takayama et al., 2000).

The vagus nerve has a multitude of target organs on the abdominal region, possibly with a compartmentalisation of the parasympathetic nervous system that allows different organs to be simultaneously modulated in different forms (Kreier et al., 2003; Teff, 2008). Even so, all the present results taken together suggest that indeed the liver is the central organ in which a facilitative parasympathetic tone allows the activation of the HISS pathway, which in term leads to increased peripheral insulin-stimulated glucose uptake.

The central role of the liver may also be glimpsed from hepatic transplant patients. These patients show decreased glucose tolerance, but similar hepatic glucose flux and hepatic glycogen content (Schneiter et al., 2000b). It is concluded that the lack of hepatic innervation leads to a decrease in glucose uptake in extrahepatic tissues[61] (Dhillon et al., 1992; Luzi et al., 1997; Sakamoto et al., 2002; Takahashi et al., 2001), expectedly the skeletal muscle (Xie and Lautt, 1996b), as has been proposed for long (Lang, 1954). Further, patients with liver disease show similarly peripheral impairment in insulin sensitivity (Lautt, 1999).

According to our hypothesis, this will be due to an impairment in HISS synthesis/release; which is consistent with the emerging recognition of the liver as having a central role in the physiology and pathophysiology of whole-body glucose homeostasis (Langhans, 2003).

In conclusion, any of the used protocols showed to be equally adequate to quantify the HPN-dependent, or, more broadly, the HISS-dependent component of insulin action. As shown, this component is the sole responsible for the increment of insulin sensitivity seen with the transition from the fasted to the fed state. The facilitative, and critical, contribution of the HPN was thus established, as well as the central role of the liver to the management of whole-body postprandial insulin sensitivity.

[61] Signs of reinnervation could be detected within a 5-years timeframe from transplant (Dhillon et al, 1992). This may be the explanation for the report of glucose metabolism amelioration 26 months after transplant (Luzi et al, 1997). Possibly, this happens faster in rats (Takahashi et al, 2001; Sakamoto et al, 2002).

This validation of the pharmacological tools available, either for parasympathetic blockade or hepatic nitric oxide inhibition, allows then to avoid the more invasive common hepatic branch vagotomy in the studies that follow.

4.2 Effect of age on postprandial insulin sensitisation

4.2.1. General context and hypothesis

Before analysing the relation between insulin resistance and some of the pathologies related to type 2 diabetes, a question was raised about the physiological changes in glucose metabolism; more precisely, those influenced by age.

It has been described that glucose intolerance develops with age mainly due to decreased skeletal muscle insulin sensitivity and responsiveness; in humans (Davidson, 1979) and in several rat strains (Goodman et al., 1983; Nakai et al., 1996; Narimiya et al., 1984; Nishimura et al., 1988; Reed et al., 1993). In time, this leads to a higher prevalence of type 2 diabetes in older individuals (Stout, 1994).

Also, the occurrence of autonomic neuropathy has been widely related to the presence of insulin resistance and type 2 diabetes (Nakano et al., 2003). But autonomic neuropathy has been usually described as a complication of type 2 diabetes (Sundkvist, 1981), arguing for a secondary role of neuropathy in the development of type 2 diabetes. However, the observation of mainly parasympathetic autonomic impairment in prediabetic subjects (Kuroda et al., 1990; Liao et al., 1995) hints to a possible role of an impairment of the HPN-dependent component of insulin action in the aetiology of diabetes. Another observation that supports this is the observation that parasympathetic neuropathy seems to precede both sympathetic and peripheral neuropathies (Nakano et al., 2003; Oikawa et al., 1986; Sundkvist, 1981).

Considering this, our **general working hypothesis** was that the HPN-dependent component of insulin action decreases with age, contributing to the reported decrease in peripheral insulin sensitivity by compromising the postprandial increment in insulin sensitisation.

4.2.2. Total insulin sensitivity in fasted versus fed male Wistar rats at 9 and 52 weeks of age

4.2.2.1 Experimental protocols

The next step in this work was then to evaluate if there is an impairment of the meal-induced increment of insulin sensitivity with age.

For this, and related studies, the model chosen was the Wistar rat strain, since it has been considered the best suited for aging-associated studies, having less confounding factors (Escriva et al., 1997) and with alterations in body composition similar to humans (Leighton et al., 1989). Also, impaired glucose tolerance with age is seen well before hyperglycaemia or ß-cell failure; complications which seem to happen generally after 12 months of age (Koricanac et al., 2004).

Using young 9 weeks old male Wistar rats as controls, we compared them with a group of 52 weeks old male animals of the same strain. Animals from both age groups were evaluated after a 16-hour fast, as before either at the fasted or fed prandial status. A single RIST was performed, to quantify either fasted or postprandial whole-body insulin sensitivity.

4.2.2.2. Results

Basal glycaemia rose with feeding, at both ages (from 84.6±2.3 in fasted, n=8, to 128.3±5.8 mg/dl in fed, n=6, at 9 weeks of age, $p<0.01$; from 89.6±6.0 in fasted, n=7, to 112.2±3.2 mg/dl in fed, n=9, at 52 weeks of age, $p<0.01$). Arterial blood pressure was not altered by feeding in either age group, but decreased from 9 to 52 weeks of age (116.7±1.5 in fasted and 117.5±3.7 in fed, at 9 weeks of age; 89.3±4.2 in fasted and 84.4±4.1 in fed, at 52 weeks of age; $p<0.001$, between age groups at each prandial state). Weight increased consistently with age (302.2±4.0 for fasted and 313.4±9.0 g for fed, at 9 weeks of age; 553.9±13.2 for fasted and 591.2±20.2 g for fed, at 52 weeks of age; $p<0.001$ between age groups at each prandial state).

For this topic, results concerning insulin sensitivity will be shown only as RIST indexes. Dynamic curves of similar groups can be found on the previous and next

topics; to the exception of 52 weeks old fasted animals. The latter however showed no changes in dynamic action when compared with the young fasted animals.

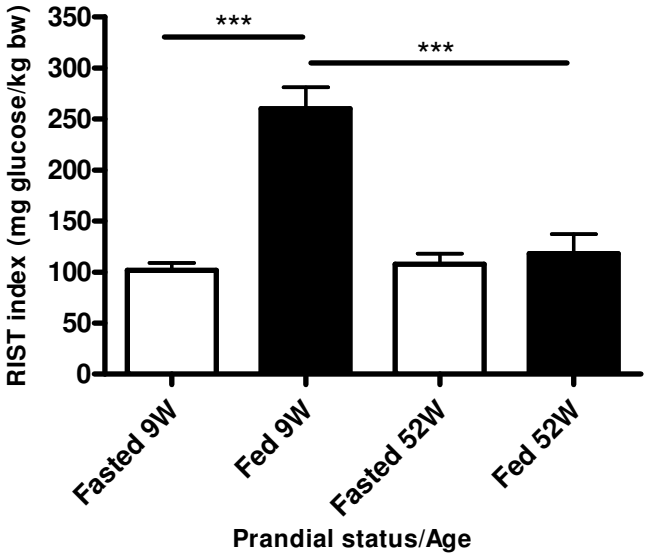


Figure 12 – Effect of age on RIST indexes of fasted and fed Wistar rats. Rapid insulin sensitivity test (RIST) indexes for total insulin action in male Wistar rats at 9 and 52 weeks of age, in the fasted (open bars) and fed (filled bars) state. The meal-induced sensitisation of insulin sensitivity, seen at 9 weeks, was lost by 52 weeks of age (*** $p<0.001$).

As before, at 9 weeks of age, Wistar rats showed a higher insulin sensitisation after a meal than in the fasting state (fasted: 101.6±7.3 vs fed: 260.0±21.1 mg glucose/kg bw, $p<0.001$). By contrast, at 52 weeks of age, this meal effect has been lost ($p<0.001$). This is illustrated by the fact that, at this age, fasting and postprandial total insulin action were similar between them (fasted: 107.8±10.3 vs postprandial: 118.0±19.1 mg glucose/kg bw), and also similar to the fasting response at 9 weeks of age (Figure 12).

4.2.2.3. Discussion

The course of development until 52 weeks of age produces, in male Wistar rats, a radical decrease in postprandial insulin sensitivity. This phenomenon seems to be due to a total loss of the increment of insulin sensitivity seen after a meal, as 52 weeks old

fed animals showed similar insulin sensitivity to the age-matched fasted ones. On the other hand, insulin action in the fasted state showed no alteration with age.

Since previous authors reported that the decrease of insulin sensitivity which develops with age happened during maturation, with a slight or no increase afterwards (Davidson, 1979; Goodman et al., 1983; Nakai et al., 1996; Narimiya et al., 1984; Nishimura et al., 1988; Reed et al., 1993), the timetable of this study was expanded to evaluate insulin sensitivity throughout maturation in more detail.

4.2.3. HPN-dependent and HPN-independent components of insulin sensitivity in fed male Wistar rats at 6, 9, 16 and 52 weeks of age

4.2.3.1. Experimental protocols

Male Wistar rats were evaluated at 6, 9, 16 and 52 weeks of age. Maturation was thus divided into two phases: from 6 to 9 weeks, corresponding to puberty, and from 9 to 16 weeks, roughly corresponding to a young adult. Although the developmental stages found in the literature vary under broad intervals, it is consensual that Wistar rats have reached their sexual maturation by this stage. The 52 weeks old group, at a midpoint in the Wistar normal 24 months life expectancy, corresponds roughly to middle-age.

On this protocol, all age groups were evaluated only on the postprandial state; but with whole-body postprandial insulin sensitivity being assessed and discerned into the HPN-dependent and –independent components. This enabled the evaluation of the possible contribution of an impairment of the HPN-dependent component to the observed loss of the increment of postprandial insulin sensitisation with age.

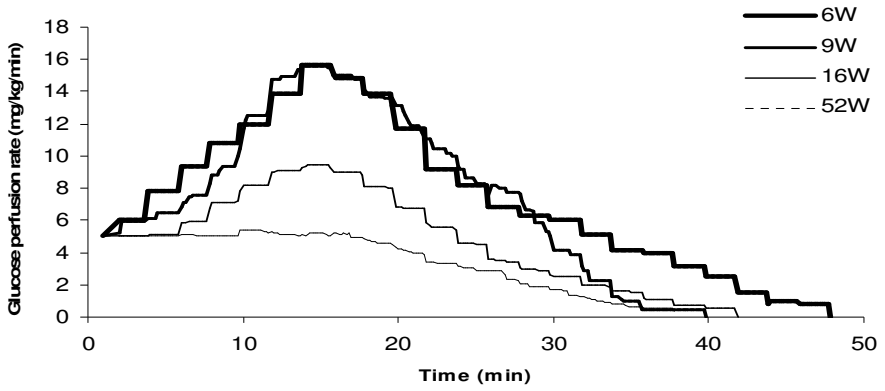
Animals were subjected to a 16-hour fasting period, followed by 1 hour free access to food. After anaesthesia, a control RIST was performed. The control RIST was followed by the administration of the muscarinic cholinergic antagonist, atropine (3 mg/kg), to block the HPN-dependent component of insulin action. After, a second, post-atropine, RIST was performed.

4.2.3.2. Results

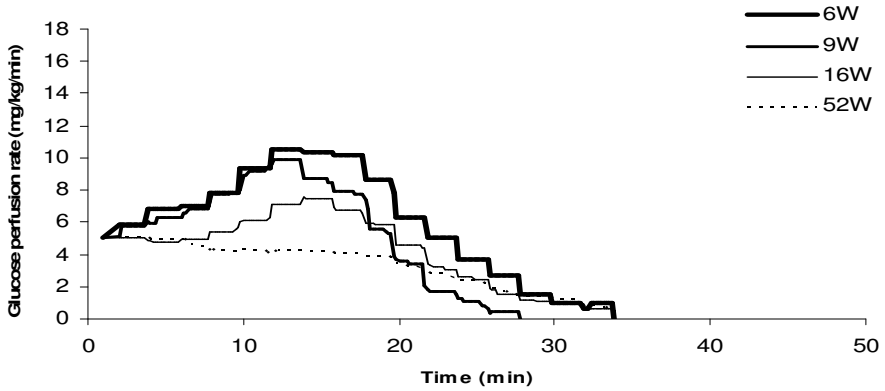
Basal postprandial glycaemia, measured as the baseline for the control RIST, was not altered by age (137.3±12.2 at 6 weeks, n=6, 125.1±7.7 at 9 weeks, n=11, 132.5±3.3 at 16 weeks, n=6, and 113.7±5.2 mg/dl at 52 weeks of age, n=13). Administration of atropine also did not alter the baseline glycaemia (129.8±13.1 at 6 weeks, 131.8±8.3 at 9 weeks, 120.3±4.3 at 16 weeks, and 108.7±2.5 mg/dl at 52 weeks of age). Arterial blood pressure was shown again to be decreased at 52 weeks of age, now in relation to all other age groups (from 113.0±1.8 at 6 weeks, to 120.5±3.3 at 9 weeks, 117.0±4.9 at 16 weeks, and 93.5±3.8 mmHg at 52 weeks of age, $p<0.001$ to 9 weeks, and $p<0.01$ to 6 and 16 weeks of age). Atropine did not significantly change these values in any age group. Body weight increased from 234.0±8.9 g at 6 weeks, to 314.5±10.8 g at 9 weeks, and to 566.0±12.9 g at 16 weeks of age. It further increased to 826.5±25.4 g at 52 weeks of age.

The analysis of the mean dynamic curves (Figure 13) provides us with a plethora of detailed information into the way insulin sensitivity, and particularly each of its components, changes with time. Also, the mean characteristics for the dynamic curves, obtained during the performed RIST and by quantification of the HPN-dependent component, are shown on Table III.

a) Total postprandial insulin sensitivity



b) Insulin sensitivity after HPN blockade: HPN-independent component of insulin action



c) HPN-dependent component of insulin action

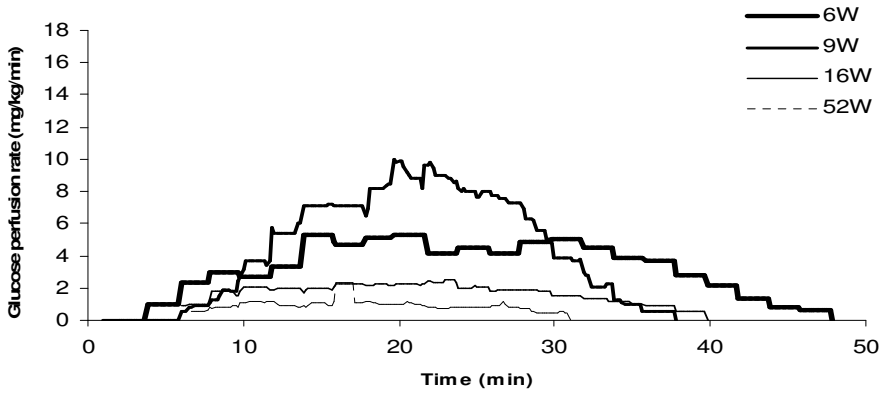


Figure 13 – Effect of age on RIST profiles of fed male Wistar rats. Mean dynamic profiles for the rapid insulin sensitivity tests (RIST) performed on control (a) and postatropine (b) conditions at 6, 9, 16 and 52 weeks of age male Wistar rats. Mean dynamic profile curve for the hepatic parasympathetic nerve (HPN)-dependent component of insulin action (c), obtained by subtracting the post-atropine curve from the control curve, at all age groups.

Age correlates with a gradual decrease of total postprandial insulin sensitivity. Looking at the respective mean dynamic curve graph for all age groups (Figure 13a), first it is observed a decrease on the duration of glucose uptake (from 44.8±1.8 at 6 weeks to 33.1±1.6 min at 9 weeks of age, $p< 0.05$), which afterwards remains stable (30.6±2.4 at 16 weeks and 31.5±2.3 min at 52 weeks of age, $p<0.01$ for both vs 6 weeks of age) (Table III). Following the shortening of total insulin action, from 9 weeks of age onwards there is a gradual decrease of action peak magnitude: unchanged from 6 to 9 weeks of age, with, respectively, 16.5±1.2 and 16.1±0.7 mg glucose.kg^{-1}.min^{-1}, then decreasing to 10.7±0.7 mg glucose.kg^{-1}.min^{-1} at 16 weeks, $p<0.001$ versus 9 weeks, and then decreasing again to 6.3±0.5 mg glucose.kg^{-1}.min^{-1}, $p<0.001$ versus 16 weeks.

For the action of insulin *per se*, assessed by the post-atropine RIST (Figure 4b), there is a decrease of peak magnitude only at 52 weeks ($p<0.001$ vs 6 and 9 weeks, $p<0.05$ vs 16 weeks of age). All other RIST characteristics are unchanged with age (Table III).

A more interesting behaviour can be glimpsed through the HPN-dependent component dynamic curves. At 6 weeks of age there is still a low maximum action, i.e. peak magnitude, which is compensated by a longer duration of action. With puberty, from 6 to 9 weeks of age, we see an increase in peak magnitude (from 6.8±0.8 to 10.8±1.0 mg glucose.kg^{-1}.min^{-1}, $p<0.05$) and a decrease in duration (from 39.0±2.4 to 25.4±1.7 min, $p<0.05$). Since the onset of the HPN-dependent component is similar between all age groups, this decrease in duration is due to a sooner offset in action (from 44.5±1.8 at 6 weeks to 32.0±1.0 min at 9 weeks of age, $p<0.05$). With further age, the duration of the HPN-dependent component remains the same, while peak magnitude decreases (to 3.6±0.8 at 16 weeks and 4.2±0.9 at 52 weeks of age, $p<0.001$ vs 9 weeks).

Table III – Effect of age on dynamic curve main properties of fed male Wistar rats. Dynamic profile characteristics of the control rapid insulin sensitivity test (RIST), post-atropine RIST and hepatic parasympathetic nerve (HPN)-component of total insulin action of 6, 9, 16 weeks old fed Wistar rats (* $p<0.001$ vs 9 weeks, # $p<0.001$ vs 16 weeks, ¥ $p<0.05$ vs 6 weeks, $ $p<0.01$ vs 6 weeks, § $p<0.001$ vs 6 weeks, £ $p<0.05$ vs 16 weeks).

		6 weeks old (n=6)	9 weeks old (n=11)	16 weeks old (n=6)	52 weeks old (n=13)
Control RIST	Peak	16.5±1.2	16.1±0.7	10.7±0.7*	6.3±0.5$^{\#}$
	Peak time	14.2±0.8	13.3±0.7	13.9±0.7	11.9±1.1
	Duration	44.8±1.8	33.1±1.6$^{¥}$	30.6±2.4$^{\$}$	31.5±2.3$^{\$}$
Postatropine RIST	Peak	10.8±0.8	9.8±1.1	8.6±0.5	5.7±0.4$^{£}$
	Peak Time	12.8±0.7	10.3±1.0	12.8±0.7	12.4±0.9
	Duration	31.2±2.6	23.4±1.7	29.0±2.2	30.2±2.0
HPN-dependent component	Peak	6.8±0.8	10.8±1.0$^{¥}$	3.6±0.8*	4.2±0.9
	Peak time	14.2±1.9	16.0±1.0	13.8±2.0	15.7±2.1
	Onset	5.5±1.3	6.7±1.3	8.2±1.2	10.5±1.7
	Offset	44.5±1.8	32.0±1.0$^{¥}$	25.6±3.4§	31.2±2.8$^{¥}$
	Duration	39.0±2.4	25.4±1.7$^{¥}$	17.3±3.7§	20.0±2.3§

The area under each dynamic curve provides us the RIST index for each component and age. Total insulin sensitivity, quantified by the control RIST index, decreased progressively with age (from 351.8±27.3 at 6 weeks to 297.4±18.5 at 9 weeks, 221.7±24.3 at 16 weeks, and 135.6±12.8 mg glucose/kg bw at 52 weeks of age; $p<0.01$ between 6 and 16 weeks of age, $p<0.05$ between 16 and 52 weeks of age) (Figure 14).

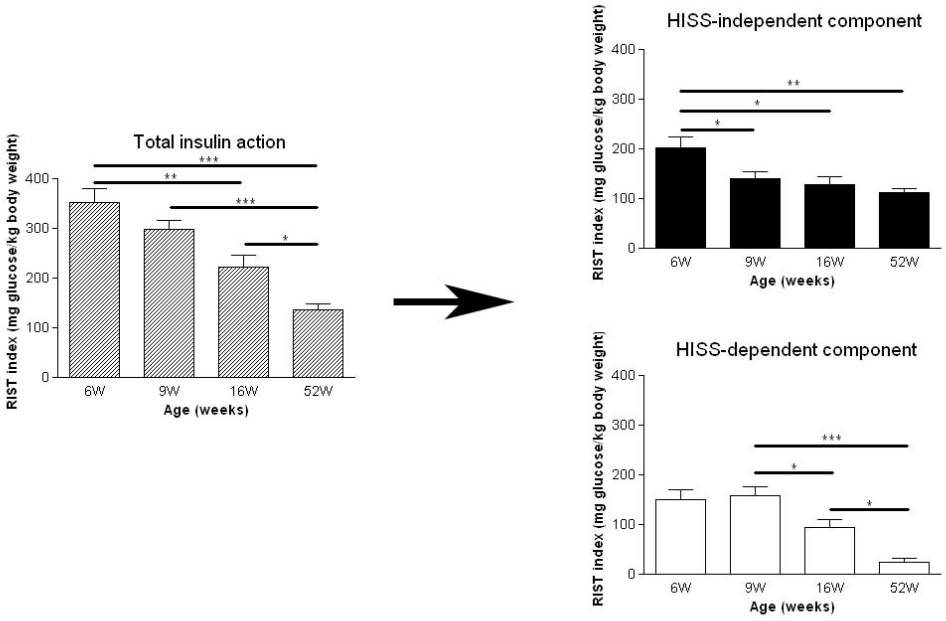


Figure 14 – Effect of age on RIST indexes of fed male Wistar rats: HPN-dependent and –independent components. Rapid insulin sensitivity test (RIST) indexes for total insulin action and its two components in male Wistar rats at 6, 9, 16 and 52 weeks of age. The hepatic parasympathetic nerve (HPN)-independent component decreased from 6 to 9 weeks and remained unchanged thereafter, whereas the HPN-dependent component decreased radically from 9 to 52 weeks of age (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

The two components that constitute total insulin action had different contributions to the observed decrease of total insulin sensitivity (Figure 14). While the HPN-independent component (the post-atropine RIST) decreased from 6 to 9 weeks (201.6±21.6 to 139.6±14.8 mg glucose/kg bw, $p<0.05$), and remained constant thereafter (127.1±17.2 at 16 weeks and 111.0±9.4 mg glucose/kg bw at 52 weeks of age), the HPN-dependent component remained constant from 6 to 9 weeks of age (from 150.2±19.2 to 157.8±17.7 mg glucose/kg bw), was decreased by 16 weeks (94.6±15.3 mg glucose/kg bw, $p<0.05$), and further diminished by 52 weeks of age (24.8±6.8 mg glucose/kg bw, $p<0.05$).

4.2.3.3. Discussion

The present study uncovered that loss of postprandial insulin sensitivity with age is progressive from 6 to 52 weeks, from puberty to middle-age, in Wistar male rats. To this loss of total insulin sensitivity contributes at puberty a shorter action duration and then, gradually, a lower peak action of insulin sensitivity. Furthermore, it was found that the HPN-dependent and –independent components have different contributions to this decrease of insulin sensitivity with age.

The RIST index data for the HPN-independent component show that the action of insulin *per se* decreases from 6 to 9 weeks of age, and remains stable thereafter. Paradoxically, the correspondent dynamic curves show only statistical difference on peak magnitude with age, and only at 52 weeks of age. However, a methodological note should be made regarding the presentation of results for the HPN-independent component; a note which may be able to dispel this conundrum.

Mean dynamic curves are calculated by taking the values of each time point from all the RIST dynamic plots for the animals in each age group, and plotting the mean of those values. The dynamic profile characteristics were determined not by analysing those mean dynamic curves for their characteristics data, but by calculating the mean of the values of those characteristics from each original dynamic curve. The discrepancy in significance among both methods of presentation seems due to the alterations of this component during age being felt more in the shape of the curve than on its key characteristics.

Compellingly, also the analysis of the evolution of the HPN-dependent component benefits from comparing different modes of presenting the data generated by the RIST. Looking at the RIST indexes, the HPN-dependent component remains unchanged from 6 to 9 weeks of age and decreases steadily thereafter. However, this masks the profound change that seems to occur in HPN-dependent component dynamics during that early stage. Indeed, at 6 weeks of age, action peak magnitude is almost half that at 9 weeks of age; but this is counterbalanced by a longer duration at the pubertal stage. As a consequence, the total amount of glucose perfused, given by the area under each curve, is the same; albeit curve dynamics being distinct.

After, from 9 weeks onwards, action duration remains the same; so the decrease seen at 16 weeks of age is due to a lower peak action. A further decrease at 52 weeks of

age is again explained by a different curve shape, since peak magnitude is the same, as easily seen in Figure 13c.

These results highlight the importance of the loss of a parasympathetically facilitated mechanism. Indeed, although the structure of the rat vagus nerve does not seem to deteriorate, not even in old age (Soltanpour and Santer, 1996), there are reports of an age-related functional reduction in parasympathetic activity (Buwalda et al., 1991; Espinola et al., 1999); by both decreased basal vagal tone and lower sensitivity to parasympathetic control[62] (Brodde et al., 1998; Stratton et al., 2003). According to the present study, the effect of this impairment starts to be evident at least from 9 weeks of age. By 1 year of age, it has already led to the almost absolute abrogation of the HISS-dependent component of insulin action.

The fact that humans also show a decrease of parasympathetic tonus with age (De Meersman and Stein, 2007; Lehofer et al., 1999; Pfeifer et al., 1983) leads to the hypothesis that the HPN-dependent component is also important in the gradual glucose intolerance seen in elderly humans[63] (Davidson, 1979; Patarrao et al., 2008; Patarrao et al., 2007; Rowe et al., 1983).

It is also interesting that the autonomic nervous system seems as compromised in non-diabetic elderly people as in younger diabetic patients (Bramann and Aleff, 1992). This leads to the notion that, in prediabetes, there is an acceleration of the parasympathetic degeneration seen with physiological ageing.

The HPN impairment may play an important role in other pathological states also related to both insulin resistance and aging. Indeed, several age-related diseases seem to be linked with insulin resistance (Facchini et al., 2001). Parasympathetic tonus decrease seems to be involved in such clinical conditions as chronic liver diseases (Hendrickse and Triger, 1993), obesity (Peterson et al., 1988), essential hypertension (Quadri et al., 1994), and type 2 diabetes (Carnethon et al., 2003a; Carnethon et al., 2003b). This again leads tentatively to a connection between the dysfunction of the HPN and its influence in the HISS mechanism (Lautt, 2004), the gradual decrease in insulin sensitivity observed in humans starting in the third or fourth decade of age

[62] Cumulatively, changes with age in muscarinic receptors density and/or affinity may also contribute to a lower parasympathetic tone (Brodde et al, 1998).
[63] Furthermore, in the last years, the use of the RIST has been validated in humans (Patarrao et al, 2007), and the HISS mechanism has been shown to be extremely relevant to postprandial insulin sensitivity in humans (Patarrao et al, 2007; Patarrao et al, 2008).

(DeFronzo, 1981), and the higher prevalence of impaired glucose tolerance, type 2 diabetes, and hypertension in aged people (Stout, 1994).

The 52 weeks of age male group is at a midpoint in the Wistar normal 104 weeks life expectancy. In this middle-age stage, male Wistar rats have shown here to be still normoglycaemic, which is supported by others (Berthelier et al., 1997). This preserved normoglycaemia seems to happen with (Berthelier et al., 1997) or without (Zecchin et al., 2003) an increase in fasting insulinaemia by 52 weeks of age. However, it would be expected that, postprandially, blood glucose in these animals is being maintained within normal values by gradually increasing insulinaemia with age; as seen elsewhere in fed and gavaged Wistar rats (Bracho-Romero and Reaven, 1977; Codina et al., 1980). This proposition is consistent with the known compensatory hyperinsulinemia that is reported to develop during decreased total insulin sensitivity with age; at least until 52 weeks of age, after which ß-cell failure and other processes related to senescence have been described to possibly lead to unchecked hyperglycaemia (Berthelier et al., 1997; Koricanac et al., 2004).

Other authors also report still unchanged postprandial insulinaemia at 52 weeks of age (Davidson, 1979; Yoshino et al., 1979) or even a decreased in insulinemia (Reaven et al., 1979; Willeit et al., 1997). This stresses this group as being in a cut-off age. A cut-off phase where middle-age reveals to be a developmental state that favours a breakdown in glucose metabolism control; this by finally being unable to compensate for the impairment of insulin sensitivity that was gradually established. Interestingly, human studies may supply two additional pieces of information to help consider the various factors that contribute to the differential outcomes seen in this developmental stage.

Firstly, reaching 60 years of age seems to mark in middle-aged humans a critical time in terms of glucose tolerance (Fink et al., 1983). Indeed, human glucose disposal seems to be slowly, but gradually, impaired, until a rapid deterioration manifests from 50 years of age onwards, independent of the development of diabetes (Davidson, 1979). Although still able to secrete more insulin, this hyperinsulinemia becomes unable to counter the progressively installed insulin resistance, and a sudden drop in peripheral glucose disposal is seen at this time (Davidson, 1979; Fink et al., 1983). Secondly, it has been reported that parasympathetic function decreases after 30-40 years of age (Piccirillo et al., 1998a; Piccirillo et al., 2001).

Thus, middle-age is revealed as a time in development where the natural progression of insulin resistance leads to a metabolic breakdown. To this, the decrease of parasympathetic activity seems to be intimately connected. The gradual decrease of the HPN-dependent component, which becomes practically nonexistent by this age, can be expected to lead to an extra burden on the direct action of insulin on peripheral tissues, thus quickening metabolic deterioration. Consistent with this hypothesis, it has been suggested (Piccirillo et al., 1998a) that a lesser impairment of vagal function can contribute to a longer maintenance of a proper glucose tolerance.

The unchanged postprandial glycaemia observed herein also strengthens the notion that the development of insulin resistance by decrease of the HPN-dependent component can happen well before the settling of the diabetic state. Middle-aged male Wistar rats have a near shutdown of HPN-dependent insulin action while retaining the direct action of insulin on target tissues seen in young 9 weeks old animals.

On the other hand, the changes seen during the pubertal stage, also with no reflection on postprandial glycaemia, seem to be related instead both to a decrease in the HPN-independent component, in a phase with no changes in fasting insulinaemia (Vital et al., 2006), and to changes in HPN-dependent component dynamics but with maintained efficacy. This hints to developmental changes in the HISS mechanism during puberty; as well as changes in intracellular components of insulin signalling without needing to induce changes in blood insulinaemia (Vital et al., 2006).

Consistent with previous observations that loss of the HPN-dependent component causes a decrease of glucose uptake only in the skeletal muscle (Xie and Lautt, 1996b), with no alteration in liver glucose flux, age-related insulin resistance also does not seem to interfere with hepatic glucose flux (Berthelier et al., 1997).

Age-related insulin resistance is described to impair specifically the peripheral tissues, mainly skeletal muscle (Barnard et al., 1992; Barnard and Youngren, 1992; Goodman et al., 1983; Nishimura et al., 1988). However, as previously described, the liver has an important role in the management of peripheral insulin sensitivity, through the HISS mechanism (Xie and Lautt, 1996b). This is most relevant in the case of liver transplants.

Liver transplantation implies that all hepatic nerves are transacted (Colle et al., 2004). Despite that, liver transplant hosts are afflicted with peripheral, but not hepatic,

insulin resistance (Schneiter et al., 2000b). Not surprisingly, postprandial glucose metabolism in aged rats is not further impaired in cases of liver transplant (Kissler et al., 2005). This is consistent with a lack of hepatic parasympathetic inflow to the liver, which is not worsened by the ablation of the hepatic nerves. Similarly, peripheral glucose uptake is not further impaired, since the HISS mechanism is already impaired by age.

The present observation that total postprandial insulin sensitivity decreases gradually and steadily from 6 to 52 weeks of age is in apparent contrast with the reported development of insulin resistance only in the maturation stage (Davidson, 1979; Goodman et al., 1983; Nakai et al., 1996; Narimiya et al., 1984; Nishimura et al., 1988; Reed et al., 1993).

Considering that the previous studies found in the literature related to animals fasted for 4 to 14 hours (Goodman et al., 1983; Nakai et al., 1996; Narimiya et al., 1984; Nishimura et al., 1988; Reed et al., 1993), the differences between those studies and the present results are explained by a partial, or even total, biological shutdown of the HPN-dependent component with fasting. Indeed, activation of the parasympathetic nervous system occurs at the onset and during food ingestion (Uijtdehaage et al., 1992). Accordingly, the HPN-dependent component is highly modulated by the prandial state, and increasing fasting periods have been shown to produce a time-dependent inhibition of the HPN-dependent component, promptly reversed by refeeding (Latour and Lautt, 2002b; Lautt et al., 2001; Sadri et al., 2006). Thus, the previous studies on the effect of age on insulin sensitivity in whole-body glucose uptake can only be compared with our data for the HPN-independent component of insulin action, as quantified by the post-atropine RIST index.

In this respect, our observation that the HPN-independent component decreases from 6 to 9 weeks of age and remains stable thereafter is consistent with those studies by others in fasted Wistar rats (a fasting time of about 13 hours, which, according to prior observations (Lautt et al., 2001), is enough to produce a considerable inhibition of the HPN-dependent component of insulin sensitivity). These conclude that insulin-stimulated glucose uptake decreases somewhere between 4 and 12 weeks (Nakai et al., 1996) or 8 and 16 weeks of age (Nishimura et al., 1988), with no decrease or only a slight decrease with further age.

The time between 9 and 16 weeks of age also coincides with the greatest rate of body weight gain. Weight gain has been suggested as a possible determinant or confounding factor for the decrease of insulin sensitivity (Barzilai and Rossetti, 1995; Berthelier et al., 1997; Leighton et al., 1989). Although a difference has not been found when analysing insulin action according to weight or fat-free mass (Nishimura et al., 1988) in fasted Wistar rats, previous studies have found a relation between obesity and insulin resistance in fed animals (Afonso et al., 2007a).

While others have seen the same body weight increase profile until 16 weeks, they have further reported that, however, fat mass and lean body mass vary differently with age; fat mass is reported to rise faster than lean body mass during maturation, from 8 to 16 weeks (Barzilai and Rossetti, 1995)[64].

The early rise in fat mass has been related to the insulin resistance seen in maturation, especially to abdominal fat (Coon et al., 1992; Kohrt et al., 1993). Since the fat pads were not weighed here, no consideration can be made to prove or disprove this hypothesis. However, since this is not a continuous process, i.e., the HPN-independent component remains similar in a phase of greater weight gain (between 9 and 16 weeks of age) and the HPN-dependent component decreases further in a time where weight gain is more stabilised (between 16 and 52 weeks of age), a direct causal effect of fat mass on insulin sensitivity is hard to evaluate; unless it is considered that there is a threshold of fat mass that influences negatively insulin sensitivity (Barzilai and Rossetti, 1995), but even that would not explain entirely the behaviour of the HPN-dependent component with age.

One approach to clarify this question would be to avert the unequal rise in fat/fat-free mass. Indeed, caloric restriction diets have been used to prevent fat mass increase (Heilbronn and Ravussin, 2003). The lack of decrease of insulin sensitivity with age in animals fed on these diets has been cited as further indication of the relation between obesity and age-related insulin resistance (Escriva et al., 2007). However, this seems to happen, not by maintained muscle glucose uptake, but by a compensatory increase in adipocyte glucose uptake (Park et al., 2006).

Furthermore, age and obesity are reported to have a synergistic effect on decreasing insulin sensitivity (Catalano et al., 2005; Das, 2004); hinting to this two

[64] This seems to happen again at a late age, associated with senescence, but then with a loss of lean body mass, and rise or maintenance of fat mass (Barzilai and Rossetti, 1995).

conditions acting on insulin sensitivity through different mechanisms (Lonnroth and Smith, 1986), or, at least, at different occasions.

Age is reported to lead to a post-receptor defect in insulin signalling, since the insulin receptor machinery seems to be intact in terms of affinity and binding (Fink et al., 1983; Rowe et al., 1983); a decrease in insulin sensitivity but not in insulin responsiveness (Rowe et al., 1983). It has been proposed that the loss of insulin sensitivity in aged Wistar rats may be explained by any factors that decrease the capacity of insulin to induce glycogen synthesis in the skeletal muscle, without affecting glucose transport into the cell (Leighton et al., 1989; Nishimura et al., 1988). On the other hand, it has been proposed, in fasted rats, that age-related decrease of insulin sensitivity is due to a decrease in glycolysis (Barzilai and Rossetti, 1995). Furthermore, in humans, when glucose uptake was matched between young and old subjects, it was also seen only an impairment in the intracellular oxidative pathway (Gumbiner et al., 1992).

Taking into account the present results, a third alternative can be forwarded: that to the decrease in insulin sensitivity with age contributes in large proportion the impairment of a pathway that either modulates the performance of the insulin receptor, itself still functional during ageing, or that possesses its own receptors which then influence intracellular insulin signalling with additive effect. In accordance with the previous paragraph, the latter would hint then to a role of the HPN-dependent component on glycolysis, rather then on whole glucose uptake.

Interestingly, age has a direct lowering effect on hepatic GSH (Leeuwenburgh and Ji, 1996). This decrease of hepatic GSH can have a direct effect on the expression of the HPN-dependent component since it has been shown to be essential for HISS release/action (Guarino et al., 2003). It may also be the coincidental link between loss of HISS-dependent action and impairment of the intracellular glycolytic pathway with age, since GSH decrease also interferes with –SH enzymes, namely with several glycolytic enzymes (Bartoc et al., 1975; Burch et al., 1981; Chen et al., 1994b; German, 1993; Tiedge et al., 1997)[65].

[65] The sensitivity to –SH is also determinant in the postprandial activity of glucokinase. The potentiation of glucokinase activity (Chen et al, 1994b) with refeeding (Burch et al, 1981), consistent with the enzyme function as a "glucose sensor" (German, 1993), increases insulin secretion, in a mechanism seemingly modulated by the redox status of the enzyme sulfhydryl groups (Tiedge et al, 1997). Lower concentrations of protective thiol compounds, such as GSH, lead to a decrease in glucokinase catalytic

Insulin action changes with age may also sprung as a reflection of the involvement of insulin in the endocrine regulation at each age of the balance between emphasis either on growth, sexual maturation and reproduction, or longevity (Tatar et al., 2003). This is expected to be of particular relevance to glucose homeostasis during puberty, but not ageing (Velasco et al., 1998); which is consistent with our results for the decrease of HISS-independent component of insulin action solely through puberty. In accordance with our results, fasting insulin sensitivity was reported to decrease during puberty, independently of gender and alterations in body composition (Cook et al., 1993).

Although glucose tolerance has been described as remaining unaltered through puberty, this has been simultaneously related to a decrease in insulin sensitivity compensated by an increase in insulin secretion (Bloch et al., 1987). Furthermore, this insulin resistance was found to affect specifically peripheral glucose metabolism; a probable side-effect of the insulin resistance destined to facilitate protein anabolism (Amiel et al., 1991).

Puberty is a time of great endocrine/neural regulatory changes, which are also reflected in changes in nutrient energy fluxes (Hannon et al., 2006). During this stage, the insulin-like growth factor 1 (IGF1) plays an important role in the crosstalk between growth hormone and insulin (Dominici et al., 2005). It is believed that a common insulin/IGF1 signalling pathway has evolved to specialisation (Tatar et al., 2003); with IGF1 as the mediator of the anabolic and mitogenic activity of growth hormone (Laron, 2001), and insulin as the main regulator of nutrient metabolism (Tatar et al., 2003). Nonetheless, it has been observed that IGF1 is able to stimulate insulin sensitivity (Clemmons, 2004), while an increase in growth hormone peripheral action reduces insulin sensitivity (Cook et al., 1993).

During puberty, there is an overexpression of growth hormone (Velasco et al., 1998), related to a stage of intense body growth (Moran et al., 2002). The decrease of insulin sensitivity in peripheral tissues seems to be related to the chronic effect of this increase in growth hormone, and is compensated by hyperinsulinemia. Additionally,

activity. Likewise, fasting may lead to glucokinase inactivation through SH side group oxidation (Tiedge et al, 1997).

IGF1 seems to regulate the balance between insulin and growth hormone actions[66] (Brown-Borg, 2008; Chandrashekar et al., 2004; Chiba et al., 2008; Dominici et al., 2005; Moran et al., 2002).

Considering that the HISS-dependent component is not altered in magnitude during this phase, the altered dynamics observed may be considered as an adaptation of this component to the changes taking place in relation to insulin action *per se*. Or, alternatively, the changes from a longer sustained, but lower intensity, HISS action, may be a remnant hint to the properties of a prior contribution of HISS to the modulation of perinatal insulin sensitivity. For example, it may reflect the developmental changes in the crosstalk between insulin- and amino acids-determined signals in the regulation of protein synthesis (Suryawan et al., 2007). Nevertheless, this topic warrants further investigation before being adequately interpreted.

Due to the importance of the crosstalk between insulin and growth hormone during puberty, in terms of control of sexual maturation and somatic growth, a pharmacological intervention at this stage to oppose the decrease in insulin action should not be considered lightly (Goran and Gower, 2001). Likewise, an inability to increase insulin secretion and/or decrease insulin extraction during puberty can be especially detrimental at this stage (Weiss et al., 2005). To accentuate the distinct metabolic vulnerability of puberty, it becomes evident that a dysfunction of HISS action, either inherent or acquired, would be particularly delicate. Considering the worsening metabolic profile related to insulin resistance in adolescents (Bacha et al., 2006), and the increasing prevalence of type 2 diabetes in youngsters (Alberti et al., 2004), this danger seems already palpable[67] (Arslanian et al., 2005; Saad et al., 2005).

[66] This crosstalk is also important for the determination of reproduction and ageing/longevity (Chandrashekar et al, 2004; Chiba et al, 2008). Likewise, it may explain the opposition of longevity and nutrient handling/somatic growth mechanisms (Brown-Borg, 2008). Indeed, processes that postpone senescence are related to lower metabolism, arrest growth, increased stress resistance, and repair functions. Also, this may be related to the effect of calorie restriction diets on prolonging longevity (Brown-Borg, 2008).

[67] The "stress" originated by the decrease of insulin sensitivity from the prepubertal to the pubertal stage can also become remarkably revelatory of possible inherited genetic defects, specifically in the ability of the pancreas to compensate this insulin resistance with a higher insulin secretion. Hence, a family history of type 2 diabetes seems to be additionally determinant of a worse prepubertal balance between insulin sensitivity and insulin secretion, which leads to a lower pubertal insulin sensitivity due to the lack of an adequate compensatory β-cell function (Arslanian et al, 2005). Again, this makes this transition stage into a particularly critical time, where a dysfunction of the natural changes in the HISS-dependent component may provide an added pressure for the progression of type 2 diabetes by providing a greater challenge for the compensatory insulin secretion (Saad et al, 2005).

In conclusion, age is related to a gradual decrease in postprandial insulin sensitivity. This "physiological" insulin resistance seems to be mainly related to a decrease in HISS action, probably determined by a decrease in parasympathetic nervous function.

The HISS-independent component is impaired during the pubertal stage and remains similar until middle age. The HISS-dependent component is maintained in magnitude during the pubertal stage, but undergoes dynamic changes in action, perhaps still reflection of the trans-pubertal process of development (Amiel et al., 1991). Then, the HISS-dependent component continues to gradually decrease, until almost non-existence at 52 weeks of age, being responsible for the decrease in postprandial total insulin sensitivity up to middle-age.

4.3. Gender comparison of postprandial insulin sensitivity with fed female Wistar rats at 9, 16 and 52 weeks of age. Further studies in 78 weeks old male and female Wistar rats.

4.3.1. Context and work hypothesis

For metabolic reasons, we had chosen to study only male animals. However, gender-related differences in the way insulin sensitivity changes with age have been reported (Franssila-Kallunki et al., 1992). Furthermore, the influence of the autonomic nervous system on insulin sensitivity may be felt differently depending on gender (Bonnemeier et al., 2003; Flanagan et al., 1999; Hogarth et al., 2007).

The unique pre-menopausal hormone status has been proposed to play a role in protecting females against certain metabolic complications. The loss of that status with age has been related, among others, to a decline in parasympathetic function (Dart et al., 2002) and the development of a state of hyperinsulinemia (Willeit et al., 1997).

Our **work hypothesis** was that, if the female gender was more protected from the development of insulin resistance with age than the male gender, it would be observable as a lesser decrease of the HPN-dependent component of insulin action with age.

4.3.2. Experimental protocols

To evaluate this, female 9, 16 and 52 weeks old Wistar rats were analysed on the postprandial state, achieved after a 16-hour fast followed by a 1 hour period refeeding. Female Wistar rats at 6 weeks of age showed several surgical difficulties due to animal size, which led to the exclusion of those experiments.

Further inquiries led to the use of two other groups: one male and one female, 78 weeks old Wistar rats. The fasting and refeeding protocol was similar, to guarantee the immediate postprandial state at the time of use. These later experiments were unique in the fact that glucose perfusion was begun not at 5 but at 3 mg/kg/min (see Material and Methods), to avoid overshooting the glycaemia baseline at the beginning of the RIST.

At 78 weeks of age Wistar rats show already a mortality rate of about 35%, in a period commonly related to senescence. All animals with visible signs of injury, cancer, or general discomfort were excluded from the study.

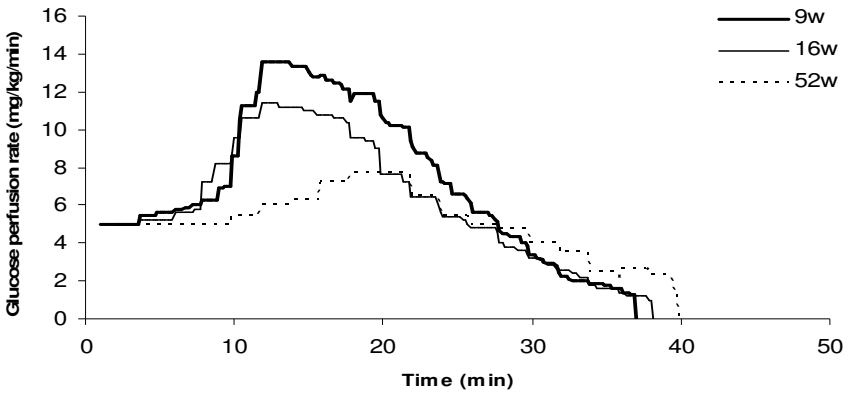
For all animals, after anaesthesia, a control RIST was performed, followed by the administration of atropine (3 mg/kg) and a second, post-atropine, RIST.

4.3.3. Results

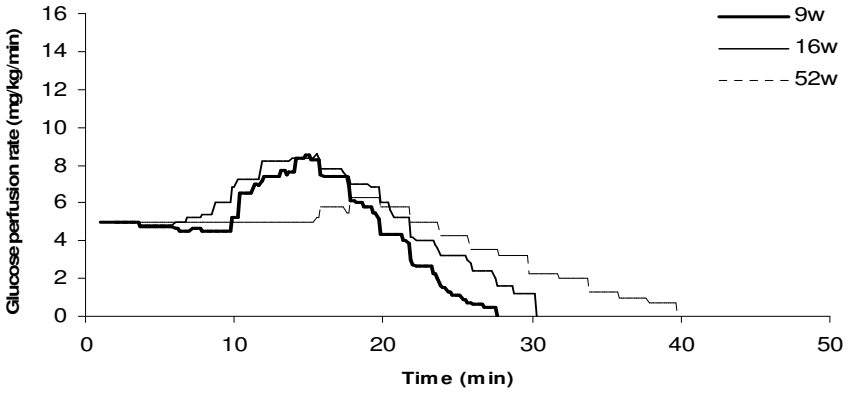
Basal postprandial glycaemia was not altered by age (111.5±4.1 at 9 weeks, n=8, 113.8±2.4 at 16 weeks, n=5, and 108.2±4.4 mg/dl at 52 weeks of age, n=5). Also, as in males, baseline glycaemia did not change after atropine administration (104.6±1.3 at 9 weeks, 105.3±5.5 at 16 weeks, and 106.2±5.2 mg/dl at 52 weeks of age). Females did not show the decrease in arterial blood pressure with age evidenced by the males (109.4±2.4 at 9 weeks, 98.0±10.6 at 16 weeks, and 103.6±3.1 mmHg at 52 weeks of age); although arterial blood pressure in females was not significantly different from those in males in any age group. Again, atropine did not change blood pressure (108.4±4.1 at 9 weeks, 97.0±11.4 at 16 weeks, and 103.4±3.9 mmHg at 52 weeks of age).

Females were always lighter compared with age-matched males. Body weight increased from 240.0±4.8 g at 9 weeks ($p<0.0001$ vs 9 weeks old males) to 317.4±17.1 g at 16 weeks ($p<0.0001$ vs 16 weeks old males), and then to 446.8±14.0 g at 52 weeks of age ($p<0.0001$ vs 52 weeks old males).

a)



b)



c)

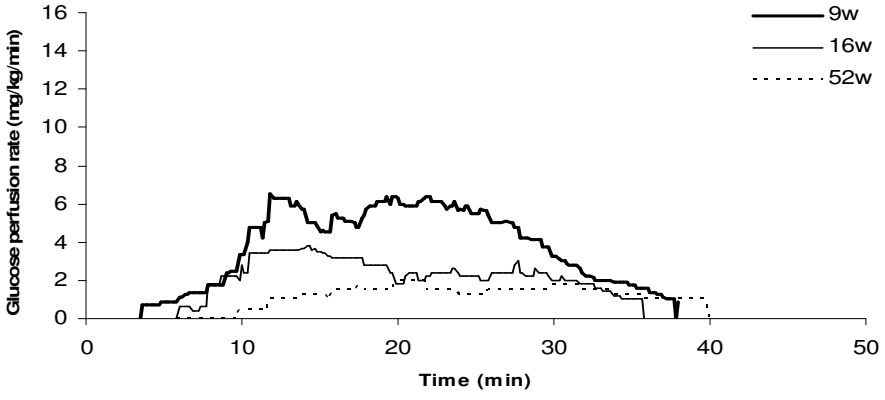


Figure 15 – Effect of age on RIST profiles of fed female Wistar rats. Mean dynamic profiles for the rapid insulin tests (RIST) performed on control (a) and post-atropine (b) conditions at 9, 16, 52 and 78 weeks of age female Wistar rats. Mean dynamic profile curve for the HPN-dependent component of insulin action (c), obtained by subtracting the post-atropine curve from the control curve, at all age groups.

Figure 15 shows the mean dynamic curves obtained for all age groups. Further, the main characteristics for the dynamic curves are presented on Table IV.

The dynamic curves for total postprandial insulin action (Figure 15a) show that a decrease in insulin sensitivity happened from 9 to 52 weeks of age. This was mainly due to a gradual lowering of peak magnitude from 9 to 52 weeks of age (becoming statistically different from 14.8±1.0 at 9 weeks to 8.3±0.6 mg glucose.kg^{-1}.min^{-1} at 52 weeks of age, $p<0.01$) (Table IV).

When the HPN-dependent component was blocked by atropine (Figure 15b), 52 weeks old females showed a lowered peak magnitude of insulin *per se* action (from 9.8±0.7 at 9 weeks to 6.6±0.4 mg glucose.kg^{-1}.min^{-1} at 52 weeks of age, $p<0.05$). This was compensated by a latter offset in action (38.8±2.4 min at 52 weeks vs 25.4±1.1 min at 9 weeks of age, $p<0.05$).

As in male Wistar rats, the effect of age on postprandial insulin action is related to a decrease in the HPN-dependent component of insulin action (Figure 15c). This is observable by a decrease in peak action (from 8.3±1.3 at 9 weeks to 2.5±0.9 mg glucose.kg^{-1}.min^{-1} at 52 weeks of age, $p<0.05$), and also to an increased flattened shape of the dynamic curve with age.

This indicates that from 9 to 52 weeks of age male and female Wistar rats show similar development alterations in the dynamic curves of total postprandial insulin action, and in the HPN-dependent and –independent components.

Table IV – Effect of age on dynamic curve main properties of fed female Wistar rats. Dynamic profile characteristics of the control rapid insulin sensitivity test (RIST), post-atropine RIST and hepatic parasympathetic nerve (HPN)-component of total insulin action of 9, 16, and 52 weeks old fed female Wistar rats (* $p<0.01$ vs 9 weeks, # $p<0.05$ vs 9 weeks, § $p<0.05$ vs 16 weeks).

		9 weeks old (n=8)	16 weeks old (n=5)	52 weeks old (n=5)
Control RIST	Peak (mg glucose.kg^{-1}.min^{-1})	14.8±1.0	12.6±0.8	8.3±0.6*
	Peak time (min)	12.5±0.9	12.4±1.1	16.8±0.6
	Duration (min)	35.1±2.1	36.3±2.6	42.3±2.5
Post-atropine RIST	Peak (mg glucose.kg^{-1}.min^{-1})	9.8±0.7	9.4±1.5	6.6±0.4#
	Peak time (min)	13.2±0.6	12.2±1.3	17.3±0.5§
	Duration (min)	25.4±1.1	29.5±1.8	38.8±2.4#
HPN-dependent component	Peak (mg glucose.kg^{-1}.min^{-1})	8.3±1.3	5.8±0.6	2.5±0.9#
	Peak time (min)	17.0±1.7	12.8±1.4	17.3±1.9
	Onset (min)	5.5±1.0	6.2±1.7	14.8±2.9
	Offset (min)	35.1±2.1	34.3±2.7	36.3±6.0
	Duration (min)	29.5±2.8	28.0±4.2	21.5±5.1

To ascertain the total amount of glucose perfused during each test, the area under each dynamic curve was calculated. The results are shown in Figure 16.

Total insulin action showed a tendency to decrease with age. However, unlike male Wistar rats, in females this decrease did not achieve statistical difference from 9 to 52 weeks of age (from 266.5±25.8 at 9 weeks to 231.9±26.2 at 16 weeks, and 201.4±16.3 mg glucose/kg bw at 52 weeks of age).

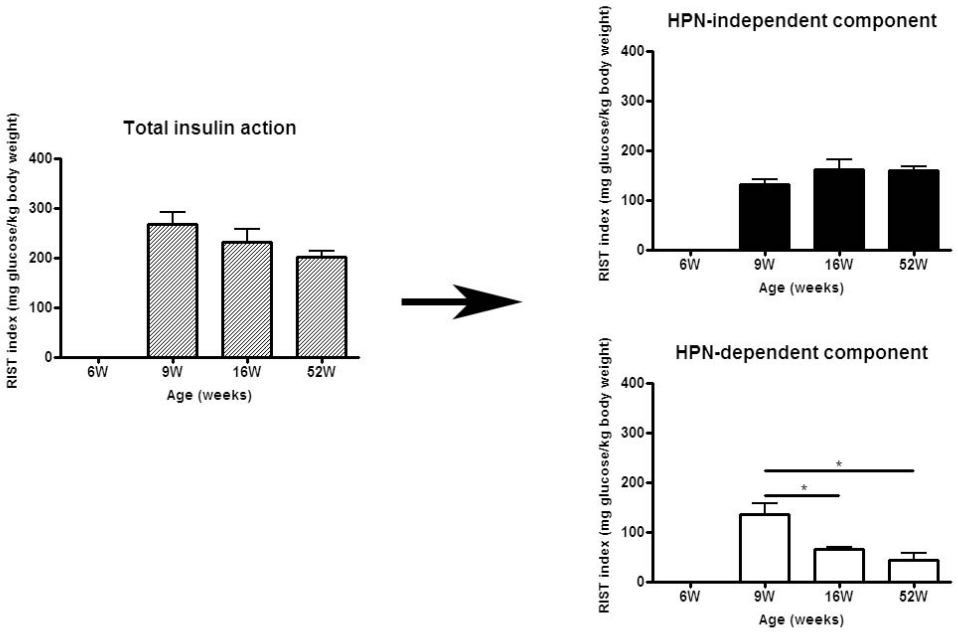


Figure 16 – Effect of age on RIST indexes of fed female Wistar rats: HPN-dependent and –independent components. Rapid insulin sensitivity test (RIST) indexes for female Wistar rats at 9, 16, and 52 weeks of age (the 6 weeks group is represented blank to help comparison with the data of male experiments). As in males, The hepatic parasympathetic nerve (HPN)-dependent component decreased with age. The HPN-independent component was unchanged by age.

When comparing these results with the ones obtained for males (Figure 14), we observe that males and females have similar total postprandial insulin sensitivity at 9 and 16 weeks of age (males: from 297.4±18.5 at 9 weeks to 221.7±24.3 mg glucose/kg bw at 16 weeks of age). However, at 52 weeks of age, the males show to be insulin resistant when compared with females (135.6±12.8 for males vs 201.4±12.6 mg glucose/kg bw for females). This seems to indicate that the loss of total insulin action with age happens slower in females than in males.

As in males, the two components of insulin action were affected differently by age in females (Figure 16). The HPN-independent component remained similar from 9 to 52 weeks of age (from 132.0±10.3 at 9 weeks to 161.7±19.9 at 16 weeks, and to 158.8±9.2 mg glucose/kg bw at 52 weeks of age). The HPN-dependent component decreased from 9 to 16 weeks of age (from 134.5±22.4 at 9 weeks to 65.6±4.4 mg glucose/kg bw at 16 weeks, $p<0.05$); after, at 52 weeks of age it did not show a further

statistically significant decrease, but evidenced still a downward trend (to 42.6±15.2 mg glucose/kg bw). In terms of behaviour, as already seen by the dynamic curves (Figure 15), male and female seem to be subjected to the same manner of developmental changes.

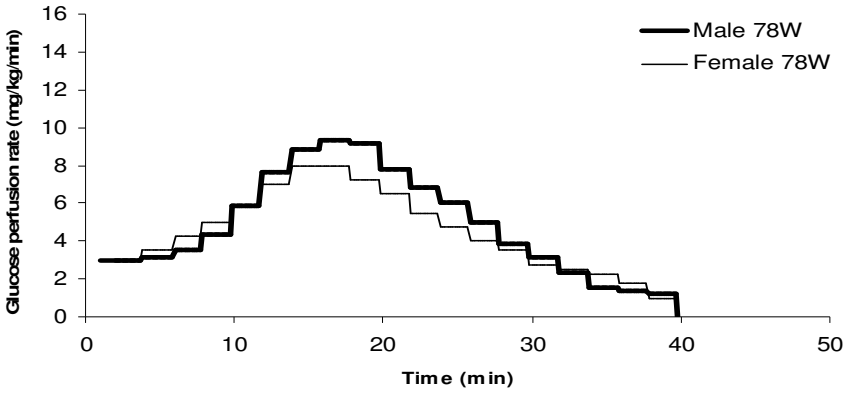
Surprisingly, comparing again males and females, the main contributor for the observed lack of decrease in postprandial insulin total action between 9 and 52 weeks old females seemed not an hypothetical preservation of the HPN-dependent component (24.8±6.8 in males and 42.6±15.2 mg glucose/kg bw in females), but rather an higher HPN-independent component at this age in females (111.0±9.4 in males vs 158.8±9.2 in females, $p<0.05$).

The previous results prompted the analysis of males and females at 78 weeks of age; although this age was firstly not considered, being related to senescence. Indeed, animals presented at this time common signs of old age (ex: degraded coating, crooked teeth, morbid obesity, stiffened vascular walls, atherosclerotic plaques, abscesses, tumours, and visual and locomotion impairments). Interestingly, hypertension was not seen even at this age. All the animals with signs of disease or general discomfort were excluded from the study.

Males had still higher body weight than females (950.0±26.3 for males, n=6, vs 547.3±21.3 g for females, n=5, $p<0.001$). None showed hypertension or hyperglycaemia. Blood arterial pressure was still normal in both genre (90.0±4.8 for females and 101.2±5.3 mmHg for males), and unchanged by atropine administration (88.0±4.3 for females and 91.8±5.7 mmHg for males. Baseline glycaemia was also normal, in both gender (99.0±6.8 for males and 98.2±2.8 mg/dl for females), not changed by atropine (96.8±3.7 for males and 95.0±2.6 mg/dl for females).

The dynamic profiles obtained at 78 weeks of age in male and female, before and after atropine administration are shown in Figure 17.

a)



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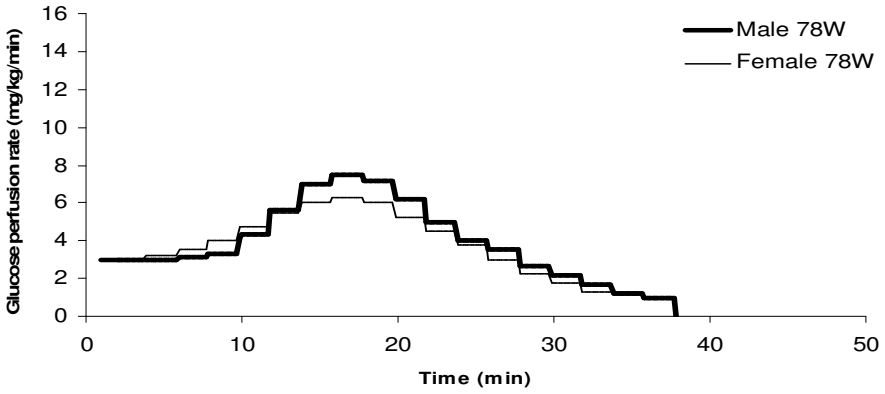

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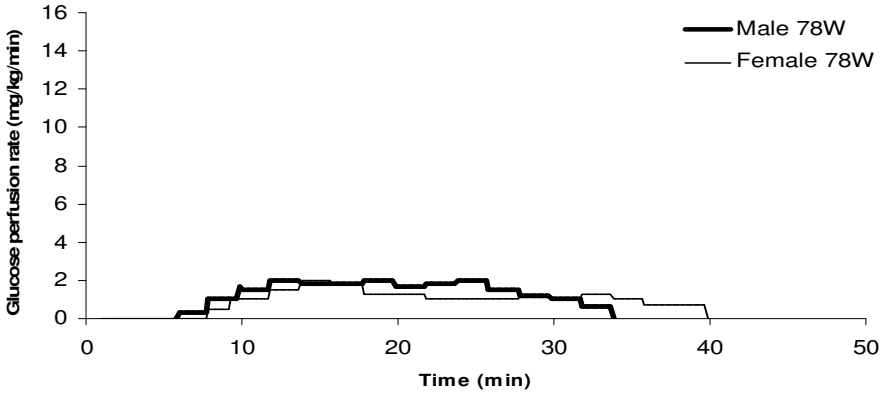


Figure 17 – RIST profiles of fed male and female Wistar rats at old age. Mean dynamic profiles for the rapid insulin tests (RIST) performed on control (a) and post-atropine (b) conditions at 78 weeks of age in male and female Wistar rats. Mean dynamic profile curve for the hepatic parasympathetic nerve (HPN)-dependent component of insulin action (c), obtained by subtracting the post-atropine curve from the control curve.

Both gender showed qualitatively the same kind of developmental changes that had already been evident in their gender-matched group at 52 weeks. Also, both gender showed similar dynamic curves for total insulin action and for the HPN-independent and –dependent components (Figure 17), as well as similar characteristics (Table V).

Table V – Dynamic curve main properties of fed male and female Wistar rats at old age. Dynamic profile characteristics of the control rapid insulin sensitivity test (RIST), post-atropine RIST and hepatic parasympathetic nerve (HPN)-component of total insulin action of 78 weeks old fed male and female Wistar rats.

		Male 78 weeks old (n=6)	Female 78 weeks old (n=5)
Control RIST	Peak (mg glucose.kg^{-1}.min^{-1})	9.7±0.9	8.3±1.3
	Peak time (min)	14.5±0.8	13.8±0.8
	Duration (min)	37.2±1.8	40.3±1.0
Post-atropine RIST	Peak (mg glucose.kg^{-1}.min^{-1})	8.0±0.7	6.5±0.6
	Peak time (min)	15.5±1.0	14.3±1.3
	Duration (min)	33.8±2.3	36.8±2.5
HPN-dependent component	Peak (mg glucose.kg^{-1}.min^{-1})	3.8±0.5	2.4±0.6
	Peak time (min)	15.4±2.7	12.8±0.4
	Onset (min)	9.2±1.7	5.2±0.5
	Offset (min)	31.8±3.3	35.1±4.0
	Duration (min)	22.7±3.1	28.6±3.7

When the area under the control RIST dynamic curve was calculated, both gender showed similar insulin sensitivity (185.5±24.3 for male and 173.7±14.5 mg glucose/kg bw for females)(Figure 18). Furthermore, female showed now a statistically significant decrease with aging (from 266.5±25.8 at 9 weeks to 173.7±14.5 mg glucose/kg bw at 78 weeks of age, $p<0.05$), as well as the males (from 297.4±18.5 at 9 weeks to 185.5±24.3 mg glucose/kg bw, $p<0.05$).

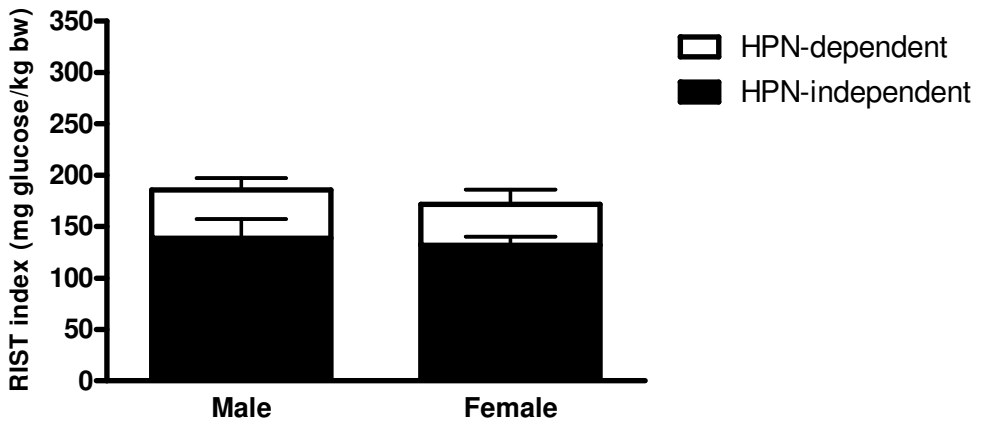


Figure 18 –RIST indexes of fed male and female Wistar rats at 78 weeks of age: HPN-dependent and –independent components. Rapid insulin sensitivity test (RIST) indexes for male and female Wistar rats at 78 weeks of age showed similar total insulin sensitivity between gender at this age, as well as a similar contribution of both components between gender.

Both components of total insulin action showed similar values among gender (Figure 18). The HPN-independent remained unchanged by aging (138.6±18.8 for males and 131.7±8.6 mg glucose/kg bw for females), while the HPN-dependent remained impaired (46.9±11.8 for males and 40.0±14.2 mg glucose/kg bw for females). In males, the HPN-dependent component at 78 weeks of age, albeit showing a tendency to be higher than at 52 weeks of age, was still decreased in relation to the 16 weeks of age group ($p<0.05$).

4.3.4. Discussion

Aging has shown to lead to a consistent decrease of total postprandial insulin sensitivity in both genders of Wistar rats. From 9 to 78 weeks of age, the action of insulin *per se* remains similar in male and female, so this development of insulin resistance seems to be solely due to a decrease of the HPN-dependent component.

The HPN-dependent component of insulin action decreased radically in both gender during the end of puberty. After that, it decreased more in males and showed a tendency to decrease in females. This seems to be related to a more radical decrease of the HPN-dependent component in females by 16 weeks of age. Indeed, consistent with this, it has been reported that gender influences differently the decrease of parasympathetic activity with aging, and that this difference tends to disappear when

middle-age is reached (Bonnemeier et al., 2003; De Meersman and Stein, 2007; Kuo et al., 1999).

Estrogen, which has been described to promote parasympathetic function and, through nitric oxide, to limit sympathetic drive (Liu et al., 2003; Rosa Brito-Zurita et al., 2003), is however reported to decrease only after one year of age, well after the sudden decrease in the HPN-dependent component observed in females. Nonetheless, it may have a role in nerve growth and survival with age (Dart et al., 2002; Lavi et al., 2007).

The slightly better profile of the HPN-dependent component in old aged, 78 weeks of age animals, especially among the males included in this study, need not be contradictory. By 78 weeks of age the mortality rate is already quite substantial, as well as animals excluded from the study for possible confounding ailments. This leads to the involuntary selection of animals able to maintain a better metabolic profile at this age. Consistently with this hypothesis, neither males nor females showed hyperglycaemia or hypertension at 78 weeks of age.

Interestingly, this data also finds a parallel with human studies. Indeed, although parasympathetic function was shown to decrease with age (Piccirillo et al., 1998a; Piccirillo et al., 2001), centenarian subjects showed increased parasympathetic function in relation to middle-aged and following aged groups (Piccirillo et al., 1998a). Beyond the reported conclusion of a U-shaped curve in parasympathetic function with age (Piccirillo et al., 1998a), first decreasing and then increasing again in tonus, one may propose the alternative conclusion that those individuals that were able to reach old age were precisely the ones where a maintained activity of the parasympathetic nervous system prevented the dramatic metabolic deterioration seen during normal aging.

The present results traced the developmental change in insulin sensitivity, which was shown to be similar between male and female in each of the young (9 weeks) and old aged (78 weeks) groups. This further confirms the Wistar rat strain as an adequate animal model of human aging, since these results are consistent with previous human studies (Davidson, 1979; Fink et al., 1983; Piccirillo et al., 1998a).

In relation to the present thesis, it helped to establish the preferential age of analysis to proceed with studies in pathological animals models – specifically, the 9

weeks old group, where the HPN-dependent component seems not yet impaired by age -, and to allow to focus on only one gender.

4.4 Alterations in postprandial insulin sensitivity by high-sugar diets

4.4.1. Context and general work hypothesis

The development of type 2 diabetes seems to be influenced in equal parts by the genetic background and by the cumulative effects of lifestyle (Facchini et al., 2001). As sure as a balanced diet and exercise can postpone the development of the disease in people with a genetic disposition, and family history of diabetes (Eriksson et al., 1989; Lopez et al., 2008; Ten and Maclaren, 2004), also even a good genetic background may not be enough to prevent the deleterious effect of an unchecked lifestyle. In this concern, diet composition seems to play an important role in the development of insulin resistance and type 2 diabetes (Hu et al., 2001).

A thoughtfully planned diet composition has been for long recognised to ameliorate the glycemic control of diabetic patients; and, when uncared for, to have a potential negative impact even on healthy individual (Himsworth, 1934). Since then, Western diets have been changing; and Portugal has followed this phenomenon rapidly, even among other Mediterranean countries (Chen and Marques-Vidal, 2007).

One of the main characteristics in diet changes has been the increased consumption of refined carbohydrates. This is considered a major factor in the current increase of type 2 diabetes and the occurrence of the diverse facets of the metabolic syndrome, moreover on their rising incidence on children and young adults (Gross et al., 2004; Yoo et al., 2004); and this is evermore relevant in Portugal since it has an high percentage of daily soft drinks consumption among youngsters, in European terms (Vereecken et al., 2005). Likewise, the increased intake of sugar-sweetened drinks is also related to an increased deterioration of glucose metabolism with age (Reaven et al., 1983) and risk of cardiovascular problems (Dhingra et al., 2007) in adults.

Several studies have established that high-sugar feeding, including high-sucrose diets, lead to the decrease of whole-body insulin sensitivity (Daly, 2003), mainly in skeletal muscle (D'Alessandro et al., 2000; Hulman and Falkner, 1994; Kim et al., 1999; Pagliassotti et al., 1994), similarly in male and female (Kim et al., 1999). However, the relevance of the HPN, which has been shown to regulate glucose homeostasis in the

postprandial state, has never been assessed in studies of insulin resistance induced by sucrose feeding.

Furthermore, this seemed an excellent model for the evaluation of the impact of nutritional factors on postprandial insulin action since it has been described as being free of the confounding effects of weight gain and hypertension, common in other models of diet manipulation (Afonso et al., 2007a; Elliott et al., 2002; Valensi, 2005).

Our **general work hypothesis** was that the highly regulated HPN-dependent component of postprandial insulin action would be very sensitive to environmental disruption by a high-sucrose diet.

4.4.2 Effect of a high-sucrose diet on Wistar and Sprague-Dawley rats

4.4.2.1. Experimental protocols

Since it has been shown that differences in strain characteristics between Wistar and Sprague-Dawley rats can lead to variations in whole-body insulin sensitivity (Gaudreault et al., 2001), these two strains, the most commonly used for insulin-stimulated glucose measurements, were used in this study.

Wistar and Sprague-Dawley were analysed at 9 weeks of age. For the last 6 weeks, both strains were divided into two groups. Besides both groups being fed a standard solid diet, one of them had access to a bottle of water while the other had access to a bottle of water and an additional bottle of a 35% sucrose solution. In the high-sucrose-diet fed group, bottles were changed sides in the cage each time they were refilled to avoid habituation. Water and sucrose solution volumes consumed were measured to establish adherence to the high-sucrose solution supplement.

Postprandial total insulin action was quantified by a RIST control. Following that, atropine was administered to block HPN influence on glucose metabolism, and a second RIST was performed to quantify the HPN-independent component of insulin action.

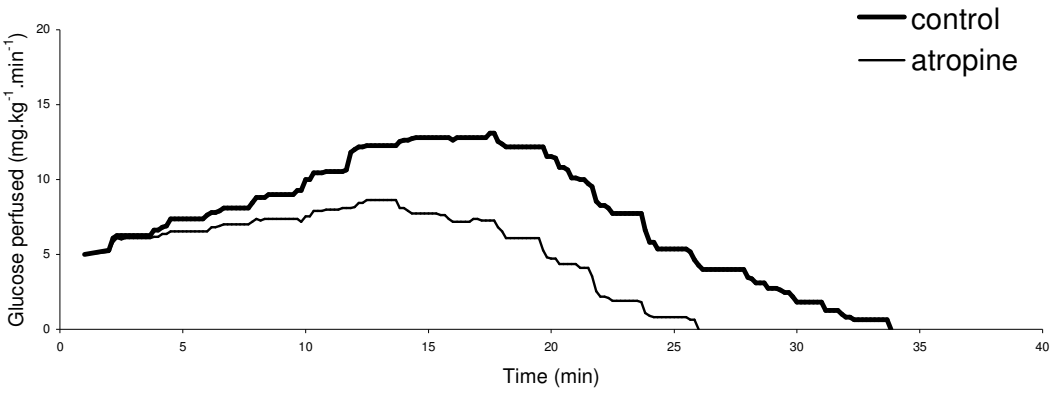
4.4.2.2. Results

Wistar rats with access to a high-sucrose solution bottle plus a water bottle showed, with experiment time, preference for consumption of the sucrose solution (from 23.1±5.4 to 35.6±2.9 ml/day, $p<0.05$) in detriment of only water (from 32.5±4.5 to 8.8±1.6 ml/day, $p<0.001$). These values did not change when the bottles were switched places. Rats with just a water bottle ingested 45.8±2.5 ml/day.

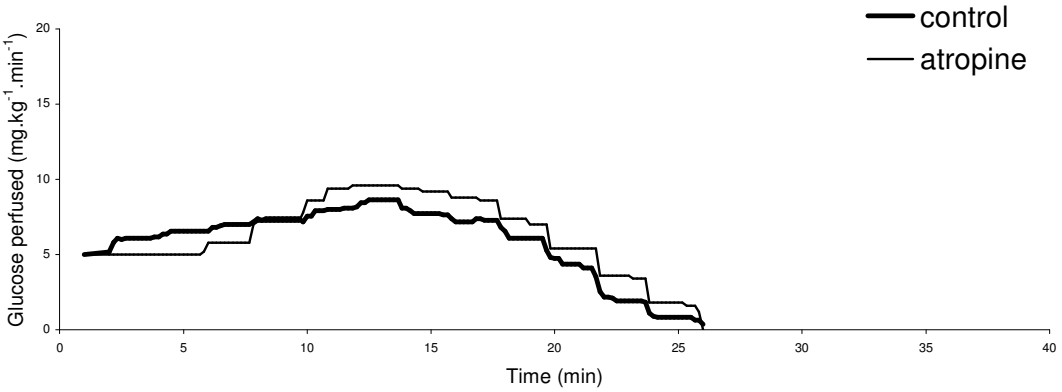
The availability of a high-sucrose solution did not produce alterations in body weight (338.5±18.1 for standard-fed, n=8, and 340.8±18.6 g for high-sucrose, n=10). Likewise, basal postprandial glycaemia was not changed either by diet (125.1±7.7 with standard diet and 136.9±4.0 mg/dl with high-sucrose diet) or atropine (131.8±8.3 with standard diet and 134.3±4.4 mg/dl with high-sucrose diet). Arterial blood pressure was also unchanged by diet (119.5±0.5 with standard diet and 111.8±5.3 mmHg with high-sucrose) or atropine (122.5±7.5 with standard diet and 112.4±5.7 mmHg with high-sucrose diet).

The mean dynamic profiles of total postprandial insulin action for standard- and high-sucrose-fed Wistar rats are shown below, on Figure 19, as well as the dynamic profiles obtained after atropine administration and by calculation of the HPN-dependent component. On Table VI are detailed the main characteristics of the aforementioned profiles.

a) Standard diet



b) High-sucrose diet



c) HPN-dependent component

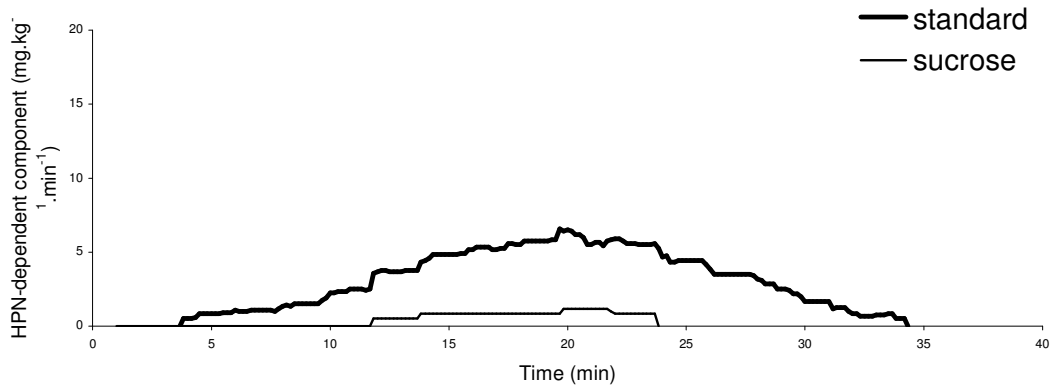


Figure 19 – Effect of high-sucrose supplementation on RIST profiles of male 9 weeks-old Wistar rats. Dynamic profiles of the control (bold lines) and postatropine (thin lines) rapid insulin sensitivity test (RIST) for standard-fed (a) and high-sucrose-fed (b) Wistar rats. c Dynamic profile of the hepatic parasympathetic nerve (HPN)-dependent component of insulin action, obtained by subtracting the post-atropine curve from the control curve, of the standard-fed (bold line) and high-sucrose-fed (thin line) Wistar rats.

There was a clear decrease in the dynamic profile of the control RIST with exposure to a high-sucrose diet. This decrease was seen both in maximal total response to insulin (peak action decreased from 15.9±0.9 in the standard-fed to 11.7±1.3 mg glucose.kg^{-1}.min^{-1} in the sucrose-fed, $p<0.01$) and in the duration of total insulin action (from 35.8±1.8 in the standard-fed to 26.8±0.5 min in the sucrose-fed, $p<0.01$)(Table VI).

The dynamic profiles for the post-atropine RIST in standard-fed and sucrose-fed Wistar rats were practically superimposed, which shows that the present high-sucrose diet had no effect on the HPN-independent component of insulin action.

The previous further shows that it is solely a decrease of the HPN-dependent component of insulin action that is responsible for the observed diminished total insulin action. Peak action of the HPN-dependent is radically compromised by consumption of the high-sucrose supplement (from 10.0±1.1 in standard-fed to 1.5±0.6 mg glucose.kg^{-1}.min^{-1}, $p<0.001$), as well as the duration of action (from 26.6±2.1 in standard-fed to 8.3±2.0 min in sucrose-fed, $p<0.01$). The decrease in duration of action was due to an earlier offset of the HPN-dependent component (from 35.4±1.9 in standard-fed to 22.9±2.1 min in sucrose-fed, $p<0.01$).

Table VI – Dynamic curve main properties of standard and high-sucrose fed Wistar rats. Dynamic profile characteristics of the control RIST, the HPN-independent component (post-atropine RIST), and the HPN-dependent component of total insulin action (* $p<0.01$ standard-fed vs sucrose-fed, # $p<0.001$ standard-fed vs sucrose-fed).

		Standard-fed	Sucrose-fed
Control RIST	Peak	15.9±0.9*	11.7±1.3*
	Peak time	12.3±0.5	12.6±0.8
	Offset	35.8±1.8*	26.8±0.5*
HPN-independent	Peak	10.8±1.1	10.2±1.2
	Peak time	11.1±0.8	11.8±0.8
	Offset	24.9±2.1	26.8±0.5
HPN-dependent	Onset	7.2±1.0	8.9±1.7
	Peak	10.0±1.1#	1.5±0.6#
	Peak time	15.7±1.1	16.1±1.9
	Offset	35.4±1.9*	22.9±2.1*
	Duration	26.6±2.1*	8.3±2.0*

Figure 20 shows the previous results but now representing them by the area delimited by the dynamic profiles. By the RIST indexes for Wistar rats, the bars on the left of the figure, it is seen again that the standard-fed group had higher total insulin sensitivity than the sucrose-fed group (305.6±34.1 for standard-fed vs 193.9±13.7 mg glucose/kg bw for the sucrose-fed; $p<0.05$). The RIST indexes obtained after atropine administration were similar in the two groups (140.4±22.1 for standard-fed and 167.4±10.8 mg glucose/kg bw for sucrose-fed).

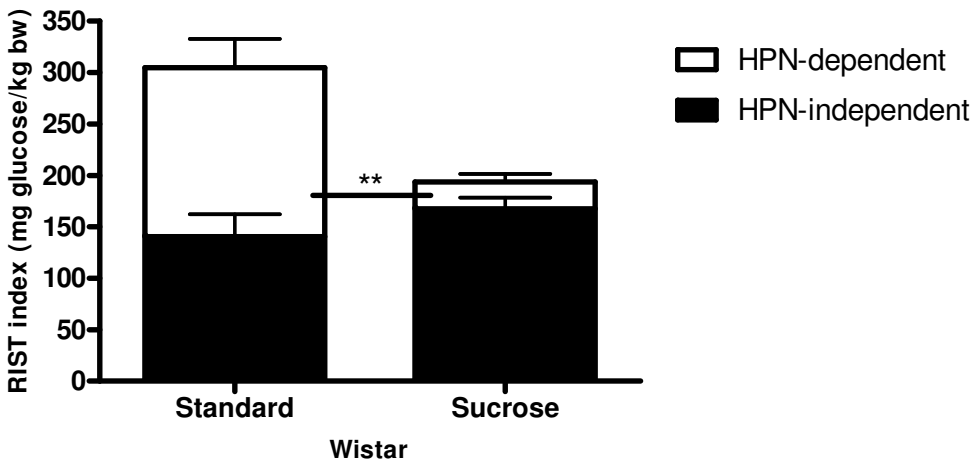


Figure 20 – RIST indexes of standard and high-sucrose fed Wistar rats. Rapid insulin sensitivity test (RIST) indexes for the hepatic parasympathetic nerve (HPN)-independent (filled) and HPN-dependent (open) components of insulin action on 9 weeks old Wistar rats. The sum of both components represents the control RIST index for each protocol. In both strains, insulin sensitivity was significantly higher for the standard-fed diet group than for the high-sucrose group (** $p<0.01$). A lower HPN-dependent component accounted for this difference.

The HPN-dependent component of insulin action was significantly lower in the sucrose-fed than in the standard-fed group (164.3±28.1 vs 26.5±7.5 mg glucose/kg bw; $p<0.0001$). This means that the contribution of the HPN-dependent component to total insulin action was diminished in sucrose-fed Wistar rats (from 52.1±6.6% in standard-fed to 12.7±3.3% in the sucrose-fed group; $p<0.0001$).

The Sprague-Dawley rats also did not presented changes in body weight with high-sucrose diet (353.9±16.3 for standard-fed, n=15, and 384.3±6.1 g for high-sucrose, n=5). Basal glycaemia was again unchanged by diet (118.9±3.5 with standard diet and 109.0±4.7 mg/dl glucose with high-sucrose diet), as well as arterial blood pressure (92.3±7.0 with standard diet and 92.0±2.6 mmHg with high-sucrose diet).

The dynamic profiles obtained for Sprague-Dawley rats were similarly shaped to the control RIST, post-atropine RIST and HPN-dependent component in Wistar rats. So the results are shown as RIST indexes (Figure 21).

Figure 21 – RIST indexes of standard and high-sucrose fed Sprague-Dawley rats. Rapid insulin sensitivity test (RIST) indexes for the hepatic parasympathetic nerve (HPN)-independent (filled) and HPN-dependent (open) components of insulin action on 9 weeks old Sprague-Dawley rats. The sum of both components represents the control RIST index for each protocol. In both strains, insulin sensitivity was significantly higher for the standard-fed diet group than for the high-sucrose group (** $p<0.01$). A lower HPN-dependent component accounted for this difference.

The standard-fed Sprague-Dawley rats had, like what is shown in Wistar, a higher total insulin sensitivity than the sucrose-fed Sprague-Dawley rats (196.4±5.9 for standard-fed vs 95.5±16.3 mg glucose/kg bw for sucrose-fed; $p<0.01$) (Figure 21). Since the HPN-independent component was similar in both diet groups (84.7±6.0 for standard-fed and 61.0±10.1 mg glucose/kg bw for sucrose-fed), the reduced insulin action observed in sucrose-fed Sprague-Dawley rats was due to a decrease of the HPN-dependent component (111.7±9.5 for standard-fed vs 35.3±21.4 mg glucose/kg for the sucrose-fed group; $p<0.01$).

Accordingly, the contribution of the HPN-dependent component to the total insulin sensitivity was lower for the sucrose-fed group (56.0±3.7 for standard-fed and 28.8±13.8% for sucrose-fed; $p<0.05$).

Although the absolute values were lower in Sprague-Dawley than in Wistar rats, the percentage contribution of the HPN-dependent component was similar between the two strains for the standard-fed and sucrose-fed groups.

4.4.2.3. Discussion

It has been shown here that the availability of a supplement rich in the disaccharide sucrose leads to decreased total postprandial insulin sensitivity. Furthermore, this development of insulin resistance is solely due to an impairment of the HPN-dependent component of insulin action. This happened identically in both Wistar and Sprague-Dawley rats; even though Wistar show higher insulin-stimulated glucose uptake than Sprague-Dawley, the partial contribution of each component of insulin action is similar in the two strains for either standard-fed or high-sucrose-fed animals.

These results support the notion that an environmental manipulation as simple as a high sucrose supplement, which was used to show the effect of the increased public consumption of soft drinks and other sweetened products (Gross et al., 2004), leads to a dramatic impairment of the HPN-dependent component of insulin action. This increase in refined carbohydrate consumption, together with a decrease in fibre consumption, is reported to play a crucial role in the increased prevalence of type 2 diabetes (Gross et al., 2004; Henry et al., 1991).

Sucrose-enriched diets have been previously reported to lead to skeletal muscle insulin resistance (D'Alessandro et al., 2000; Hulman and Falkner, 1994; Kim et al., 1999; Pagliassotti et al., 1994). However, none of the previous studies took into consideration that insulin-stimulated glucose uptake consists of two discernable components, one of which is modulated by the activation of the hepatic parasympathetic nerves (Lautt, 1999), and the prandial status (Latour and Lautt, 2002b; Lautt et al., 2001).

Presently, the administration of atropine, a muscarinic antagonist, only decreased postprandial insulin sensitivity in standard-fed animals. High-sucrose-fed animals showed similar pre- and post-atropine RIST profiles, and subsequently indexes. Furthermore, the insulin sensitivity of post-atropine standard-fed animals was not statistically different from that obtained for pre- and post-atropine sucrose-fed rats, thus showing that the decrease in whole-body insulin sensitivity evidenced by sucrose-fed rats is entirely a reflection of the impairment of the HPN-dependent component of insulin action. It also indicates that this component is essentially absent from 9 weeks

old rats given access to a 35% liquid sucrose solution in complement to standard rat chow and regular water. Further, this supplement became the almost exclusive source of drinking fluids, showing a clear behavioural adherence to sweetened beverages.

The RIST methodology measures whole-body insulin-stimulated glucose uptake. High-sucrose diets are sometimes reported to induce hepatic insulin resistance besides skeletal muscle insulin resistance (Pagliassotti et al., 1994; Storlien et al., 1988), which could hamper the conclusion drawn that related the sucrose-induced insulin resistance observed solely to a decrease in glucose uptake at the skeletal muscle. However, those reports have come from studies in fasted animals, at low doses of insulin. Higher doses of insulin, even in the fasted state, seem to be able to adequately suppress hepatic glucose output, thus revealing decreased insulin sensitivity only at the skeletal muscle (Pagliassotti et al., 1994). The level of insulinaemia reached in the present study, by being in the postprandial state and performing a test after the bolus administration of insulin, should be enough to assure the latter situation.

This is consistent with the described action of the HPN-dependent component mainly on skeletal muscle (Lautt, 2005; Xie and Lautt, 1996b). And since there was no alteration in body weight or arterial pressure, obesity and hypertension may be excluded as confounding factors, in accordance with previous authors (Pagliassotti et al., 1994; Podolin et al., 1998; Santure et al., 2002; Santure et al., 2003).

The disaccharide sucrose is completely hydrolysed, after digestion by the enzyme sucrase, into equal amounts of the monosaccharides glucose and fructose, and absorption, at the small intestine, happens similarly either with sucrose or with an equimolar mixture of glucose and fructose (Dahlqvist and Thomson, 1963). However, after a high-sucrose meal, fructose is metabolised much more rapidly than glucose (Daly et al., 2000). More, fructose has been suggested to be the moiety responsible for sucrose-induced insulin resistance (Thresher et al., 2000).

Although fructose has been widely advised as a favourable carbohydrate substitute in the diet of diabetic subjects (Gerrits and Tsalikian, 1993), for its capacity to acutely produce lower postprandial glucose and insulin responses (Shiota et al., 2002) at catalytic amounts (Moore et al., 2001; Shiota et al., 2002), the continuous ingestion of this monosaccharide may induce metabolic adaptations that ultimately lead to a deterioration in glycemic control.

After digestion and absorption, fructose is rapidly taken up by the portal blood flow and transported mainly to the liver. Small amounts are metabolised in the small intestinal mucosa and the kidney, and almost none at all by the peripheral tissues (Hallfrisch, 1990). In the liver, fructose is phosphorylated by fructokinase and gives rise to an increase in the triose phosphate pool. This is done by bypassing the rate-limiting step of glycolysis, the enzyme phosphofructokinase (Mayes, 1993). The greater availability of intermediates created favours the glycolytic pathway; with a higher production of lactate (Mayes, 1993), seen even when catalytic amounts of fructose are used (Moore et al., 2001).

The concomitant presence of fructose and glucose, as when derived from sucrose, induces hepatic glycogen deposition of glucose (Hallfrisch, 1990; Mayes, 1993), which does not happen when fructose is given alone (Hallfrisch, 1990). Thus, after a high-sucrose content meal, while the glucose moiety is diverted to hepatic glycogen deposition, fructose is quickly metabolised by the liver (Daly et al., 2000) and predominantly driven into the production of lactate.

Feeding a high-sucrose diet leads to increased intestinal absorption of both products of hydrolysis, fructose and glucose (Michaelis et al., 1975; Reiser et al., 1975; Riby et al., 1993), and the long term consumption of sucrose or fructose primes the organism for these changes by optimizing even more the enzymatic machinery involved (Mayes, 1993; Reiser et al., 1981). Fructose is transported, when present at low concentrations, by the GLUT 5 (Ushijima et al., 1995). The disaccharide transport effect of sucrose (Michaelis et al., 1975; Michaelis and Szepesi, 1974) leads to increased fructose absorption by action of the enzyme sucrase (Ushijima et al., 1995) and transient insertion of GLUT 2 into the enterocyte apical membrane (Kellett and Brot-Laroche, 2005; Kellett et al., 2008), when fructose concentration is higher than 30 mmol/L (Ushijima et al., 1995), usually after meals (Kellett et al., 2008).

Also, the aforementioned beneficial lower levels of post-meal glucose and insulin response to fructose ingestion have been reported to become similar to other sugars when there is a prolonged consumption of fructose within mixed meals (Hallfrisch, 1990).

In time, sucrose supplementation leads to postprandial hyperinsulinemia (Mayes, 1993), with an initial compensatory increase in the first peak of insulin secretion to maintain normoglycemia and then further deterioration in insulin kinetics with time of

exposure (Chicco et al., 2003). Also, GLUT2 permanent apical localisation is related to insulin resistance induced by sucrose/fructose ingestion and diabetes (Kellett et al., 2008). Hyperinsulinemia may thus be favoured in an attempt to re-establish the ability of insulin to bind to insulin receptors in the enterocyte needed to internalise GLUT2 (Tobin et al., 2008); which normally prevents transepithelial sugar absorption, to minimise postprandial glucose excursions. Impaired insulin action induced by fructose-containing diets lead to three-fold GLUT2 protein and permanent high apical GLUT2 insertion (Kellett et al., 2008).

Other long term effects include increased hepatic lipogenesis and increased circulating triglyceride through very-low-density lipoproteins hepatic secretion. Together with the rise in lactate production, the hypertriglyceridemia can be a possible contributor to the development of skeletal muscle insulin resistance (Chicco et al., 2003; D'Alessandro et al., 2000).

The present results show that the exposure to a high-sucrose diet leads primarily to an impairment of the HPN-dependent component of insulin action. This was obtained by simply providing the animals with a liquid supplement of 35% of sucrose. Changes in the direct action of insulin on peripheral tissues may eventually occur, product of the above mentioned adaptations to the high-sucrose diet (Chicco et al., 2003), but they will be secondary to the loss of the postprandial increment in insulin action. Indeed, alterations of fasting insulin sensitivity are reported to occur when the content of sucrose in the diet is higher than 60 %, or the content of fructose higher than 34% (Daly et al., 1997), while acute effects of sucrose on enzymatic activities are already seen when it constitutes 20 to 30 % of total diet (Michaelis and Szepesi, 1974).

Likewise, the reports of hypertension in high-sucrose/fructose diets (Preuss et al., 1998; Reaven and Ho, 1991) seem to be related to higher/longer sugar doses than the one available in the present study (Preuss et al., 1998), which is consistent with the lack of change in arterial blood pressure reported herein. The sodium and potassium content of the diet may also be a confounding factor in the heterogeneity of hypertension development with high-sugar diets[68] (Bezerra et al., 2001; Claxton and

[68] The development of hypertension by sugar loading may be a consequence of the development of hyperglycaemia. Hyperglycaemia induces overactivity of the sympathetic nervous system, which favours the rise in blood pressure. On the other hand, insulin-stimulated nitric oxide has been reported to oppose this mechanism by suppressing sympathetic activity (Claxton and Brands, 2002). The impairment of nitric oxide in conditions associated with insulin resistance, hyperglycaemia, and oxidative stress may thus bring about the rise in blood pressure. Since the present methodology was able to render the animals

Brands, 2003; Johnson et al., 1993). Nonetheless, in the present study hypertension has been shown not to be an etiological factor to the insulin resistance induced by high-sucrose feeding through the decrease of the HPN-dependent component.

For all the above, it seems likely that the ability of sucrose to induce a decrease in insulin sensitivity is mediated by a direct metabolic effect of fructose on the HPN-dependent component of insulin action (Brito et al., 2008). Other effects, as hypertension and changes in lipid profile, are most likely consequences of further metabolic deterioration of long-lasting high-sucrose diets (Ahrens, 1974).

The present finding highlights the importance, to the study of high-sucrose-fed animals, of reports of decreased parasympathetic vagal activity in high-fructose-fed rats (Miller et al., 1999).

Recently, an increasing emphasis is being placed on a causal effect of an autonomic imbalance in the metabolic syndrome (Kreier et al., 2003). In this imbalance, brought about by environmental factors, the decrease of parasympathetic activity seems to be the determining factor in the development of insulin resistance, which favours the progression to type 2 diabetes mellitus (Lindmark et al., 2003). An increase in sympathetic autonomous system activity may also play a role, aggravating this imbalance (Kreier et al., 2003). In this respect, high-sucrose diets seem to be able to induce an increase in central sympathetic outflow (Bunag et al., 1983; Freitas et al., 2007). However, this is secondary to the impairment of insulin sensitivity (Bezerra et al., 2001; Hwang et al., 1987), and nonetheless may not yet be present in the model studied herein, since sympathetic overactivity is described to be related to the manifestation of hypertension (Bunag et al., 1983; Valensi, 2005), which is presently absent.

This rising imbalance against parasympathetic activity is consistent with the present study, an example of how an environmental alteration, such as diet composition, can lead quickly and radically into postprandial insulin resistance through an impairment of hepatic parasympathetic activity.

This also underlines the importance of postprandial insulin assessment, in comparison with the standard evaluation on the fasted state, as providing an early warning to the development of insulin resistance and, from there, of other potential

insulin-resistant without becoming hyperglycaemic and/or hypertensive, this question seems definitely to be related to a further metabolic degradation, unrelated to the development of insulin resistance

metabolic complications. Indeed, considering that HPN-dependent component action is vestigial in the fasted state (Lautt et al., 2001), the standard analysis would yield no sign of impairment after the 6 weeks of high-sucrose supplementation, when by then it has been shown here that almost 50% of total postprandial insulin sensitivity is lost. Furthermore, sucrose-induced insulin resistance in skeletal muscle has been related to the impairment of a humoral factor, which would explain why only *in vivo*, and not *in vitro*, studies show skeletal muscle insulin resistance in sucrose-fed rats when compared to starch-fed rats (Kim et al., 1999). This again hints to the incorporation of the HPN as a functional step of the broader HISS pathway.

In further support to the relevance of the postprandial state in the evaluation of the impact of dietary manipulations, high-sucrose diets lead to transient peaks of hyperglycaemia and hyperinsulinaemia after meal ingestion (Daly, 2003; Daly et al., 1998). And it is now believed that those episodes of hyperglycaemia are extremely relevant to the development of metabolic complications (Wright et al., 2006), even in individuals with otherwise achieved glycaemic control (Del Prato, 2002; Tuomilehto and Del Prato, 2001).

In resume, it seems that the applied model uncovered the primary step of sucrose-induced insulin resistance. At a time when body weight, glycaemia, blood pressure and direct metabolic effects of insulin on peripheral target tissues are not yet compromised, both Wistar and Sprague-Dawley rats showed consistently an entire loss of the increment of insulin action sensitisation seen after a meal. As shown before, this increment is signalled through a HPN-dependent pathway, which function seems to be quickly impaired by a high-sucrose diet. This defect in the postprandial capability of the organism to deal with the increased influx of glucose and fructose will undoubtedly lead to several complications and metabolic degradation.

It becomes thus expectable, and has been described (Chicco et al., 2003; Pagliassotti et al., 1996; Pagliassotti et al., 1994), that the duration of exposure may vary the effects of the high-sucrose diet. So, after obtaining such a radical result simply by providing the animals with an additional bottle of sucrose solution for 6 weeks, it became relevant to study the time-course of the impact of the high-sucrose diet on insulin sensitivity.

4.4.3. Effect of the duration of exposure to a liquid high-sucrose diet in Sprague-Dawley rats

4.4.3.1. Experimental protocols

Since it was observed that age is also a critical factor for the status of the HPN-dependent component of insulin action, the sucrose-diet was introduced at different ages, with the animals being analysed at the same final age. So, they were analysed at 12 weeks of age, with sucrose being introduced, depending on the group, 2, 6 or 9 weeks before.

Sprague-Dawley rats were divided into four groups. All were given standard solid food and a bottle of water. At 3, 6 or 9 weeks of age the bottle of water was substituted by a bottle of a 35% sucrose solution. The standard-diet group had plain water for the entire experiment.

4.4.3.2. Results

The group submitted to 9 weeks of sucrose supplementation group showed, in comparison with the standard-fed group, higher basal glycaemia (106.3±3.3 for standard-fed, n=9, vs 142.3±8.8 mg/dl for 9 weeks sucrose supplemented diet, n=11; $p<0.05$) and higher body weight (459.1±4.7 for standard-fed vs 643.1±25.9 g for 9 weeks sucrose supplemented diet; $p<0.0001$). For all other exposure times, sucrose-fed showed similar values to standard-fed rats for basal glycaemia (120.1±4.7 for 2 weeks, n=7, and 109.0±4.1 mg/dl for 6 weeks, n=5) and body weight (460.0±9.8 for 2 weeks and 489.2±29.3 g for 6 weeks of sucrose diet). Arterial blood pressure was similar between the standard-fed and all sucrose-fed groups (101.9±2.9 for standard-fed vs 97.9±3.7 for 2 weeks, 92.3±7.0 for 6 weeks, and 92.0±2.6 mmHg for 9 weeks of high-sucrose diet).

Figure 22 integrates the data for the total insulin action and for each of the components that constitute it: HPN-dependent and –independent components of postprandial insulin action.

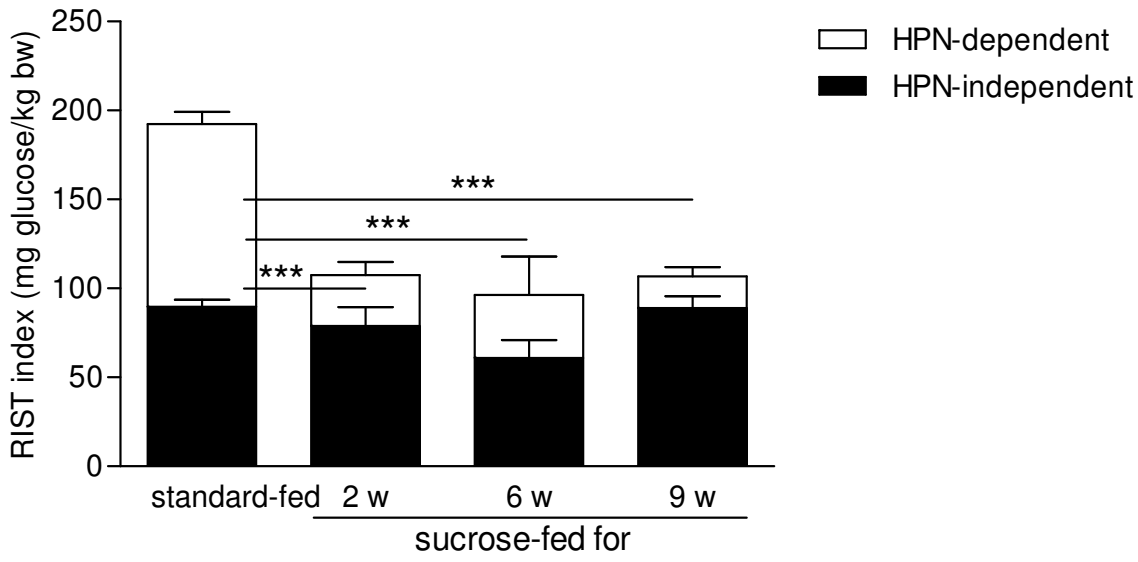


Figure 22 – Effect of exposure time to high-sucrose diet in Sprague-Dawley rats. Rapid insulin sensitivity test (RIST) indexes for the hepatic parasympathetic nerve (HPN)-independent (filled) and HPN-dependent (open) components of insulin action of Sprague-Dawley rats on a high-sucrose diet for different periods. The sum of both components represents the control RIST index. Insulin sensitivity was significantly higher for the standard-fed diet group than for the sucrose-fed group. The HPN-dependent component accounted for this variation (*** $p<0.001$).

All sucrose-fed groups had similar total insulin sensitivity (106.2±12.5 with 2 weeks of sucrose diet, n=7, 95.5±16.3 with 6 weeks of sucrose diet, n=5, and 106.6±8.4 mg glucose/kg bw with 9 weeks of sucrose diet, n=11), which was lower than in the standard-fed (192.4±4.9 mg glucose/kg bw; $p<0.001$).

Since there was no statistical difference in post-atropine RIST index between standard-fed and any sucrose-fed group (89.6±4.1 for the standard-fed and 78.9±10.6 with 2 weeks of sucrose diet, 61.0±10.1 with 6 weeks of sucrose diet, and 88.8±6.7 mg glucose/kg bw with 9 weeks of sucrose diet), the decrease of total insulin sensitivity was due to lower HPN-dependent component in any sucrose-fed group (102.7±6.9 for standard-fed vs 28.5±7.6 with 2 weeks of sucrose diet, 35.3±21.4 with 6 weeks of sucrose diet, and 17.9±5.4 mg glucose/kg bw with 9 weeks of sucrose diet; $p<0.001$).

Accordingly, the contribution of the HPN-dependent component to total insulin sensitivity was lower in all sucrose-fed groups than in the standard-fed group (52.5±2.6% for the standard-fed vs 26.4±6.2% with 2 weeks sucrose diet, 28.8±13.8%

with 6 weeks of sucrose diet, and 17.9±5.4% with 9 weeks sucrose diet; $p<0.01$, $p<0.05$ and $p<0.001$, respectively).

4.4.3.3. Discussion

The insulin resistance caused by the liquid high-sucrose diet, through impairment of the HPN-dependent component of insulin action, is fully expressed after only 2 weeks of exposure to the source of sucrose. In contrast, the HPN-independent component is unchanged, even after 9 weeks of exposure to the high-sucrose diet.

The group subjected to the longer period of sucrose exposure (9 weeks) showed a higher weight gain in relation to the standard-fed group. We can therefore exclude weight gain as a confounding factor since there was no additional increase of insulin resistance in this group. The added influence of obesity did not seem to have an additional impact on the HPN-dependent component, already impaired, or on the HPN-independent component. It was more likely an additional effect of the high-sucrose diet; possibly through increased lipogenesis derived from the augmented metabolism of fructose (Chicco et al., 2003; Mayes, 1993). Interestingly, dietary interaction in obese children showed a closer relation between carbohydrate metabolism and sucrose intake than between the former and body weight (Kahle et al., 1982).

In addition, since basal hyperglycaemia was only observed in the rats exposed to the sucrose supplement for 9 weeks, we conclude that the impairment of the HPN-dependent component is not the result of high blood glucose levels, because sucrose-fed rats exposed for 2 or 6 weeks were similarly insulin resistant, despite being normoglycaemic. However, it is expected that the rise in blood glucose will eventually lead to defects by protein glycation (Gerrits and Tsalikian, 1993).

The excess weight gain seen by the time postprandial hyperglycaemia is attained may be related with the surplus time that glucose is available in circulation after a meal, increasing the quantity diverted for adipose tissue accumulation.

The high-sucrose diet has been shown to have a quick detrimental influence on insulin sensitivity. This supplementation has been given in all previous protocols in liquid form. However, questions have been raised concerning the differential effect that the way of delivery may have on protecting against these deleterious effects, or even have a beneficial effect on insulin sensitivity (Baschetti, 1999).

4.4.4. Effect of liquid vs solid high-sucrose diet in Sprague-Dawley rats

4.4.4.1. Experimental protocols

The physical form in which sucrose is made available may be an important factor on the magnitude of the metabolic effects it elicits (Crapo and Henry, 1988; Kanarek and Orthen-Gambill, 1982). To study if the way of delivery of the sucrose supplement bore any influence on the way this diet impaired insulin sensitivity, Sprague-Dawley rats were divided into three groups. One received standard-solid-diet and water, other received standard-solid-diet and a sucrose solution, and the third group received high-sucrose-solid-diet and water. Since the objective was to study the possible protective role of the solid form, the time of exposure chosen was the longest previously used.

4.4.4.2. Results

The groups given access to a high-sucrose diet, either in the solid meal composition or by a liquid supplement, showed higher body weight standard-fed animals (441.6±42.9 for standard-fed, n=7, vs 577.5±22.3 g for solid high-sucrose, n=6, $p<0.05$, and 622.3±21.8 g for liquid high-sucrose, n=5, $p<0.01$). Concerning basal glycaemia, only the liquid high-sucrose group showed an increase in relation to the standard-fed (138.0±9.8 for liquid high-sucrose vs 108.7±4.2 mg/dl, $p<0.05$), with the solid high-sucrose group showing normal levels (111.7±2.8 mg/dl). Arterial blood pressure was unchanged (98.6±5.0 for standard-fed, 100.4±3.2 for solid high-sucrose, and 95.5±2.6 mmHg for liquid high-sucrose).

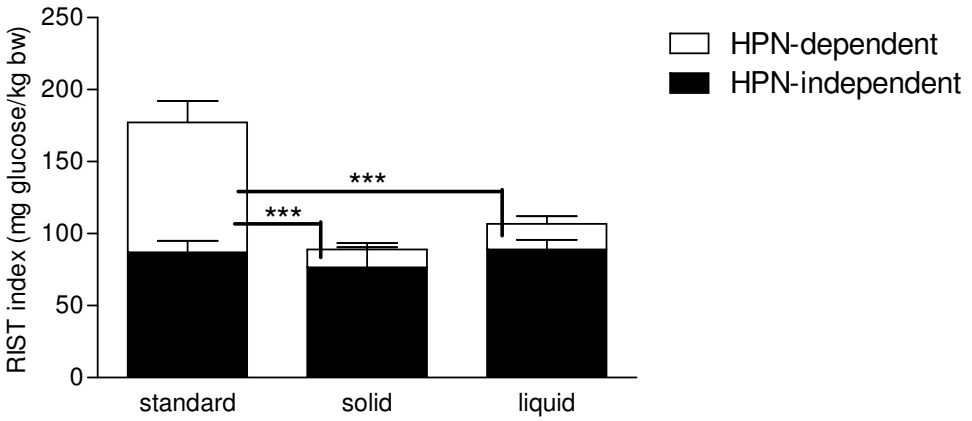


Figure 23 – Effect of delivery form of high-sucrose diet in Sprague-Dawley rats. Rapid insulin sensitivity test (RIST) indexes for the hepatic parasympathetic nerve (HPN)-independent (filled) and HPN-dependent components of insulin action of Sprague-Dawley rats fed either a standard diet or given a liquid or solid sucrose supplement. The sum of both components represents the control RIST index. Insulin sensitivity was significantly higher for the standard diet for any of the high-sucrose-fed groups. The HPN-dependent component accounted for this difference (*** $p<0.001$).

Both liquid- and solid-sucrose-fed groups had similarly lower total insulin sensitivity than the standard-fed group (177.3±12.7 for standard-fed vs 100.7±10.6 for liquid-sucrose-fed, and 88.2±14.6 mg glucose/kg bw for solid-sucrose-fed; $p<0.05$ and $p<0.001$, respectively) (Figure 23).

After atropine administration, the RIST indexes obtained were similar among all groups (86.9±8.0 for standard diet, 79.4±6.5 for liquid-sucrose diet, and 76.5±14.1 mg glucose/kg bw for solid-sucrose diet). The HPN-dependent component was lower in both sucrose-fed groups than in the standard-fed group (90.4±14.7 for standard diet, vs 21.5±8.0 for liquid-sucrose diet, and 12.3±4.7 mg glucose/kg bw for solid-sucrose diet; $p<0.001$). Thus, the contribution of the HPN-dependent component was also lower in sucrose-fed animals than in the standard-fed group (48.5±6.9% with standard diet, vs 18.9±6.1% with liquid-sucrose diet, and 14.2±6.9% with solid-sucrose diet; $p<0.001$).

4.4.4.3. Discussion

The exposure to a high-sucrose intake during 9 weeks has again shown to produce insulin resistance, as well as other changes. Only the liquid high-sucrose group showed an exaggerated blood glycaemic level, which was accompanied by a higher weight gain. However, the normoglycaemic solid high-sucrose group evidenced already a higher body weight than the standard-fed. Albeit this differences in outcome, insulin sensitivity was similarly impaired by both forms of sucrose feeding (solid vs liquid).

The hyperglycaemia observed in the liquid high-sucrose group may be due to the faster availability of sucrose to be hydrolysed in the small intestine by sucrase when given already in a solution. This would also lead to an increase in recruitment of enterocyte GLUT2, leading with continued exposure to maintained inception of these glucose transporters on the apical wall (Kellett et al., 2008). This exacerbate transport of sugars through the enterocyte brings forth higher levels of circulating glucose and fructose. The same amount of sucrose, arriving more sporadically and subject to a slower digestion, as with the solid meal, might not produce such an extreme exposure.

Fructose has usually been perceived by the general consumer as having beneficial effects due to its supposedly healthy fruit origin and to the fact that diabetics are encouraged to replace other sugars in their diet by fructose. Furthermore, the use of high-fructose and high-sucrose sweeteners in the food industry has increased (Henry et al., 1991).

The detrimental effects of a high-sucrose supplement shown herein, both by the magnitude and quickness of impact, illustrate the necessity of a closer scrutiny and care into the consumption of such products; moreover when children and youngsters are a preferential marketing target for most of them. But also adults may be affected, since people with worse metabolic profiles may be more sensitive to the detrimental effects of sucrose (Daly et al., 1997; Michaelis and Szepesi, 1974), and since dietary manipulations with sucrose have been shown to deteriorate even further the reduction in insulin-stimulated glucose uptake seen with age (Berdanier et al., 1979; Lautt et al., 2009; Reaven et al., 1983).

The fact that only the HPN-dependent component of insulin action, a pathway highly modulated by the prandial status, was affected at this point further argues for the

importance of the evaluation of this component as an early marker of peripheral insulin resistance.

4.5. Hepatic insulin sensitising substance (HISS) mechanism status and role on insulin sensitivity in an animal model of essential hypertension

4.5.1. Hepatic parasympathetic nerves (HPN)/hepatic nitric oxide (HNO) branch of the HISS pathway in hypertension

4.5.1.1. General context and work hypothesis

Hypertension seems to be associated with insulin resistance and compensatory hyperinsulinemia; and further with type 2 diabetes and other features of the metabolic syndrome (DeFronzo and Ferrannini, 1991; Ferrannini et al., 1987; Frontoni et al., 2005; Landsberg, 2005; Reaven, 1993). The precise causal relations between these pathologies are still shrouded by uncertainty. Reasons have been forwarded for an effect of insulin resistance both on the aetiology and on the complications of hypertension (DeFronzo and Ferrannini, 1991; Frontoni et al., 2005; Katakam et al., 1998; Reaven, 1991; Sowers, 2004).

Insulin resistance and sympathovagal imbalance are believed to be important factors in the connection between hypertension and diabetes (Frontoni et al., 2005). Furthermore, the disruption of the parasympathetic response has been reported to worsen glucose tolerance in individuals diagnosed with hypertension (Chen et al., 1998). Insulin resistant states are also related to dysfunctional responses of the autonomic nervous system to insulin, with increased sympathovagal balance (augmented sympathetic reactivity and unresponsive parasympathetic control); that could influence cardiovascular problems (Bergholm et al., 2001). Autonomic dysfunction seems even to exist at the prehypertensive stage (Lucini et al., 2002; Paolisso et al., 2000; Weston, 2000). These provide us clues to believe in the involvement of the HPN-dependent component in the relation between insulin resistance and hypertension.

Essential hypertension is a common cause of cardiovascular and cerebrovascular complications. Of idiopathic origin, a set of genetic, psychological and dietary factors seem to concur to its development (Das, 2001). The development of hypertension has been related to the presence of obesity (Reaven et al., 1996). However, since obesity has been shown to have in itself a detrimental effect on insulin sensitivity (Afonso et al., 2007a; Afonso et al., 2007b; Montani et al., 2002), the two factors had to be separated.

Thus, the spontaneously hypertensive rat (SHR) was used to evaluate the relation between insulin resistance and essential hypertension. The SHR is a genetic pathological model widely used for these studies because it presents similar symptoms to the corresponding human pathology (Dickhout and Lee, 1998; Lerman et al., 2005).

Insulin resistance in essential hypertension may also be connected to a nitric oxide dysfunction (Nava et al., 1998). Since hepatic nitric oxide (HNO) is also a metabolic intervenient in the hepatic insulin sensitising substance (HISS) hypothesis signalling pathway, in which the HPN seem to play a crucial role, we also studied this component, by use of a nitric oxide synthase inhibitor; administered directly in the liver, through the portal vein. As before, no studies had previously been reported on the SHR that address the relation of the HPN or HNO to changes in postprandial insulin sensitivity.

Furthermore, contradicting results put in question (Klimes and Sebokova, 1997) both the condition of insulin resistance and which of its common normotensive controls should be used – Wistar or Wistar Kyoto (WKY). Whereas several studies show a decreased insulin-stimulated disposal in SHR (Hulman et al., 1991; Katayama et al., 1997; Mondon and Reaven, 1988), others report a similarity (Frontoni et al., 1992; Katayama et al., 1997; Natalucci et al., 2000) or even an increase (Buchanan et al., 1992; Tsutsu et al., 1989), in comparison to WKY or Wistar.

Considering this, besides studying the contributions of the HPN- and HNO-dependent and –independent components of insulin action in hypertension-related insulin resistance, the aim of this study was to compare these characteristics among all three strains. The meal-induced sensitisation of insulin action was also assessed in this non-obese animal model of essential hypertension.

Our **working hypothesis** was that hypertension is associated with insulin resistance in SHR, and that insulin resistance is related with an impairment of the HPN-

dependent component of postprandial insulin action. We **also hypothesised** that other steps of the HISS pathway will show similar impairment.

4.5.1.2. Experimental protocols

Nine weeks old male Wistar, WKY and SHR rats were analysed on the postprandial state. All animals were under continuous anaesthesia.

In a first batch of animals from the three strains, after a control RIST, atropine (3 mg/kg) was administered to enable the differentiation of the HPN-dependent and –independent components. A second RIST was performed, postatropine, providing the quantification of the HPN-independent component of insulin action.

In a second batch of animals, the control RIST was performed, followed by the intraportal (ipv) administration of Ng-methyl-arginine (L-NMMA; 0.73 mg/kg), to prevent the production of HNO.

4.5.1.3. Results

4.5.1.3.1. Assessment of the hypertensive condition

The SHR had higher arterial blood pressure than Wistar or WKY (159.2±3.7 for SHR, n=29, vs 118.1±3.7 for Wistar, n=18, and 106.7±2.5 mm Hg for WKY, n=20; $p<0.001$; pooled measurements done from all animals at the beginning of experiments), confirming the hypertensive condition in those animals. These values were not significantly altered with the administration of iv atropine (145.0±12.3 mmHg for SHR 114.8±7.7 for Wistar, and 95.0±13.2 for WKY). With ipv L-NMMA, any change of blood pressure induced during the perfusion was transient, and no alteration was observed by the time the post-L-NMMA RIST was began (143.6±5.4 mmHg for SHR, 110.3±8.9 for Wistar, and 90.0±7.3 for WKY).

4.5.1.3.2. Other metabolic parameters

All strains presented similar body weight (313.8±15.4 for Wistar, 283.3±11.0 for WKY and 278.4±9.5 g for SHR). Glycaemias were also similar among strains (128.4±11.0 for Wistar, 133.0±6.9 for WKY, and 123.1±7.2 mg/dl for SHR), and not

altered by atropine (131.8±8.3 for Wistar, 112.6±7.9 for WKY, and 130.5±8.2 mg/dl for SHR) or L-NMMA administration (132.8±9.8 for Wistar, 143.2±9.0 for WKY, and 136.9±7.5 mg/dl for SHR).

4.5.1.3.3. Insulin sensitivity before and after parasympathetic blockade in hypertension

The two normotensive control strains for SHR, Wistar and WKY, showed very different results. Indeed, whereas SHR showed to be insulin resistant when compared with Wistar (296.1±16.9 for WIS, n=12, vs 217.8±19.8 mg glucose/kg bw for SHR, n=13; $p<0.01$), WKY showed similar total insulin action to SHR (209.1±13.6 for WKY, n=12), thus also being insulin resistant when compared with Wistar ($p<0.01$) (Figure 24).

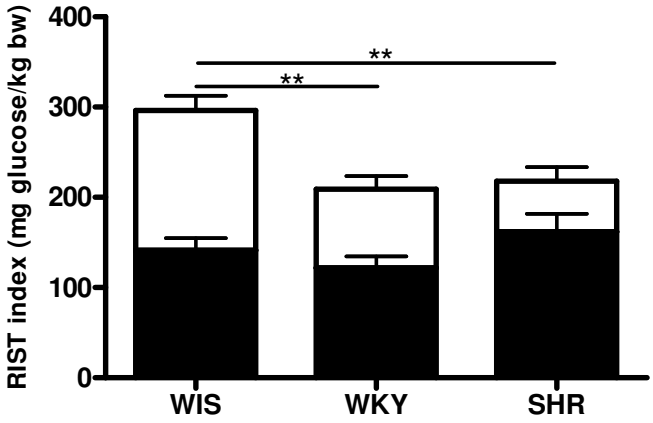


Figure 24 – Effect of hypertension on postprandial glucose metabolism in comparison to two normotensive controls; before and after muscarinic antagonism. Rapid insulin sensitivity test (RIST) indexes for the hepatic parasympathetic nerve (HPN)-independent (filled) and HPN-dependent (open) components of total insulin action for 9 weeks old male Wistar (WIS), Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR). The sum of both components represents the control RIST index for each strain. Insulin sensitivity was significantly higher for WIS than for WKY (** $p<0.01$) or SHR (** $p<0.01$). The HPN-dependent component accounted for these differences ($p<0.05$ for WKY and $p<0.001$ for SHR, not shown on the graph).

The RIST indexes obtained after atropine administration did not show any significant difference between the three strains (141.3±13.6 for Wistar, 121.8±12.8 for WKY and 161.8±19.7 mg glucose/kg bw for SHR) (Figure 24).

The SHR presented a lower HPN-dependent component of insulin action than Wistar (55.9±16.0 for SHR vs 154.8±16.4 mg glucose/kg bw for Wistar, $p<0.001$), and with WKY showing an intermediate value, also statistically different from Wistar (87.1±14.6 mg glucose/kg bw, $p<0.05$).

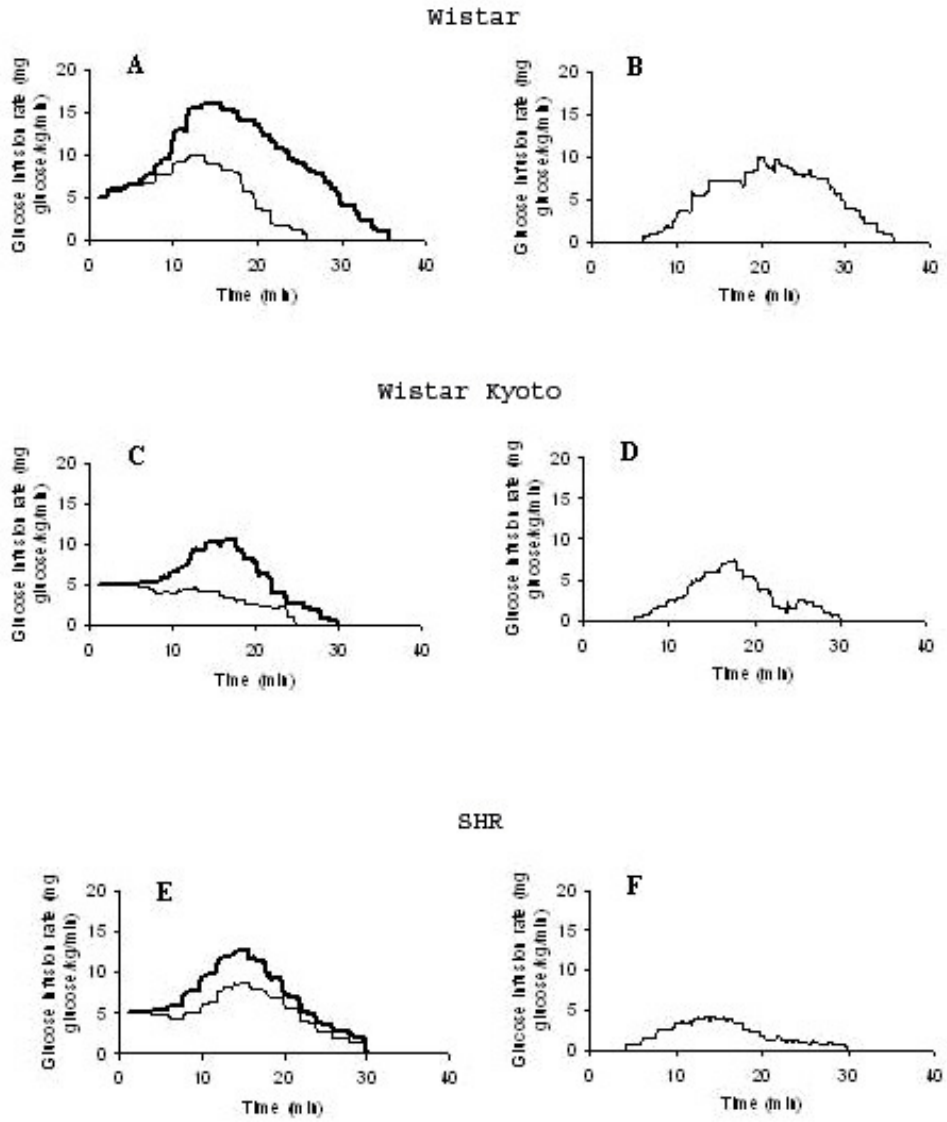


Figure 25 – RIST profiles of spontaneously hypertensive rats (SHR), and their normotensive controls Wistar and Wistar Kyoto; at 9 weeks of age. A, C and E, Dynamic profiles of the control (bold line) and postatropine (thin line) RISTs of Wistar, Wistar Kyoto, and SHR, respectively. B, D and F, Dynamic profile of the hepatic parasympathetic nerve (HPN)-dependent component of insulin action obtained after subtracting the post-atropine curve from the control curve of Wistar, Wistar Kyoto and SHR, respectively.

The characteristics of the dynamic profiles of the RISTs obtained before and after atropine administration (HPN-dependent component) as well as the HPN-dependent component (Figure 25) are described in Table VII.

Table VII – Dynamic curve main properties of normotensive Wistar and Wistar-Kyoto (WKY), and spontaneously hypertensive rat (SHR). Dynamic profile characteristics of the control rapid insulin sensitivity test (RIST), the hepatic parasympathetic nerve (HPN)-independent component (postatropine RIST), and the HPN-dependent component of total insulin action (* $p<0.05$ between Wistar and WKY, # $p<0.05$ between Wistar and SHR, & $p<0.01$ between Wistar and WKY).

		Wistar	WKY	SHR
Control RIST	Peak	16.2±0.7*	11.7±1.7*	13.3±1.2
	Peak time	11.9±0.5	13.4±1.6	12.7±0.6
	Offset	34.9±1.3	29.1±1.7	30.4±1.7
HPN-independent	Peak	11.3±1.3*	6.3±0.7*	9.6±1.1
	Peak time	10.7±0.8	12.5±2.7	12.1±0.9
	Offset	23.4±2.0	22.1±2.8	27.3±1.9
HPN-dependent	Onset	7.7±1.2	7.1±0.6	6.4±1.0
	Peak	10.9±1.1#	8.4±1.2	6.5±1.0#
	Peak time	16.2±1.3#	15.5±1.0	12.1±1.1#
	Offset	34.4±1.4#&	25.5±1.5&	26.3±1.8#
	Duration	26.7±1.7*#	18.4±1.8*	19.9±2.0#

For the characteristics related to the control and postatropine RISTs, the only significant differences found were surprisingly between Wistar and WKY. In relation to Wistar, the WKY strain showed a decrease in maximal (peak) response both in the control RIST (from 16.2±0.7 in Wistar to 11.7±1.7 mg glucose.kg^{-1}.min^{-1} in WKY, $p<0.05$) and in the postatropine RIST (from 11.3±1.3 in Wistar to 6.3±0.7 mg glucose.kg^{-1}.min^{-1} in WKY, $p<0.05$). Since SHR showed values that are intermediate for these characteristics, it produced no statistical difference.

The duration of action of the HPN-dependent component was similarly decreased in WKY and SHR, in relation to Wistar rats (from 26.7±1.7 in Wistar to 18.4±1.8 min in WKY, $p<0.05$, and 19.9±2.0 min in SHR, $p<0.05$). This was due to an

early offset of action of this component (from occurring at 34.4±1.4 min in Wistar to 25.5±1.5 min in WKY, $p<0.01$, and to 26.3±1.8 min in SHR, $p<0.05$).

Other alterations of the main characteristics of the dynamic profiles were only seen in SHR. Peak action was decreased (from 10.9±1.1 in Wistar to 6.5±1.0 mg glucose.kg^{-1}.min^{-1} in SHR, $p<0.05$), as well as maximum response happened slightly earlier in SHR (from 16.2±1.3 in Wistar to 12.1±1.1 min in SHR, $p<0.05$).

4.5.1.3.3. Role of hepatic nitric oxide on insulin sensitivity in essential hypertension

Again, both WKY and SHR showed lower total insulin sensitivity indexes than Wistar (225.3±23.8 for WKY, n=8, and 238.2±10.6 for SHR, n=16, vs 298.9±8.6 mg glucose/kg bw for Wistar, n=6, $p<0.05$ in both cases).

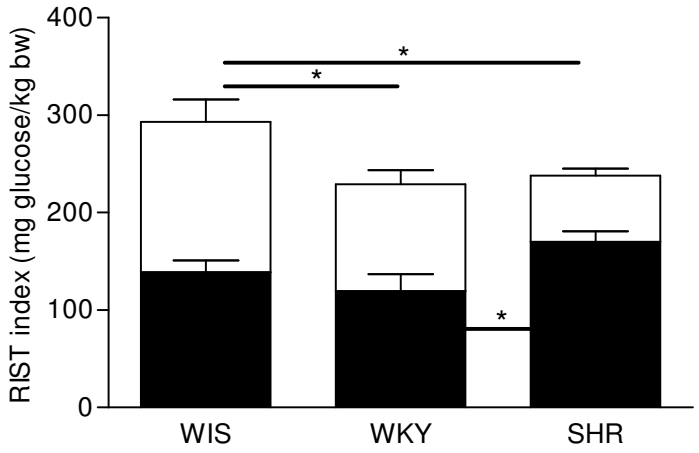


Figure 26 – Effect of hypertension on postprandial glucose metabolism in comparison to two normotensive controls; before and after nitric oxide inhibition. Rapid insulin sensitivity test (RIST) indexes for the hepatic nitric oxide (HNO)-independent (filled) and HNO-dependent (open) components of total insulin action for 9 weeks old male Wistar (WIS), Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR). The sum of both components represents the control RIST index for each strain. Insulin sensitivity was significantly higher for WIS than for WKY or SHR (* $p<0.05$). The HNO-dependent component accounted for these differences ($p<0.05$ for WKY and $p<0.001$ for SHR, not shown on the graph).

The HNO-independent component, quantified by the RIST after ipv *L*-NMMA administration, showed a significant increase in the SHR when compared with the WKY (151.2±6.8 for Wistar; 123.3±19.1 for WKY vs 170.0±10.9 mg glucose/kg bw for SHR, $p<0.05$) (Figure 26).

As in the previous protocol, the HNO-dependent component was lower in SHR than in Wistar (68.2±7.1 for SHR vs 147.2±13.6 mg glucose/kg bw for Wistar, $p<0.001$), and with WKY showing an intermediate value, here also statistically different from Wistar (102.0±13.5 mg glucose/kg bw, $p<0.05$).

The data for the dynamic profiles for this protocol, similar to the ones obtained in the previous protocol, are not shown.

4.5.1.4. Discussion

The aim of the study was to characterize several steps of the HISS-dependent and –independent components of postprandial insulin sensitivity in an animal model of essential hypertension, the SHR, and two normotensive controls, Wistar and Wistar Kyoto.

Insulin resistance was found when comparing SHR and Wistar. This impairment was solely due to a dysfunction of the HISS-dependent component. However, the WKY has shown similar insulin sensitivity to SHR. This argues either for a secondary role of hypertension on the development of insulin resistance or to a separate defect on WKY that independently impairs the HISS-dependent component in that strain.

At 9 weeks of age, SHR showed already higher arterial blood pressure and had lost the tendency to be lighter than the normotensive strains, as was expected (Dickhout and Lee, 1998). Likewise, hypertensive rats showed to be normoglycaemic.

The insulinemias of the three strains have been described as being similar (Buchanan et al., 1992; Farrace et al., 1995; Swislocki et al., 1997) or augmented on the SHR (Chen et al., 1994a; Gouveia et al., 2000a; Mondon et al., 1989). The insulinemias have not been measured in the present studies. However, since the RIST constitutes a measure of the response of an exogenous bolus of insulin, this may not be a critical factor.

The lack of definition in genetics (St Lezin et al., 1992) between SHR, WKY, and Wistar, from which both the previous two strains were derived, further disturbs the analysis of this animal model. Authors trying to trace the way by which the strains have been established, and possible inconsistencies between stocks of different academic or commercial origin, have come across several difficulties (Furukawa et al., 1998; Kurtz and Morris, 1987). To try to reduce to a minimum this interference, all animals analysed herein were from a single commercial source.

According to the present studies, SHR and WKY show similar total postprandial insulin sensitivity as measured by the control RIST index. Furthermore, both strains show decreased total postprandial insulin sensitivity when compared with Wistar rats. This seems due to a decrease of the HISS-dependent component of insulin action; either quantified by blockade of the HPN or inhibition of HNO. Surprisingly, SHR show the highest absolute values of the HISS-independent component, possibly hinting to a compensatory mechanism.

Intravenous administration of the muscarinic cholinergic antagonist atropine has been reported to induce insulin resistance in a magnitude similar to specific hepatic parasympathetic denervation (Xie and Lautt, 1996a) or fasting (Lautt et al., 2001). This is consistent with the observation that food intake enhances parasympathetic tone (Uijtdehaage et al., 1992); therefore, special care has to be taken when comparing the available literature with our results.

Almost all reported results were performed in fasted animals; thus, they are strictly comparable with our HISS-independent component data. In this respect, the quantification of the HISS-independent component, given by the RIST index obtained after atropine or L-NMMA, that is, the direct metabolic effect of insulin on its target tissues, showed a tendency to be augmented in SHR when compared with WKY. This is consistent with results obtained by other authors with animals either in the fasted state or where the prandial state was not monitored (Farrace et al., 1995; Frontoni et al., 1992; Tsutsu et al., 1989).

A possible explanation for the higher HISS-independent insulin sensitivity in SHR is an excess insulin-stimulated glucose disposal accounted for by the nonoxidative glucose metabolism pathway, representing an increase of glycogen accumulation in the skeletal muscle (Farrace et al., 1995; Frontoni et al., 1992), with increased activity of

skeletal muscle glycogen synthase (Farrace et al., 1995). That insulin-stimulated glucose uptake benefits from high insulin secretion is known (Chen et al., 1994a), but this compensatory mechanism of insulin sensitivity was not seen in any other of the pathophysiological models studied herein.

The SHR has been described to more easily develop insulin resistance from stressful situations than WKY (Mondon and Reaven, 1988). This was used (Tsutsu et al., 1989) to explain the discrepancy towards studies where SHR were found to be insulin resistant in relation to WKY. Since we have observed a similar degree of insulin sensitivity in both strains, this is possibly another hint to the adequacy of the RIST technique to evaluate insulin sensitivity without inducing stress to the animals. Also, anaesthesia seems not to affect the results as others propose (Farrace et al., 1995; Frontoni et al., 1992), since we obtained, in SHR, the same augmented expression of the HISS-independent component of insulin action under maintained anaesthesia.

The HISS-dependent component showed a decrease from Wistar to WKY, and even more dramatically to SHR. That is consistent with the results of other studies where insulin sensitivity was measured before and after feeding (Gouveia et al., 2000b; Swislocki et al., 1997). There, skeletal muscle glycogen content, identical between WKY and SHR in the fasted state, increased from fasting to refeeding in WKY, whereas in SHR it remained similar to fasting levels (Swislocki et al., 1997). In addition, feeding increased muscle glycogen content much more in Wistar than in SHR (Gouveia et al., 2000b). Furthermore, in both studies, liver glycogen accumulation increased similarly in both strains, and liver glycogen total content was similar (Gouveia et al., 2000b; Swislocki et al., 1997), which indicates that the difference in postprandial insulin sensitivity between the strains is specific to the skeletal muscle. This supports the concept that, in the postprandial state, the skeletal muscle is further sensitised to glucose uptake and glycogen synthesis by a mechanism that is unable to properly function in the SHR.

As Swislocki and colleagues suggested (Swislocki et al., 1997), there seems to exist a circulating factor that increases skeletal muscle insulin-stimulated glucose uptake and glycogen accumulation. Furthermore, this factor, that is here considered to be coincident with the HISS mechanism, was seen here to be dependent on the status of the HPN and HNO.

Insulin resistance in essential hypertension seems also to be related to parasympathetic dysfunction (Chen et al., 1998). More, in SHR, a reduction in vagal neurone number (Corbett et al., 2007) may be contributing to the loss of function. It has further been proposed that hypertension is rather related to an increased sympathetic activity resulting in sympathetic/parasympathetic imbalance (Reaven et al., 1996), but, even if that is the case, it seems to be preceded by an impairment of the parasympathetic nervous system (Valensi et al., 1998).

Still considering that an increased sympathetic activity further disrupts the sympathovagal unbalance, this adrenergic overactivity would theoretically impact glucose metabolism on SHR in two ways: i) the chronic effect of long lasting hyperadrenergism would reverse the ability of catecholamines to inhibit glycogen synthase and insulin action (Farrace et al., 1995), and ii) the further imbalance away from parasympathetic function would lead to a lowering of HISS action (Lautt, 2004). Both these conditions seem to have been observed in the present study: a decrease in HISS action accompanied by an increase in HISS-independent action.

Consistent with this hypothesis, the administration of a central sympatholytic drug in human hypertensive subjects has been shown to ameliorate both the cardiovascular profile and glucose metabolism (De Luca et al., 2000; Hausberg and Grassi, 2004); further indicating that sympathetic overactivity may be influencing negatively both conditions.

Considering that WKY already show some decrease of the HISS-dependent component, there is also conceivable the existence of a common genetic factor between WKY and SHR that, at the administered insulin dose (50 mU/kg), impairs HISS action in both strains. In fact, a common genetic factor in SHR and WKY has already been proposed by others. Interestingly, it has been related to a diminished sensitivity to vagal activity (Ohnuma et al., 2000), which again argues in favour of a secondary role of hypertension in the development of insulin resistance in this animal model. This hypothesis also reinforces the highly sensitiveness of the HISS pathway to interference by genetic and environmental factors; evenmore conditions where the action of insulin per se is not changed or is even augmented.

This indicates also that, although there are fears of considerable genetic divergence between WKY and SHR (Rapp, 1987; St Lezin et al., 1992), at least they

seem to share the genetic trait that is, possibly by lowering vagal tone, favouring a decrease of the HISS-dependent component of insulin action.

The SHR show other metabolic problems that may contribute to the impairment of HISS action, such as a defective HNO synthesis/action. Defective nitric oxide synthesis has been proposed as a link between insulin resistance and hypertension (Scherrer and Sartori, 2000); bringing about also sympathetic overactivity, by removal of the central effect of nitric oxide (Sakuma et al., 1992; Souza et al., 2001).

However, nitric oxide production may be normal or even increased in the presence of hypertension. Indeed, several authors report higher levels of plasma nitric oxide in SHR (Wu and Yen, 1999) (20% higher than in WKY), but that this nitric oxide action is mechanistically ineffective (Maffei et al., 2002; Nava et al., 1998). It could thus mean that hypertension is related to preserved production, but abnormal biological effect, of nitric oxide (Piatti et al., 2000).

Angiotensin II is known to influence negatively NOS and insulin signalling in the presence of superoxide anion (Sowers, 2004). So, to the previous, may also be relevant the fact that angiotensin-converting enzyme inhibitors, given as antihypertensive drugs, are able to raise the effective action of nitric oxide (Das, 2001) and lead to an augmented response of GLUT4 (Katayama et al., 1997). However, even if antihypertensive drugs that interrupt the renin-angiotensin axis slow the metabolic degradation to type 2 diabetes compared to other drugs (Scheen, 2004), this action may not be enough to fully reinstate HISS-dependent insulin sensitivity, since other steps of the HISS pathway, like hepatic parasympathetic tone, seem to be impaired[69]. Consistent with this hypothesis, the mentioned effects of rennin-angiotensin inhibitors seemed to have no impact on insulin action or glucose tolerance (Swislocki et al., 1999).

Nitric oxide synthase is also a physiological source of superoxide anion. Interestingly, HISS action was able to be mimicked by the administration of nitric oxide donors that release both nitric oxide and superoxide anion, but not by donors that only produced nitric oxide (Guarino et al., 2003). This underlines the importance of the balance between the production of nitric oxide and superoxide anion.

[69] Angiotensin-converting enzyme inhibitors are also described to possibly have a sympatholytic effect (Hausberg and Grassi, 2004), which may additionally contribute to a removal of the negative constraints that surround the HISS pathway.

It was reported that the excess of superoxide anion in SHR may be due to a dysfunctional NOS (McIntyre et al., 1999). Also, the action of cofactors as tetrahydrobiopterin, important in controlling the balance between nitric oxide and superoxide (Cosentino et al., 1998; McIntyre et al., 1999) seem to be impaired in SHR (Hong et al., 2001), even in a prehypertensive stage[70] (Cosentino et al., 1998; Shinozaki et al., 1999). Thus, depletion of tetrabiopterin allows NOS to produce more superoxide anion and hydrogen peroxide (Brown and Borutaite, 2006; Hong et al., 2001), increasing oxidative stress[71] (Gomez-Amores et al., 2007; Power et al., 2007; Ruggenenti et al., 2009).

This increased oxidative stress may play an important role in decreasing peripheral insulin action through a decrease in HISS action, by disruption of the equilibrium between nitric oxide, superoxide anion, and glutathione production, well before the action of insulin *per se* is affected.

Other authors (Baron et al., 1995) link nitric oxide inhibition with a decrease in insulin sensitivity by way of a decrease of blood flow and nutrient availability to skeletal muscle cells. However, in that study blood pressure was kept augmented by continuous L-NMMA perfusion throughout the insulin sensitivity test. On the other hand, in the present study, the dose of L-NMMA given was such that enabled blood pressure to return to normal levels before the insulin sensitivity test was started.

Although we have seen no alteration in blood pressure in any of the strains, at least not by the time the second RIST (the post-L-NMMA RIST) was started, a case could be presented based on the hypothesis of changes in the microvasculature feeding of myocytes. However, the fact that atropine had the same effect as L-NMMA, and as hepatic parasympathetic ablation, strengthens the hypothesis that hemodynamic changes were not responsible for the change in glucose uptake. Furthermore, in healthy animals, the administration of the nitric oxide donor SIN-1 was able to revert the insulin resistance produced with L-NMMA; but only when it was given ipv and not iv (Guarino et al., 2003), despite having a clear vasodilator effect in both cases, clearly showing that insulin sensitivity was reinstated by hepatic control and not through hemodynamic

[70] Interestingly, tetrahydrobiopterin deficiency has been reported to be determined directly by decreased insulin action (Shinozaki et al, 1999).

[71] Likewise, substances known to decrease superoxide anion production, as for example L-Carnitine, lead to increased nitric oxide bioavailability (Gomez-Amores et al, 2007). This may partly explain how it ameliorates simultaneously essential hypertension, glucose tolerance, and nerve conduction (Power et al, 2007; Ruggenenti et al, 2009).

changes. The involvement of HISS in this mechanism seems further corroborated by others who report that the insulin resistance produced by L-NMMA administration corresponds to a lower glucose uptake at the skeletal muscle (Baron et al., 1995; Capaldo et al., 1991).

The notion that the physiological insulin actions on skeletal muscle glucose uptake and vasodilation should be considered separately is also supported by the fact that different doses of insulin can show or not alterations in glucose uptake on SHR in relation to WKY, but at the same time show a persistent impairment on the ability of insulin to induce skeletal muscle vasodilation (Pitre et al., 1996). However, this segregation of pathways does not exclude that haemodynamics and HISS action may be independently impaired by a common factor[72] (Lembo et al., 1992; Van De Borne et al., 1999).

A direct causal dependence between insulin resistance and hypertension is possibly denied by several other experiments. Mainly, it has been reported that lowering blood pressure is not enough to ameliorate glucose metabolism (Reaven, 1991). Likewise, an α-glucosidase inhibitor, which delays carbohydrate absorption at the small intestine[73] (Bavenholm and Efendic, 2006; Bischoff, 1995), reduces insulinaemic and glycaemic responses to a meal by giving acarbose, without changing blood pressure (Swislocki et al., 2007).

It seems thus plausible to consider that insulin resistance and hypertension may be related to similar pathophysiological mechanisms, but that they are not mutually determinant. Even considering that they may share some etiological similitude, the normalisation of either one does not necessarily mean the betterment of the other.

In conclusion, the SHR is still valuable as a model of essential hypertension, but should be used with caution in studies related to insulin resistance. Because hypertensive humans show marked insulin resistance when compared with normotensive subjects (Gouveia et al., 2000a), the similar total insulin sensitivity of

[72] Such common factor could be, for example, the increased sympathetic nervous activity produced in response to hyperinsulinaemia (Lembo et al, 1992; Van de Borne et al, 1999).
[73] Acarbose reduces specifically postprandial blood glucose excursions and contributes to a better overall glycaemic control (Bischoff H, 1995). This leads also to a lowering of risk factors associated with type 2 diabetes and cardiovascular disease, mainly those induced by blood hyperglycaemia (Bavenholm and Efendic, 2006).

WKY and SHR, as given by the control RIST indexes, suggests the use of the Wistar as the most adequate normotensive control for SHR in insulin sensitivity studies.

4.5.2. Hepatic glutathione (GSH) branch of the HISS pathway in essential hypertension

4.5.2.1. General context and work hypothesis

The optimal action of the HISS pathway is dependent upon more than the correct activation of the HPN-HNO axis. It has been found that hepatic glutathione is also critical to the synthesis/release of HISS (Guarino and Macedo, 2006); being increased through a pathway independent, but convergent, to that activated by the hepatic parasympathetic nerves and subsequent production of hepatic nitric oxide.

Glutathione (GSH) is ubiquitous in all tissues, but is found in especially high levels on the liver. Hepatic GSH synthesis is particularly dependent upon the availability of the amino acid cysteine, which is mostly derived from the diet (Lu, 1999). This fact seems paramount in the relation between postprandial insulin-stimulated glucose uptake and hepatic glutathione content (Guarino et al., 2003).

Interestingly, it has been reported that insulin stimulates hepatic GSH synthesis by inducing γ–glutamylcysteine synthetase, the rate-limiting enzyme in the γ–glutamyl cycle (Huang et al., 2000; Lu et al., 1990; Lu et al., 1992; Lu et al., 1991; Sun et al., 1996)[74,75].

The decreased bioavailability of nitric oxide may be due to increased oxidative stress (Brown and Borutaite, 2006; Hancock et al., 2001); which has been found to happen in hypertension (Touyz, 2004). Oxidative degradation of nitric oxide may happen through overproduction of superoxide (McIntyre et al., 1999; Zalba et al., 2001). The reaction of nitric oxide and superoxide anion forms peroxynitrite (Hancock et al., 2001). This scavenging of nitric oxide has been proposed as a link between insulin resistance and endothelial dysfunction (Zalba et al., 2001). The formation of

[74] Fasting and stress enhance the efflux of GSH out from the liver to the plasma. Of particular interest in this seems to be the effect of glucagon, which increases GSH efflux from the liver by cAMP-mediated membrane hyperpolarization (Lu et al, 1990; Lu et al, 1991). These factors also decrease hepatic GSH synthesis by inducing γ–glutamylcysteine synthetase phosphorilation (Sun et al, 1996).

[75] Insulin does not have any effect on GSH synthetase. This is common to the factors that, like insulin, increase the expression of the heavy subunit of γ–glutamylcysteine synthetase (Huang et al, 2000).

peroxynitrite may further increase oxidative stress (Brown and Borutaite, 2006); and be particularly relevant to the HISS hypothesis by reacting directly with glutathione, and depleting its level (Jourd'heuil et al., 2001; Wink et al., 2001). Glutathione may be also oxidized by hydrogen peroxidase, which also comes from superoxide (Brown and Borutaite, 2006). Since this presents an additional way by which the HISS-dependent component may become further impaired, the status of reduced/oxidised hepatic glutathione was subsequently studied in this animal model of hypertension.

Our **working hypothesis** was that a decrease in hepatic glutathione related to essential hypertension may contribute to a deterioration of postprandial insulin sensitivity, as GSH is essential for HISS action.

4.5.2.2. Experimental protocols

For the evaluation of glutathione levels in insulin resistance and hypertension, only Wistar and SHR were chosen. Both 9 weeks old Wistar and SHR animals were fasted for 16 hours and had access to food for 1 hour. After that, they were anesthetised and several samples of liver were collected and promptly frozen in liquid nitrogen.

Quantification of reduced glutathione (GSH) and oxidised glutathione (GSSG) was done by high-performance liquid chromatography.

4.5.2.3. Results

At 9 weeks of age, there was no difference in postprandial hepatic glutathione between the hypertensive SHR (n=7) and their normotensive control Wistar (n=6), either in the reduced form (GSH: 5.94±0.42 for Wistar and 7.14±0.95 for SHR) or the oxidised form (GSSG: 86.46±20.45 for Wistar and 148.10±32.47 for SHR). So, as expected, the ratio of the forms (GSH/GSSG) also showed no difference between normotensive and hypertensive rats (a ratio of 85.01±14.90 for Wistar and 77.97±22.71 for SHR) (Figure 27).

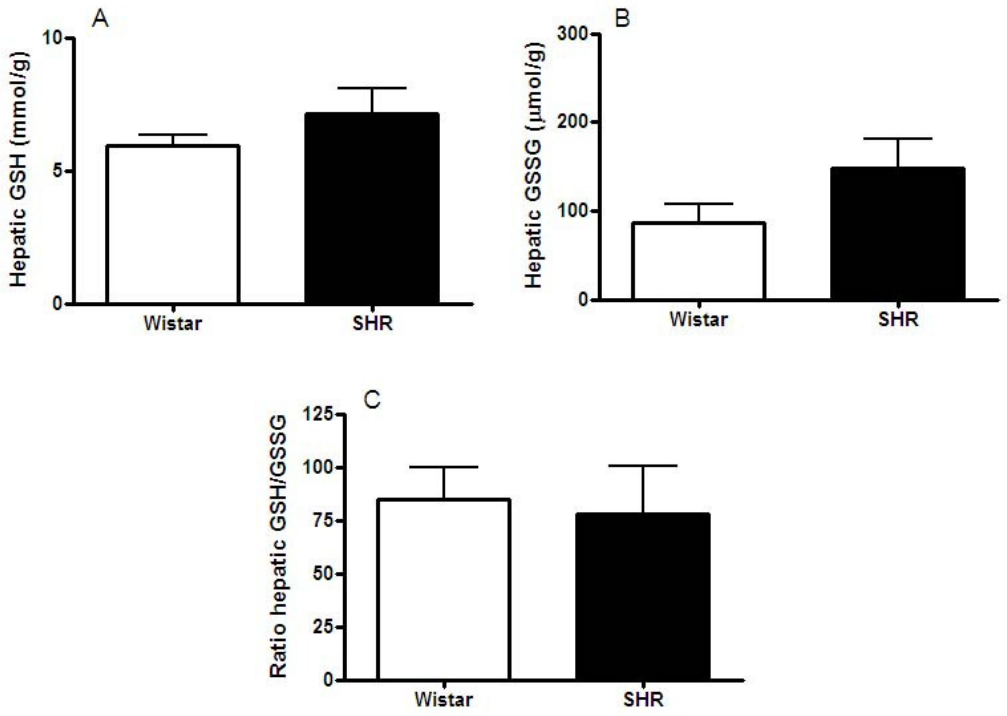


Figure 27 – Hepatic reduced and oxidised glutathione content in Wistar and spontaneously hypertensive rats (SHR). Values for hepatic (A) reduced glutathione (GSH) and (B) oxidised glutathione (GSSG). The ration between the two is also shown (C). None of these parameters show a statistically significant alteration with the presence of hypertension.

4.5.2.4. Discussion

Essential hypertension has been related to a state of increased vascular oxidative stress, inclusively by impairment of GSH availability in vascular smooth muscle cells (Wu and Juurlink, 2002). Concerning hepatic GSH metabolism in essential hypertension, studies have focused on comparing only Wistar Kyoto and SHR. In this respect, hepatic GSH content has been reported to be similar between these two strains (Binda et al., 2001; Kitts et al., 1998); however, hepatic GSH/GSSG ratio seems to decrease (Cediel et al., 2003; Gomez-Amores et al., 2007), indicating an increase in oxidative stress[76].

[76] SHR show an increase in glutathione redutase (GSSG-R), the enzyme responsible for GSH recycling, and a decrease in glutathione peroxidase (GSH-Px), which is involved in scavenging peroxides (Kitts et al, 1998; Binda et al, 2001; Cediel et al, 2003; Gómez-Amores et al, 2007). This upregulation of GSSG-R may show a response to an increase in oxidative stress, while the reduction in GSH-Px may be both a cause and a consequence of oxidative stress (Binda et al, 2001).

Besides using solely the Wistar Kyoto as the normotensive control for SHR, in these previous studies no care was given as to the prandial status; with animals being analysed in the fasted or *ad libitum* state. Thus, the present study was the first to determine the postprandial hepatic glutathione status in the hypertensive SHR; furthermore in comparison with the Wistar rat, a normotensive control that sets the SHR clearly as a model of insulin resistance.

Here was shown that SHR does not present any alteration of either reduced or oxidised glutathione, in comparison with Wistar. The GSH/GSSG ratio is logically also similar between strains. We can thus conclude that SHR do not seem to present a deficiency in GSH metabolism in the postprandial state that would be expected to contribute to the decrease in the HISS-dependent component that determines the insulin resistance in this model of essential hypertension.

Since hepatic GSH is noted to rise after a meal, probably both from the influx of dietary cysteine and from a need to match the rise in oxidative stress produced by the handling of nutrients, it is conceivable to consider that a deficiency in GSH metabolism would be sooner visible in the antioxidant function during fasting; when hepatic GSH is lower and, furthermore, the efflux of GSH to other tissues is paramount. This hypothesis seems consistent with the studies previously mentioned. Furthermore, the present study may already hint to an initial rise in postprandial oxidative stress, since SHR showed a tendency for a higher hepatic GSSG content.

Since, at 9 weeks of age, postprandial hepatic GSH seems to be equally available in SHR than in Wistar, it is consequently not likely that it constitutes a constraining factor for HISS synthesis/action in SHR. Thus, insulin resistance in essential hypertension seems to be determined by an impairment of the HPN-HNO branch of the HISS pathway[77].

[77] The present study further underscores that mechanisms such as antioxidant defence, glucose metabolism, and endothelial function may depend on the same elements but seem to be impaired in a differentiated form. This seems especially pertinent to prevent causal assumptions to be drawn from changes in interrelated, but otherwise separate, physiological processes.

4.5.3. Postprandial increment of insulin sensitivity in SHR

4.5.3.1. Context and work hypothesis

All the previous studies point to the fact that the HISS pathway is responsible for the increment of insulin sensitivity seen after a meal. Also, the insulin resistance observed in SHR was attributed to a decrease in HISS action.

In the case of the SHR then, our **work hypothesis** was that the insulin sensitivity in the fasted state was similar to the postprandial response obtained after HISS inhibition by administration of atropine or L-NMMA.

4.5.3.2. Experimental protocols

For this, a group of 9 weeks old SHR was analysed after a 17-hour fast. The animals were anaesthetised prior to being subjected to a control RIST.

4.5.3.3. Results

The RIST control index obtained in the fasted SHR (n=6) was of 150.3±22.0 mg glucose/kg bw (Figure 28), down from 229.1±10.6 (n=29) seen after meal ingestion (pooled from the results presented on items 4.5.3.1 and 4.5.3.2), $p<0.01$. This decrease was similar to the ones obtained after atropine (to 161.8±19.7 mg glucose/kg bw) and obtained after L-NMMA (to 170.0±10.9 mg glucose/kg bw)(again, see items 4.5.3.1 and 4.5.3.2).

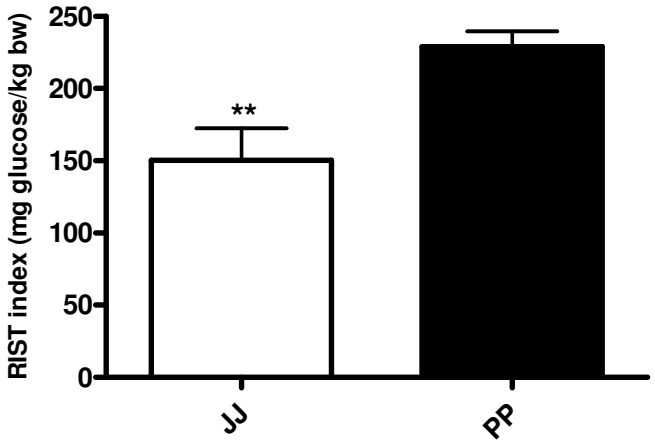


Figure 28 – Insulin sensitivity in the fasted and postprandial states, in an animal model of essential hypertension. Rapid insulin sensitivity test (RIST) indexes for fasted (JJ) and fed (PP) total insulin action for 9 weeks old male spontaneously hypertensive rats (SHR). The decrease of insulin sensitivity with fasting was similar to the one obtained with blockade of the hepatic insulin sensitising substance (HISS) pathway (** $p<0.01$).

4.5.3.4. Discussion

This shows that in SHR the postprandial increment of insulin sensitivity is entirely due to the HISS-dependent component, as already seen in healthy normotensive Wistar rats (see topic 4.1). Hence, the decrease in postprandial insulin sensitivity seen in hypertension is again shown to be due to a dysfunction of the HISS-dependent component of insulin action.

Discrepancies between reports have been tentatively explained by differences in fasting time, especially between Wistar Kyoto and SHR. Different fasting times showed different results on insulin sensitivity. After a short fasting time (6h), SHR showed decreased insulin sensitivity in relation to Wistar Kyoto (Mondon and Reaven, 1988). In contrast, after a longer fasting time (24h), SHR showed augmented glucose uptake (Frontoni et al., 2005; Frontoni et al., 1992). Further, a fasting time just slightly shorter than the present study (12-14h) showed no difference between SHR and Wistar Kyoto (Pitre et al., 1996), at an insulin perfusion known to reflect peripheral insulin sensitivity.

All these studies are consistent with the present results, considering that Wistar Kyoto still has preserved some of the HISS-dependent component action, which enables

some postprandial increment; on the other hand absent in SHR. Shorter fasting times would be insufficient to fully shutdown HISS action, thus masking the real difference between Wistar Kyoto and SHR in the fasted state: an augmented HISS-independent component in the hypertensive animals. This interpretation may also shed some light into the apparent disparities found among other published studies using the SHR.

4.6. Changes in insulin sensitivity in an animal model of essential hypertension determined by age

4.6.1. General context and work hypothesis

At 9 weeks of age, SHR showed a tendency to compensate the impairment of the HISS-dependent component by an exacerbation of the action of insulin *per se*, directly on the skeletal muscle. Furthermore, contrary to what was seen here in healthy Wistar normotensive rats, ageing was reported not to impair insulin sensitivity in the SHR (Natalucci et al., 2003).

Since maturation until 16 weeks of age was shown to be a crucial stage of development concerning changes in glucose metabolism (Ribeiro et al., 2008), it became relevant to study the influence of the continuous exposure to hypertension during this stage.

Our **work hypothesis** was that the presence of hypertension during maturation would produce an even more pronounced decrease in insulin sensitivity, by further impairing the HISS-dependent component of insulin action.

4.6.2. Experimental protocols

Wistar, Wistar Kyoto and SHR rats were used, at 9 and 16 weeks of age. After a 16 hour fasting period, all animals were fed for 1 hour and anesthetised. Total postprandial insulin action was evaluated by way of a control RIST. The HISS-dependent and –independent components were quantified by performing a RIST after the administration of atropine (3 mg/kg).

4.6.3. Results

The SHR showed similar total postprandial insulin sensitivity at 9 (n=13) and 16 (n=6) weeks of age. In contrast, while Wistar Kyoto also showed similar values of total insulin sensitivity at both ages (n=13 and n=7) (Figure 29), Wistar showed the radical decrease from 9 to 16 weeks of age already seen in this dissertation (from 297.4±18.5, n=11, to 221.7±24.3 mg glucose/kg bw, n=6, $p<0.05$).

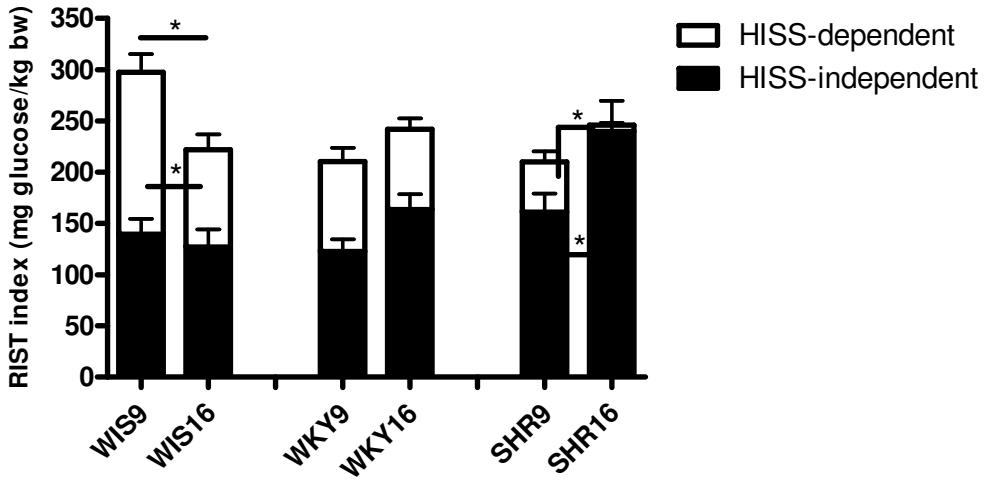


Figure 29 – Effect of maturation on spontaneously hypertensive rats (SHR) and normotensive controls. Rapid insulin sensitivity test (RIST) indexes for the hepatic insulin sensitising substance (HISS)-independent (filled) and HISS-dependent (open) components of total insulin action for 9 and 16 weeks old male Wistar (WIS), Wistar Kyoto (WKY), and SHR . The sum of both components represents the control postprandial RIST index for each strain. Total postprandial insulin sensitivity decreases in Wistar with age, due to a decrease of the HISS-dependent component (* $p<0.05$). The HISS-dependent component also decreases with age in SHR, but is partially compensated by an increase in the HISS-independent component (* $p<0.05$)

For both normotensive controls, Wistar and Wistar Kyoto, the HISS-independent component of insulin sensitivity, quantified by the post-atropine RIST, does not change with age, but while in Wistar Kyoto the HISS-dependent component is also unchanged (Figure 29), in Wistar it decreases (from 157.8±17.7 at 9 weeks to 94.6±15.3 mg glucose/kg bw at 16 weeks of age, $p<0.05$), notably becoming of similar magnitude to the one observed in Wistar Kyoto at both 9 and 16 weeks of age.

In the hypertensive strain, SHR, while the HISS-dependent component becomes practically non-existent with age (from 49.1±10.3 at 9 weeks to 6.3±2.0 mg glucose/kg bw at 16 weeks of age, $p<0.05$ to SHR at 9 weeks; and $p<0.001$ to both Wistar and Wistar Kyoto at 16 weeks of age), the HISS-independent component seems to continue rising in a compensatory manner (from 161.0±18.2 at 9 weeks to 239.7±29.9 mg glucose/kg bw at 16 weeks of age, $p<0.05$), becoming even higher than in Wistar or Wistar Kyoto ($p<0.01$ to Wistar and $p<0.05$ to Wistar Kyoto at 16 weeks of age).

4.6.4. Discussion

By 16 weeks of age, all strains – the hypertensive SHR and the normotensive Wistar and Wistar Kyoto –, had similar total postprandial insulin sensitivity. This was due to a decrease of the HISS-dependent component of insulin action in Wistar rats; which at 9 weeks of age were the most insulin sensitive strain. In the SHR there was also a radical decrease of the HISS-dependent component; but in this case a compensatory increase of the HISS-independent component permitted to maintain similar total postprandial insulin sensitivity to the one observed at 9 weeks of age. Wistar Kyoto showed no alteration in either components of insulin sensitivity with age; however, this strain had already a lower HISS-dependent component at 9 weeks of age, interestingly similar to the observed in Wistar at 16 weeks of age.

The development of insulin resistance during maturation in Wistar, solely due to a decrease of the HISS-dependent component, is consistent with previous published studies (Ribeiro et al., 2008), presented here (see item 4.2.3). The observation that maturation does not affect the HISS-dependent component in Wistar Kyoto does not seem surprising, since there is already by 9 weeks an impairment of this component (see item 4.5). The disappearance of the HISS-dependent component in the 16 weeks old SHR suggests that although hypertension seems to have a secondary role in the development of insulin resistance, its presence seems related to a radical worsening of this component of insulin action.

A previous study by others (Vera et al., 2002) regarding a comparison of only Wistar and SHR between 9 and 16 weeks of age yielded contradictory results. This may have been due to the use of data obtained in the fasting state to calculate insulin sensitivity, when HISS is physiologically shutdown. However, it seems consensual that

young SHR are insulin resistant in comparison with Wistar, and that insulin sensitivity is more affected by maturation in Wistar than in SHR.

Interestingly, further ageing is reported still not to induce deterioration in insulin sensitivity in the SHR (Natalucci et al., 2003); not even when it reaches a stage of senescence[78] (Iwase et al., 1994). Likewise, hepatic glutathione levels, which were shown in the prior item not to be impaired in SHR, seem to remain adequate within the time frame of maturation (Vericel et al., 1994).

The effect of age on decreasing the HISS-dependent component of insulin action is clearly shown on healthy Wistar rats. That Wistar Kyoto shows no alteration of the HISS-dependent component with maturation, having already at 9 weeks a similar value of this component to the one obtained on Wistar rats at 16 weeks of age, seems further indication that the effect of age *per se* on insulin sensitivity is limited to this magnitude. And if that be the case, then we can conclude that in SHR the prolonged exposure to hypertension is related to a complete impairment of the HISS-dependent component in SHR.

Concurrently, the capability of the hypertensive strain to compensate for the development of insulin resistance by increasing the HISS-independent component is maintained at least until the end of maturation. This warrants more study, and may yet come to provide a new mechanism to improve insulin sensitivity.

[78] This state of senescence was characterised by an age near the mean life expectancy (26 months) and further hinted by a loss of body weight; in all probability due to the loss of lean body mass (Iwase et al, 1994).

5. OVERALL DISCUSSION

The results presented in this thesis have reiterated the physiological importance of hepatic parasympathetic nervous system integrity to the postprandial disposal of glucose. The impacts of gender and age in this mechanism have likewise been evaluated. Also, surgical and pharmacological interventions have argued in favour of the integration of this pathway into the hepatic parasympathetic sensitising substance (HISS) hypothesis.

Lifestyle changes as simple as dietary composition have been here shown to produce radical alterations in the physiological increment of insulin sensitivity seen in the postprandial state. Furthermore, this has solidified the interpretation of the HISS pathway as a primary site of dysfunction in regard to alterations of postprandial insulin sensitivity. Correspondingly, the analysis of an animal model of hypertension, the SHR, has further demonstrated how the HISS-dependent and –independent components of insulin action can be affected *in vivo* in quite opposite ways.

5.1. Methodological considerations in the assessment of whole-body insulin sensitivity, and critical relevance of prandial status

Through the years, a multitude of different tests has been used to assess in vivo insulin sensitivity (Hermans et al., 1999). In the present studies, the quantification of whole-body insulin sensitivity was carried out using a modified euglycaemic clamp known as the rapid insulin sensitivity test (RIST) (Lautt et al., 1998). This test was specifically designed to avoid the counter-regulatory responses to the hypoglycaemia that follows an insulin bolus, as administered in a standard insulin tolerance test (ITT). The RIST has been shown to be reproducible in at least four consecutive tests in anaesthetised animals (Lautt et al., 1998), allowing for the assessment of insulin sensitivity in different conditions in the same animal, and in the same day; which is usually not possible with the ITT (Akinmokun et al., 1992) or the current golden standard test for insulin sensitivity assessment (DeFronzo et al., 1979), the hyperinsulinemic euglycaemic clamp (HIEC).

The RIST has been shown to correlate well with the ITT; except on the later segment (after 25 min). The RIST was also found to be more sensitive in detecting the HISS-dependent component of insulin action than the HIEC (Reid et al., 2002). The former might be explained by the hypoglycaemic response that follows the insulin bolus, leading to higher endogenous glucose output and tissue insulin resistance. The latter may happen due to the physiological shutdown of HISS action by fasting, since this test is performed after an overnight fast; and by the alterations produced by the constant perfusion of insulin, which physiologically has a pulsatile behaviour. The lack of pulsatility imposed by the HIEC may lead to lower insulin sensitivity (Reid et al., 2002), by desensitisation of the insulin receptors (Shanik et al., 2008). The maintained continuous hyperinsulinemia may also induce an alteration in sympathovagal autonomic balance (Van De Borne et al., 1999); which, even if the HIEC was conducted on the postprandial state, would decrease parasympathetic tonus and impair HISS action. This is supported by the observation that insulin induces the shutdown of the HISS-dependent component when the same dose is given not in a physiological-like pulse manner but in a continuous infusion (Reid and Lautt, 2004).

Since HISS action is entirely correlated with the prandial state (Lautt et al., 2001; Patarrao et al., 2008; Sadri et al., 2006), the conclusion is drawn that, if a complete assessment of whole-body insulin sensitivity is desired, it is essential to evaluate its status in the postprandial state. Furthermore, this becomes even more relevant when it is considered that postprandial alterations in glucose homeostasis seem to predate the ones seen in the fasted state. If we are to detect the precocious defects that lead to the development of type 2 diabetes, the postprandial state should be the primary goal; even, or especially, at a time when postprandial hyperglycaemia is not yet present.

Several studies have tried to look into postprandial insulin action deriving parameters from the oral glucose tolerance test (OGTT) (Abdul-Ghani et al., 2007). This was viewed as an ideal form to evaluate the roles of both insulin sensitivity and insulin secretion in the disposal of glucose. In line with the increasing importance recognised to the postprandial state, the OGTT was found to be more sensitive in detecting asymptomatic diabetes than fasting plasma glucose (Modan and Harris, 1994). More recently, it has been advocated that a meal tolerance test (MTT) would be even more adequate to evaluate the postprandial state. The presence of other nutrients, than simply glucose, in a mixed meal, produces metabolic responses of their own in regards

to insulin secretion, and tissue metabolism. Furthermore, the MTT has been found to be a more consistent and reproducible test than the OGTT[79] (White et al., 1983; Wolever et al., 1998); and thus be able to better mimic the conditions of every-day life nutritional challenges.

However, both the OGTT and the MTT are under too many variables, and represent glucose disposal, and glucose fluxes, influenced by several processes. The RIST, on the other hand, is performed at a time when plasma glycaemia has already stabilised, and several processes involved in nutrient disposal have reached a dynamic equilibrium. In this condition, where the rate of glucose appearance equals the rate of disappearance, it can be argued that the machinery responsible for cell glucose uptake is still somewhat summoned from the stimuli provided by the meal challenge (Holloszy, 2005). Thus, it seems possible to consider that the administration of a bolus of exogenous insulin, while plasma euglycaemic is maintained, will be better able to isolate and assess the tissue responsiveness to insulin in the postprandial state.

The experiments reported herein were performed with the animals under anesthesia. Still, this is expected not to impact negatively on the assessment of insulin sensitivity. Although pentobarbital anaesthesia has been reported to produce insulin resistance (Penicaud et al., 1987), previous studies done with the RIST showed no difference in glucose uptake between anesthetised and conscious rats (Latour and Lautt, 2002b). A possible explanation is that maintaining euthermia, as was done in the present methodology by using a heating pad and a rectal probe, prevents the anaesthesia-induced alterations in glucose metabolism (Holscher et al., 2008; Lang et al., 1987).

Animals in the postprandial state usually show a stabilisation of blood glycaemia at a value higher than the fasting blood glucose level. A noticeable fact on the anesthetised animals is that this postprandial blood glycaemia is maintained constant for several hours, well after it has started to fall in postprandial conscious animals. This may be due to an effect of the anaesthesia on delaying gastric emptying and on increasing the nutrients transit time through the intestine, by slower intestinal motility (Reynell and Spray, 1957).

[79] Gastroduodenal motor activity promotes a more consistent gastric emptying when both lipids and carbohydrates are present than only carbohydrates (White et al, 1983). Furthermore, an oral glucose load produces a slightly different profile of plasma glycaemia and insulinaemia as that seen after a mixed meal.

However, as much as the present results seem to support the HISS hypothesis as it was originally postulated (Lautt, 1999), alternative pathways to explain the hepatic activation of HISS have since been presented (Chen et al., 2003; Porszasz et al., 2003). The present work could not include a study of these alternative pathways; but, in light of the results obtained herein, a critical read of those reported results should be drawn next.

By the first alternative hypothesis, the nervous facilitative signal would be, not of parasympathetic nature, but a nitrergic one. This would travel through the anterior hepatic plexus, by capsaicin-sensitive sensory fibres, and lead to the synthesis of hepatic nitric oxide of neural origin (Porszasz et al., 2002).

The related papers promptly raise several methodological questions. Although recognising the importance of the postprandial status to insulin sensitivity (Zsuga et al., 2004), the referred studies analyse the fasted state (Porszasz et al., 2003), the *ad libitum* state (Peitl et al., 2005), or fail to mention clearly the actual prandial status (Porszasz et al., 2002; Zsuga et al., 2004). Although the protocol and animals used were described as being identical to ones in the present thesis, Zsuga and colleagues (Zsuga et al., 2004) showed only one third of the postprandial whole-body insulin sensitivity reported here. Since the authors do not report allowing the animals access to food after an 18 hr fasting period, this seems consistent with the present data for fasted rats. More, in another study by the same group (Peitl et al., 2005), done in *ad libitum* fed rats, the magnitude of insulin sensitivity is only slightly lower than the one observed in the present results.

Since the prandial status is critical for the magnitude of insulin sensitivity, this approach seems liable to produce confounding results. Differential prandial states introduce a greater variability by including animals with different degrees of HISS inactivation by fasting time (Lautt et al., 2001).

Other methodology differences may also have a bearing on these discrepancies. The use of the hyperinsulinemic euglycemic clamp, shown to produce an inhibition of the HISS-dependent component (Reid et al., 2002), possibly through a sympathetic shift of the sympathovagal balance (Muscelli et al., 1998), can also be a confounding factor, as well as the possible inadequate use of the RIST by performing it in conditions that assess only the HISS-independent response.

One study (Peitl et al., 2005) proposed to dispel the controversy among alternative pathways for the HISS mechanism. It aimed to focus on the direct effect of

the HPN on insulin sensitivity, by applying electrical stimulation after hepatic denervation. The fact that this produced an almost total elimination of postprandial insulin sensitivity is against any proof of principle in any other protocol. More, the study reported differential effects of atropine and hepatic denervation on insulin sensitivity; which are here shown to produce the same degree of insulin resistance on the fed state. It can be proposed that the abrogation of insulin sensitivity was by way of an increase of hepatic glucose output, and that this electrical stimulation does not mimic what was here seen in the physiological state.

Another line of inquiry has purported to provide additional information regarding the existence of hepatic factors that may be relevant to diabetes therapy (Groop, 2003). Specifically, bone morphogenetic protein 9 (BMP-9) has been presented as a possible candidate for the elusive nature of HISS[80] (Chen et al., 2003). More recently, a Brazilian group has further described details about how BMP-9 seems to affect glucose homeostasis (Caperuto et al., 2008).

BMP-9 is secreted predominantly in the liver (Miller et al., 2000), and its expression and processing is decreased by prolonged fasting (Caperuto et al., 2008). Further, it has been shown to be impaired in insulin resistant animals (Caperuto et al., 2008). Interestingly, BMP-9 has been reported as mediating increased acetylcholine synthesis and cholinergic action, at least in the central nervous system (Lopez-Coviella et al., 2000), and to need the combined arrival of glucose and insulin to the liver to be adequately expressed (Caperuto et al., 2008). However, BMP-9 influence on glucose homeostasis seems to differ from HISS in a most fundamental property. Administration of BMP-9 antibodies to fasted rats induces insulin resistance (Caperuto et al., 2008), when it was here observed that HISS manipulation in fasted animals did not produce any alteration on fasting insulin sensitivity; which is consistent with the hypothesis that HISS is only secreted in the postprandial state.

Both the inadequacy between BMP-9 and HISS action characteristics, and the methodological confounders in the studies supporting the nitrergic hypothesis, and the discrepancies to the results presented herein, argue against the validity of any of these

[80] Throughout this initial paper (Chen et al, 2003), HISS is referred to more as a possible category of hepatic factors able to control glucose homeostasis than to a unique factor. Thus, BMP-9 is presented more as *a* HISS than as *the* HISS. Only after (Caperuto et al, 2008), is it presented as corresponding exactly to HISS, with the same mechanism proposed by Lautt (Lautt, 1999).

alternative proposals in explaining the postprandial insulin sensitivity potentiation mechanism.

The sequence of events that constitute the HISS mechanism have been well described in what relates to the signal generation in the liver and target location in the skeletal muscle (Guarino et al., 2004; Guarino and Macedo, 2006). This provides a metabolic pathway that theoretically can be inhibited/stimulated at several points (Sadri and Lautt, 1999; Xie and Lautt, 1994). As it is, this was confirmed in the present thesis. The given doses of the muscarinic cholinergic antagonist, atropine, and the nitric oxide synthase inhibitor, L-NMMA, were able to produce the same degree of insulin resistance as the surgical ablation of the HPN; also without changing the mean postprandial glycaemic value. Furthermore, it was shown that, both in control strains and in pathological models of insulin resistance, postprandial insulin action assessed after HISS blockade corresponds to the fasting insulin action.

5.2. Modulation of insulin sensitivity by gender and developmental stage

Postprandial whole-body insulin sensitivity was here shown to be greatly dependent on age. More, depending on the developmental stage, this effect is due to alterations in the magnitude and/or dynamic profile[81] of either the action of insulin *per se* or the modulation of insulin responsiveness[82] by HISS (Kahn, 1978; Zhou et al., 2006). To add another layer of complexity to the control of insulin sensitivity, and to the concurrent experimental design on this phenomenon, ageing seems to have a similar impact on male and female, but this effect progresses at different rates with age in each gender.

[81] The analysis of the dynamic profiles obtained provided here adittional metabolic information. A similar proposition has been made for the OGTT, which usually is analysed at the 2-hours endpoint (Zhou et al, 2006).

[82] Throughout this thesis, as in most of the bibliography, the effect of insulin on stimulating cell glucose uptake is generally addressed to as "insulin sensitivity". However, since it corresponds to the study of a single dose of exogenous insulin (50 mU/kg), and not to a dose-response curve, it should be more correctly named "insulin responsiveness" (Kahn et al, 1978). In a strict sense, "insulin sensitivity" is rather related to the dose of insulin needed to produce half of the maximal insulin-stimulated glucose uptake (also known as the ED_{50}); "insulin responsiveness" is related to the maximal effect achieved by insulin. According to the presented hypothesis, HISS acts on skeletal muscle by potentiating insulin-stimulated glucose uptake, i.e. increasing the tissues responsiveness to insulin (augmenting insulin signalling either at the insulin receptor or at the intracellular signalling pathway). Nonetheless, for conceptual reasons, the choice was made to maintain the general term, inclosing this disclaimer.

At 78 weeks of age, which corresponds to old age in humans[83], Wistar rats show a similar marked decrease of whole-body insulin sensitivity in both genders, in comparison to young animals. However, this impairment is achieved more rapidly in males than in females. This may explain the observation sometimes alluded to that females are protected against the detrimental effects of age on insulin sensitivity. Indeed, female Wistar rats showed similar insulin sensitivity than males during maturation, but where less affected by middle-age. As pointed out, when old age was achieved, the females where as insulin-resistant as the males. These observations also stress the fact that, when conducting gender studies, the choice of subjects age can critically influence the results obtained concerning insulin sensitivity and glucose tolerance.

When this progress of insulin resistance is analysed by discriminating between the HISS-dependent and –independent components, more information surmises. During the earlier stage of sexual maturation, the direct effect of insulin on glucose uptake, i.e. the HISS-independent component, decreases. Considering that insulin plays multiple roles in physiology, and that one of the most important seems to be related with the control of growth, this may represent a reflex of changes in other pathways coordinated by insulin (Suryawan et al., 2001). Likewise, the HISS-dependent component changes during this phase of maturation, but not in magnitude. Maturation rather has an impact on HISS action dynamics: from 6 to 9 weeks of age, animals showed a remodelling of HISS action to a shorter duration but higher peak action. After 9 weeks of age, HISS action retains this duration, and decreases gradually in peak action.

The development with further ageing of the two constituents of postprandial insulin action shows further differences. While the HISS-dependent component decreases gradually, the HISS-independent component is essentially maintained; which argues for a preservation of the basic machinery associated with insulin signalling transduction. This seems to be consistent with human studies, which indicate that the prevalence of impaired fasting glucose does not rise with age, unlike impaired glucose tolerance which does[84] (Qiao et al., 2005). The present studies lead to the conclusion

[83] Representing, in Wistar rats, three quarters of their two-years mean life expectancy.

[84] Furthermore, an index of insulin resistance calculated solely based on fasting values, and reflecting hepatic, but not peripheral, insulin sensitivity, was found to be weakly influenced by age (Qiao et al, 2005). Again, this is consistent with the presented results, and the interpretation regarding the involvement of the HISS mechanism, primordially as a peripheral sensitizer, in the postprandial state.

that the gradual decrease of the HISS-dependent is the main responsible for the decreased postprandial insulin sensitivity with ageing.

The loss of parasympathetic autonomic nerve function with age, reported elsewhere (Sloan et al., 2008), becomes especially relevant in the context of the HISS mechanism. Similarly, the observation that this parasympathetic function and a favourable sympathovagal balance are preserved in healthy centenarians (Paolisso et al., 1999), who also present rather sustained glucose tolerance, again points to the critical importance that this pathway plays on the control of whole-body insulin sensitivity[85] (Bergman et al., 2007).

5.3. Particular susceptibility of HISS action to the detrimental effect of modern dietary habits

The critical effect of a HPN-dependent component decrease was again uncovered by a simple dietary manipulation. This diet change was intended to reflect the increase consumption of refined carbohydrates, even more important since it affects especially children and youngsters. This was achieved simply by providing the access to a source of sugared water.

The sucrose supplement quickly produced the near abrogation of the HISS-dependent component. Indeed, this effect was obtained with a mere two weeks of exposure to the 35% sucrose solution. Interestingly, the HISS-independent component of insulin action was unaffected, even after 9 weeks of exposure to the high-sucrose diet.

Knowing that fructose is absorbed after being dissociated into glucose and fructose, and that fructose-rich diets have been implicated in the impairment of parasympathetic vagal function (Miller et al., 1999), it might be concluded that it is the fructose content that is producing the decrease in parasympathetic function and, alone or in conjunction with other processes, impairing HISS action.

[85] Rather than looking solely for characteristics that predispose for ageing-related metabolic deregulation, the genetic makeup of high-longevity individuals is being studied in an attempt to identify favourable genotypes which confer protection against the influence of age-related disease genes (Bergman et al, 2007). It can be expected that genes that contribute to preserve or augment parasympathetic function must be among this group.

Diet-induced insulin resistance has been proposed to precede other aspects of the metabolic syndrome (Barnard et al., 1998), this puts into a renewed perspective the proposition that what the changes in modern lifestyle habits are doing is merely accelerating the effect of ageing on glucose homeostasis (Levi and Werman, 1998), and favouring the appearance of other pathologies. The present results highlight the hypothesis that this happens through the impairment of the HISS pathway control on postprandial insulin sensitivity.

5.4. Clarification of the relation between insulin resistance and hypertension through the study of the HISS-dependent and –independent components

Hypertension is indicated as a common element of the metabolic syndrome. However, even before this cluster develops, when defects of insulin sensitivity first become visible, hypertension is one of the associated diseases that reach full expression before glucose intolerance progresses (Facchini et al., 2001; Shanik et al., 2008). To study the relation between insulin resistance and hypertension, the SHR, the most common animal model for hypertension studies, was used.

Early contradictory studies led to the present use of two normotensive controls, the Wistar and the Wistar Kyoto. This showed that the normotensive Wistar Kyoto were as insulin-resistant as the SHR, when compared with Wistar rats. This argued for a secondary role of hypertension in this animal model of insulin resistance. When the two components of insulin action were studied, it became evident that here too the decrease of postprandial insulin sensitivity was solely due to an impairment of the HISS-dependent component. This impairment was even more marked in the hypertensive animals. This was not immediately seen when total postprandial insulin action had been assessed, since in this strain there is a compensatory increase of the HISS-independent component. The HISS pathway dysfunction seems thus to be connected to a genetic factor common among Wistar Kyoto and SHR; which effect is aggravated concomitantly with the presence of hypertension.

Interestingly, the results provided by the analysis of the Wistar Kyoto may show an unexpected relevance in regard to human pathophysiology, and the HISS connection; especially in the case of young normotensive offspring of hypertensive parents. These healthy offspring tend to present decreased insulin sensitivity (Beatty et al., 1993;

Pazarloglou et al., 2007; Vlasakova et al., 2004), and to develop glucose intolerance (Ferrari et al., 1991). Indeed, normotensive offspring of hypertensive subjects have been shown to have lowered parasympathetic activity (Davrath et al., 2003; Piccirillo et al., 2000). This fact has also been shown to be independent of the development of obesity (Allemann et al., 1993), a common confounding factor in the relation between insulin resistance and hypertension; which was here excluded by the choice of SHR animal model. This suggests the presence of a HISS action dysfunction in these individuals; further aggravated when hypertension becomes present.

The search for genes related to the predisposition to type 2 diabetes has been pursued for quite some time now (Beck-Nielsen and Groop, 1994; Ridderstrale and Groop, 2009). It seems thus opened the hypothesis that this search might be extended to the genetics of the HISS-dependent component of insulin action. The genetic analysis of the SHR strain has already been initiated (Pravenec et al., 2004).

The development of hypertension has been related to a previous poorer autonomic nervous system control (Chandler and DiCarlo, 1998; Schroeder et al., 2003), that may determine the decrease of HISS action. Indeed, the most relevant factor to the development of hypertension during maturation seems to be an increased contribution of the sympathetic nervous system (Zicha and Kunes, 1999), which would be expected to interfere also with the parasympathetic-controlled HISS pathway. However, the hypertensive state has similarly been associated with the disruption of the physiological action of compounds also involved on the HISS pathway; as nitric oxide and glutathione (Lepori et al., 2001). That HISS action worsens from the Wistar Kyoto to the SHR lifted the hypothesis that these other dysfunctions could have a synergistic effect on producing insulin resistance.

The dysfunction of nitric oxide in insulin resistant states is considered a conundrum. Although nitric oxide action is decreased, its production seems rather to be increased, in type 2 diabetics (Chien et al., 2005); and even in prediabetic, insulin-resistant offspring of type 2 diabetics (Piatti et al., 2000). This may be explained by an impairment of nitric oxide bioavalability (Hollenberg, 2006). More, nitric oxide seems to have a dual role; either being responsible for protecting tissues through neutralising reactive oxygen species or for favouring oxidative stress by increasing reactive nitrogen oxide species, apparently depending on its concentration (Wink et al., 2001). The later,

deleterious, effect may be related to the triggering of inflammatory and immunological responses[86] (Sarela et al., 1999; Wei et al., 2005).

However, in the present work, hepatic nitric oxide inhibition did not produce a further impairment of insulin sensitivity than the one attributable to its participation on the HISS pathway. Again, a causal relation is difficult to be established.

Here, insulin sensitivity after nitric oxide blockade was assessed at a time when the effect of the inhibitor on blood pressure had already waned. The idea that the involvement of nitric oxide in insulin sensitivity is mostly segregated from its effect on hemodynamic control seems to be supported by several reversal studies. Changes of blood flow achieved with a nitric oxide donor do not ameliorate glucose uptake (Natali et al., 1998). Analogously, other drugs induce changes in insulin sensitivity but not on blood pressure in hypertensive (Santure et al., 2000).

Glutathione is another compound with multiple physiological functions. It has been proposed that control of GSH efflux by insulin and glucagon may provide a balance between the several functions of GSH on glucose metabolism, antioxidant defence, and cysteine reservoir for use in other tissues (Lu et al., 1991). Nonetheless, control of GSH synthesis in the liver may also provide a balance between glucose metabolism, antioxidant defence, and acute-phase proteins synthesis within the liver proper (Lu et al., 1991). However, albeit hepatic GSH has been shown to be essential for the postprandial activation of the HISS pathway, the presented results suggest that it is not limiting in the dysfunction of the HISS-dependent component seen in hypertension.

After maturation, all three strains present similar total postprandial insulin sensitivity. In SHR, with the time of exposure to hypertension, the HISS-dependent component virtually disappears; which is accompanied by a further compensatory increase in the HISS-independent component. In the normotensive Wistar Kyoto, no alteration in either the HISS-dependent or –independent components was observed. This

[86] This would also support the hypothesis of a balance between the constitutive and inducible isoforms of nitric oxide synthase, which would influence insulin-stimulated glucose uptake, through the modulation of HISS action. Indeed, livers from cirrhotic patients show decreased constitutive NOS activity, while inducible NOS activity is similar to normal individuals (Sarela et al, 1999). The increased iNOS is expected only in later disease stages, in parallel with an inflammatory response (Wei et al, 2005).

was not surprising, when the result for the other normotensive control was considered. Indeed, in Wistar the HISS-independent component remained unchanged, while the HISS-dependent component decreased, to a value similar to the one presented by Wistar Kyoto at both 9 and 16 weeks of age. Thus, again the progression of insulin resistance in these pathologies seems to be an acceleration of the normal ageing deleterious effect on HISS-dependent insulin sensitivity, either the organism is able to compensate or not for that insulin resistance.

Interestingly, autonomic nervous system control, which is lower even during the development of hypertension, deteriorates with age more in normotensive than in hypertensive subjects (Schroeder et al., 2003). This may contribute, and even explain, the relative age alterations of the HISS-dependent component of insulin action between the three animal strains.

It has been described that insulin leads, in elderly individuals, to vasoconstriction; when in young individuals it produces vasodilation through the production of nitric oxide in the endothelium (Hausberg et al., 1997). This led several researchers to postulate that the decrease of whole-body insulin sensitivity with age is related to a lack of proper distribution of insulin and glucose to the skeletal muscle cells (Baron et al., 2000). The vascular properties of insulin would thus regulate the ability to exert its metabolic functions. Other authors maintain that insulin indeed plays a role in the regulation of blood pressure, but that this effect is not a determinant in skeletal muscle glucose uptake (Scherrer et al., 1994).

The results presented here show that the action of insulin *per se* on tissue glucose uptake, both in normal and disease animal models, is essentially maintained or even augmented. In contrast to this preservation, the neurohumoral mechanism by which whole-body is potentiated after a meal ingestion was revealed to be extremely vulnerable. Consequently, these results support the notion of separately controlled effects of insulin on glucose metabolism and blood pressure control.

5.5. The HISS pathway as a new paradigm in the cluster of hepatic disease, autonomic dysfunction, insulin resistance, and type 2 diabetes

It is well established that liver disease is accompanied by defects in whole-body glucose homeostasis (Tsochatzis et al., 2009), and that it can be a determining factor in the metabolic syndrome cluster (Haffner, 2006). Surprisingly, the initial stages of liver dysfunction are related not with hepatic insulin resistance, as would be expected, but to a decrease in peripheral insulin sensitivity; having been attributed to a decrease in glucose uptake and glycogen synthesis in the skeletal muscle (Selberg et al., 1993). Only after, defects in hepatic glucose output suppression and insulin secretion determine the progression to type 2 diabetes (Perseghin et al., 2000). Interestingly, this same behaviour is uncannily similar to the progress seen from impaired glucose tolerance to type 2 diabetes; specifically when insulin hypersecretion fails or is unable to further compensate for the insulin resistance in skeletal muscle and adipose tissue (Kido et al., 2000).

The progression of liver disease seems to be also accompanied by radical changes in autonomic nervous system control. The increase in parasympathetic nervous tone from fasting to the postprandial state is quickly lost. On the other hand, the sympathovagal balance is preserved in early stages of the disease; both the basal ratio during fasting as well as the decrease of this ratio with food ingestion. However, in latter stages, there is an imbalance towards greater sympathetic dominance, which is even worsened in the postprandial state (Miyajima et al., 2001).

This is consistent with the observation that liver injury leads to HISS dysfunction (Ming et al., 2006). The elements that can impact on HISS action are multiple, as shown herein. Further, they seem to have a cumulative effect. For example, a state of emotional stress leads to a worsened metabolic state of hypertensive subjects (Piccirillo et al., 1998b). Indeed, loss of parasympathetic tone has been proposed as a possible mechanistic link between the stressful outcome of low socioeconomic status and poor health (Hemingway et al., 2005; Sloan et al., 2005). Similarly, the state of insulin resistance in essential hypertension can be further aggravated by nutritional factors (Uchida et al., 1997).

The aetiological importance of the decrease in hepatic parasympathetic nerve function to this cluster may perhaps be illustrated by the fact that insulin resistant, but

yet glucose tolerant, offspring of type 2 diabetic patients have been reported to show already an impairment of the parasympathetic nervous system (Frontoni et al., 2003).

Taking into account the present results, the relation between the health status of the liver and hepatic parasympathetic nerves, and glucose homeostasis, seems to be far from coincidental. Additionally, the dysfunction of other steps of the HISS pathway may contribute to a general impairment of this mechanism.

Recent studies, with administration of drugs that prevented the rise of oxidative stress, were able to avoid the loss of the HISS-dependent component of insulin action seen with ageing (Lautt et al., 2008). This importance of oxidative stress with ageing has been recognised to impair cysteine availability and/or GSH synthesis (Liu and Choi, 2000) in parallel with insulin resistance (Droge, 2005; Droge and Kinscherf, 2008); which is consistent with the hypothesis of HISS decrease with age. Interestingly, the degradation of nerve conduction seen in glucose intolerance states (Viswanathan et al., 2004) seems to be prevented by a pharmacological intervention able to address the aforementioned problem (Soneru et al., 1997).

Likewise, the impairment of HISS action in animals provided a high-sucrose diet was recently reverted by pharmacologically mimicking both feeding signals (Lautt et al., 2009). Interestingly, although full recovery of HISS action was only possible with the simultaneous administration of both drugs (bethanechol and n-acetylcysteine), the parasympathetic agonist showed to produce by itself a partial improvement of postprandial insulin sensitivity. Other authors showed that high-sucrose diets produce a decrease in nitric oxide bioavailability at the onset of insulin resistance (Blouet et al., 2007b), with postprandial glucose disposal being then ameliorated by dietary cysteine (Blouet et al., 2007a; Blouet et al., 2007c).

Although the chemical nature of the HISS molecule remains elusive, some of the characteristics of its formation and action mechanism may be speculated upon.

The two feeding signals are essential for the possibility of HISS action, either by determining its formation (as seems to be the case with cysteine to GSH synthesis) and/or release. However, the hypoglycaemic effect of HISS is felt on whole-body homeostasis only when circulating levels of insulin are increased. This raises the hypothesis that HISS formation is dependent upon a processing of insulin itself;

possibly related to the combination of GSH and nitric oxide in the liver. Indeed, insulin chains have been shown to be able to undergo nitrosylation (Santos et al., 2006).

Insulin is known to only be able to determine some of its functions when degraded, after being internalised while bound to the insulin receptor (Fawcett et al., 2001). Insulin degradation is promoted by IDE. Further, hepatic IDE activity is increased by rising circulating insulin levels (Pivovarova et al., 2009). Thus, the rise in insulin in the postprandial state leads to an increase of the availability of insulin chains within hepatocytes; where they might be nitrosylated by increased GSH and nitric oxide. The ultimate fate of insulin within the hepatocyte, degradation or reprocessing, may be determined by the modulation of IDE by nitric oxide (Cordes et al., 2009).

In response to a meal, insulin levels rise. This insulin can produce alterations in the hepatocyte membrane; leading to the formation of caveolin-rich membrane domains, where insulin receptors are also located (Balbis et al., 2004; Vainio et al., 2002). After, activated insulin receptors migrate from the lipid rafts in microvilli to clathrin-coated pits, where they are internalised to endosomes (Carpentier et al., 1993).

The eNOS isoform has also been reported as being co-localised with caveolin at the intracellular side of the hepatocyte membrane (Jobgen et al., 2006; Viaro et al., 2000). Postprandial acetylcholine release by hepatic parasympathetic nerves can bind to muscarinic receptors in the cell membrane and lead to the intracellular release of calcium. This activates calmodulin, which releases the eNOS from being bound to caveolin, and allows the production of NO (Shah et al., 2007). Additionally, increases in insulin and cysteine promote the synthesis of hepatic GSH (Kim and Reaven, 2004; Kwon and Stipanuk, 2001). Thus, insulin would be cleaved by endosomal IDE in concert with PDI (Jung and Thomas, 1996), with GSH as a co-substrate (Turano et al., 2002) and a determinant of the processing pathway followed by insulin (Morgan et al., 1985); with the simultaneous nitrosylation of the insulin fragments. Insulin molecules and fragments are then released into the bloodstream when the insulin receptor is recycled to the cell surface (Duckworth et al., 1998).

This proposed mechanism would also explain certain defects in the HISS pathway. For example, increased lipid deposition may induce changes in the membrane lipid content, altering the relation of the insulin receptor with the lipid rafts (Vainio et al., 2005). Also, in liver disease, eNOS seems to be translocated away from the hepatocyte membrane to the nuclei (McNaughton et al., 2002), which could entail a

decrease in HISS synthesis. That hepatic IDE activity is inhibited by excessive glycaemia (Fawcett and Duckworth, 2009) hints to a possible mechanism by which a marginally impaired postprandial glucose disposal would exacerbate HISS dysfunction in a vicious cycle. GSH may also have a secondary role in HISS synthesis, by preserving IDE and PDI activities (Carbone et al., 2005). Indeed, they seem easily disruptible by thiol-alkylating compounds (Neant-Fery et al., 2008); which makes it particularly sensitive to oxidative stress.

Additionally, conditions where postprandial hepatic insulin extraction is altered in response to peripheral insulin resistance (Hojlund et al., 2006), as ageing (Basu et al., 2006; Matveyenko et al., 2008), hypertension (Burattini et al., 2009; Salvatore et al., 1992), obesity (Valera Mora et al., 2003), and liver disease (Goto et al., 1995; Letiexhe et al., 1993) and transplantation (Schneiter et al., 2000a), may contribute to change intrahepatic insulin availability and synthesis of HISS.

The mode of transportation of HISS through the bloodstream to reach the target tissue, the skeletal muscle, is less clear, although it can be proposed to be in association with a carrier protein. Indeed, nitric oxide is able to circulate as an S-nitroso adduct of serum albumin (Di Simplicio et al., 2003; Stamler, 2004; Stamler et al., 1992).

Once reaching the skeletal muscle, the HISS molecule would act either directly on the insulin receptor[87], or through a specific receptor. In this respect, it has been shown that surface-bound PDI catalyses the transfer of the nitric oxide carried by extracellular S-nitrosothiols into intracellular thiols (Ramachandran et al., 2001; Zai et al., 1999). Consistent with the HISS hypothesis, endogenously-produced nitric oxide has been shown to induce effects in organs distant from the organ site of expression (Elrod et al., 2008).

It seems thus possible that HISS is produced in the liver, in result of the balance between several nutrient signals, and that it travels through the bloodstream to ultimately potentiate insulin-stimulated glucose uptake at the skeletal muscle.

[87] The insulin fragments that are released through retroendocytosis have been reported to maintain receptor binding and biological activity (Duckworth, 1998).

5.6. Conclusion and implications

Type 2 diabetes is today a global problem (Yach et al., 2004). Each 10 seconds two more patients are diagnosed worldwide; with a particular impact on the working age population, in developed and developing countries alike (Siegel and Narayan, 2008). There has been a debate about the feasibility of doing a population-wide screening for undiagnosed diabetes and prediabetes (Waugh et al., 2007). The present results argue that a crucial risk factor for metabolic deregulation, the dysfunctional HISS action (Lautt, 2007), is settled well before the emergence of alterations in blood glycaemia, arterial pressure, or body weight. The early detection of this dysfunction in asymptomatic individuals would most likely be ultimately cost effective, and would greatly decrease the morbidity and mortality that derive from type 2 diabetes and accompanying pathologies.

Furthermore, the increased pressure to sanction HbA1c status as the criteria for the diagnosis of diabetes (ADA, 2010), and the attempt to eradicate the concept of prediabetes - with its different aetiologies that may impact on the success of preventive measures (Faerch et al., 2009) - from the clinical practice (TIEC, 2009), raises the concern that interventions will be applied more and more at a latter stage of glucose homeostasis deregulation; perhaps when HISS function, and postprandial incremental insulin sensitivity, are already mainly irrecoverable.

The insulin signalling pathway in peripheral tissues was here shown to be more robust than most studies lead us to believe for the last three decades. Well before changes of the direct action of this energy regulatory hormone on insulin-sensitive tissues occurs, the postprandial potentiation provided for by the HISS pathway is impaired, or even absolutely lost.

Although genetic defects have been shown to be able to determine the impairment of the insulin pathway at a distal point common to both HISS-dependent and –independent components (Afonso et al., 2007b), other genetic factors, and the majority of environmental manipulations, lead to whole-body insulin resistance primarily by the dysfunction of the HISS-dependent component of insulin action (Afonso et al., 2007a; Lautt, 2007; Ribeiro et al., 2008; Ribeiro et al., 2007; Ribeiro et al., 2001; Ribeiro et al., 2005).

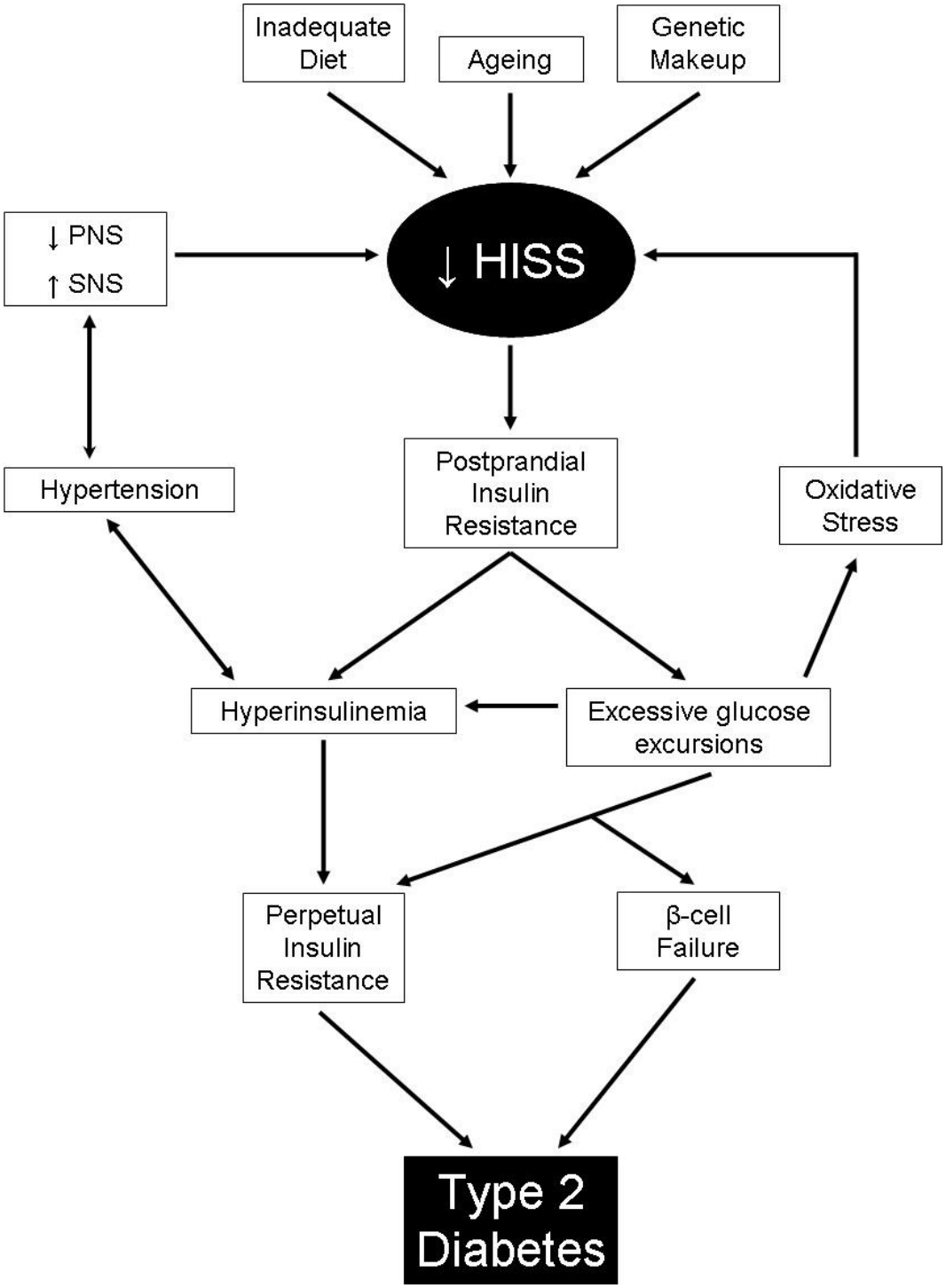


Figure 30 – Integrative view: from hepatic insulin sensitising substance (HISS) dysfunction to progression to type 2 diabetes. Conditions highlighted in the present studies, as inadequate diet, ageing, and possibly genetic predisposition, lead, by several mechanisms, like parasympathetic dysautonomia, to HISS dysfunction. This induces selective postprandial insulin resistance; with slower disposal of absorbed glucose in peripheral tissues, excessive glucose excursions, and hyperinsulinemia. Oxidative stress may worsen HISS action. Hypertension is likewise related to hyperinsulinemia and a detriment in parasympathetic autonomic function. Exposure to postprandial glucose intolerance favours the development of perpetual insulin resistance. Insulin needs and glucotoxicity contribute ultimately to the collapse of β-cells, and establishment of type 2 diabetes.

Figure 30 integrates the dysfunction of the HISS mechanism with the progression to type 2 diabetes, with special emphasis on the pathologies addressed in the present thesis.

Behavioural interventions, like nutritional control and physical activity, have been at the forefront of the effort for delaying the development of type 2 diabetes; especially in individuals with recognised risk factors (Carnethon et al., 2003a; Roumen et al., 2008). The fact that these activities have been described to ameliorate parasympathetic function (Hirsch et al., 1991; Nagai and Moritani, 2004; Wichi et al., 2009) provides a link between them and HISS action; and highlights even more eloquently the impact of lifestyle in a time where overall glucose metabolism seems still normal.

Drug therapy has also become an important support for addressing the development of type 2 diabetes (Cefalu, 2007; Hays et al., 2008). However, it has been described as being directed to effects rather than causes of type 2 diabetes, and to have several side effects associated (Moller, 2001). It should be undoubtedly more favourable to direct these efforts address the earlier defects in glucose homeostasis, as seems the case with the impairment of the HISS-dependent component of insulin action by genetic and environmental factors.

The HISS mechanism was shown to be responsible for the increment of insulin sensitivity seen in the postprandial state. Here, both the hepatic parasympathetic nerves and hepatic nitric oxide were confirmed as vital elements in this pathway.

The loss of HISS action happens naturally during the ageing process. Additionally, nutritional factors can accelerate that loss. Likewise, genetic factors seem to be able to diminish HISS action in healthy animals; with a further lessening if hypertension is present.

Further, the analysis of these pathological animal models has lead to the conclusion that the dysfunction of the HISS pathway predates the development of obesity and hyperglycaemia.

For all this, the observed HISS dysfunction seems to lay at the ontogeny of insulin resistance, and of the alteration of postprandial glucose fluxes; which may be at the base of type 2 diabetes and the metabolic syndrome. This strengthens the notion that

the HISS pathway is an attractive target for behavioural and pharmacological interventions; especially those that show to be able to ameliorate autonomic parasympathetic nervous function.

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