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FAST-TO-FED SHIFT IN GLUCOSE HOMEOSTASIS: CLUES TO AN EARLIER DETECTION OF HUMAN PREDIABETIC STATES

Faculdade de Ciências Médicas

Universidade Nova de Lisboa

Lisboa

2010

Dissertation presented to obtain the PhD degree in "Ciências da Vida, especialidade de Fisiologia" at the Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Supervised by Professors M. Paula Macedo and W. Wayne Lautt

FCT Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Ciência.Inovação 2010
Programa Operacional Ciência e Inovação 2010
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Realizado com o apoio da Fundação para a Ciência e Tecnologia (SFRH/BD/5806/2001) e co-financiado pelo Programa Operacional Ciência e Inovação 2010 (POCI 2010), no âmbito da formação avançada para a Ciência – Medida IV.3 (III Quadro Comunitário de Apoio).

To my parents and Bruno

"Research is what I'm doing when I don't know what I'm doing."

Wernher von Braun

PUBLICATIONS

The original results and conclusions presented in this thesis have been partially published in the following peer-reviewed papers.

- Rita S. Patarrão, W. Wayne Lautt, J. Schafer and M. Paula Macedo, Glucagon causes HISS-dependent insulin resistance (*in preparation*).

- Rita S. Patarrão, W. Wayne Lautt, Ricardo A. Afonso, Rogério T. Ribeiro, Ana B. Fernandes, José M. Boavida and M. Paula Macedo, Overweight subjects have impaired postprandial but not fasting insulin sensitivity due to a compromise of the HISS-dependent component (*in preparation*).

- Rita S. Patarrão, W. Wayne Lautt, Maria P. Guarino, Ricardo A. Afonso, Rogério T. Ribeiro, Ana B. Fernandes, José M. Boavida and M. Paula Macedo, Meal-induced insulin sensitisation and its parasympathetic regulation in humans. *Can J Physiol Pharmacol*, 86: 880-888 (**2008**).

- Rita S. Patarrão, W. Wayne Lautt, Maria P. Guarino, Ricardo A. Afonso, Rogério T. Ribeiro, Ana B. Fernandes, José M. Boavida and M. Paula Macedo, A new technique to assess insulin sensitivity in humans: the rapid insulin sensitivity test (RIST). *Proc West Pharmacol Soc*, 50: 105-109 (**2007**).

- Celina Santos, Ricardo A. Afonso, Maria P. Guarino, **Rita S. Patarrão**, Ana B. Fernandes, Maria P. Macedo and Jorge Caldeira, In vitro nitrosation of insulin A and B-chains. *Eur J Mass Spectrom*, 12: 331-338 (**2006**).

ACKNOWLEDGEMENTS

Neste momento tão importante da minha vida científica gostaria de salientar que a realização e concretização desta tese de doutoramento, só foi possível graças à presença e envolvimento de várias pessoas e instituições.

À Fundação para a Ciência e Tecnologia, pelo seu suporte financeiro.

À Faculdade de Ciências Médicas e à Faculdade de Medicina de Manitoba, Winnipeg, Canadá, mais especificamente, ao Departamento de Farmacologia e Terapêutica, por todas as facilidades concedidas para a realização do trabalho experimental em animais de laboratório.

Ao Instituto Português de Oncologia de Lisboa e à Associação Protectora dos Diabéticos de Portugal pela cedência das instalações e por todas as facilidades concedidas para a realização do trabalho experimental em humanos.

À Professora Doutora Maria Paula Macedo, minha orientadora e, acima de tudo amiga. Estou certa de não dispor de palavras suficientes para exprimir toda a minha gratidão, pela orientação de todo o meu trabalho científico. Foi com ela que dei os meus primeiros passos na ciência, e sem ela a realização desta dissertação jamais seria possível. A sua forma de estar perante a Ciência é única, e contagia qualquer um, mesmo nos momentos mais difíceis. Um muito obrigada, Profezinha!

To Wayne, I would like to express my heartfelt thanks for all the knowledge, the scientific discussions, and for giving me the opportunity to learn and share so much science in his lab, across the ocean. His enthusiasm in research as a co-supervisor is "healthy". My personal and scientific life without him would not be the same. He is simply the best co-supervisor in the world that someone may have!

Ao Professor Doutor Pedro Costa, por me ter recebido e acolhido no Departamento de Fisiologia e por ter sido um interlocutor disponível e arguto.

Ao Professor Doutor Miguel Mota Carmo, para além da grande amizade criada, pelo seu contributo na minha vida pessoal, académica e científica, e pela sua preocupação constante que manifestou desde sempre.

À Professora Doutora Maria da Graça Morais, pela confiança demonstrada ao convidar-me como docente do Departamento de Bioquímica e por toda a sensatez com que sempre me aconselhou.

Aos Professores Doutores António Bensabat Rendas, Maria Amália Botelho e Miguel Seabra, sempre interessados demonstrando grande entusiasmo no meu percurso científico.

Aos membros dos Departamentos de Fisiologia, Fisiopatologia e Bioquímica da Faculdade de Ciência Médicas, nomeadamente, Professora Doutora Ana Isabel Santos, Professora Doutora Alexandra Ribeiro, Professor Doutor Carlos Filipe, Dra. Mafalda Nascimento, Sofia Ramos, Dra. Iolanda Caires, Isabel Ribeiro da Silva, Dra. Ana Sofia Tavares, Helena Almeida, Teresa Fortunato, Firmina Lebre e Augusta Marques pelo acolhimento e pela cooperação.

Aos membros do Biotério da Faculdade de Ciência Médicas e da Faculdade de Medicina de Manitoba, nomeadamente à Gracinda Menezes, Mariana Lavajo, Ludmila, e Gery Nolette, por todo o apoio prestado na manutenção e manipulação de todos os animais de laboratório utilizados nas muitas experiências realizadas.

Ainda na Faculdade de Ciências Médicas um obrigado muito especial à Professora Doutora Paula Videira, Dra. Luísa Barata e Dra. Mafalda Rodrigues.

Aos Professores Doutores Jorge Caldeira e Celina Santos, do Departamento de Química da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa, a quem agradeço terem tornado o "mundo da Química" um pouco mais cor-de-rosa.

A todos os membros do Laboratório da Associação Protectora dos Diabéticos de Portugal por todo o apoio prestado. Uma palavra muito especial para a Dra. Zulmira Peerally que, para além da grande amizade criada, esteve sempre a meu lado com uma constante precisão e rigor, aquando da realização das inúmeras quantificações bioquímicas e, na posterior interpretação dos resultados obtidos.

Aos Doutores José Manuel Boavida, Rui Duarte, Luís Gardete Correia e ao Professor Doutor João Raposo, da Associação Protectora dos Diabéticos de Portugal, por todas as reuniões científicas que tivemos em conjunto e pelo grandioso apoio na área clínica da Diabetes.

Gostaria de prestar um especial agradecimento a todos os colegas de trabalho, de Winnipeg e de Lisboa, que comigo partilham ou partilharam toda a minha vivência no laboratório.

À Ana Fernandes, além de ser uma grande colega, é uma grande, grande amiga. A sua capacidade científica e de lidar com uma hipótese de trabalho é "espectacular". A sua sinceridade é acima de tudo especial e muito construtiva. Os seus comentários e a forma

como estes foram discutidos ao longo destes anos, trouxeram uma grande "mais-valia" para a elaboração desta dissertação. Obrigada, Iana!

À Maria Guarino, um obrigado muito especial. Jamais esquecerei a nossa amizade e todos os nossos momentos partilhados. As nossas discussões científicas serão sempre recordadas com um carinho muito especial.

Ao Ricardo Afonso, apesar de inúmeras vezes as nossas ideias e pontos de vista serem distintos, nunca deixámos de os discutir e disfrutar das suas consequências. A nossa colaboração e entreajuda mútua tem sido muito construtiva e saudável!

Ao Rogério Ribeiro, pela partilha científica e por todo o apoio informático ao longo destes anos.

À Inês Lima, Fátima Martins e Iva Lamarão, pela enorme "lufada" de ar fresco e "energia renovável" que souberam trazer ao laboratório.

I thank Josh Schafer, my "Canadian brother", for all of his time dedicated to me, towards science. It was good working with a person like Josh, especially in a lab far away from home. "Rita, do you have your timer?"

I am grateful to Dallas Legare for his attention and fantastic technical training.

To Zhi Ming and David Sontag for their friendship and camaraderie.

Karen Sanders, I have no words to described her superb efficiency, competence and capacity to organize such sort of things.

To all my Canadian friends, especially, Samantha, Christy, Kristin, Carmen, Clatus, Marc and "Bosco", a supreme acknowledgment. Thank you all for being there, and for all special support that you gave me!!

À Nina Correia, Filipa Duarte-Ramos, e Nair Bonito, amigas e ex-colegas de laboratório, por todos os bons e menos bons momentos passados juntas.

À Gisa e à Milu, pela enorme amizade.

Para além da minha vida científica, existem pessoas sem as quais a minha vida não faria sentido.

Aos meus pais, pois sem eles eu não estaria cá, e não os presentearia com esta dissertação. Esta etapa da minha vida também é vossa! Queria aqui deixar o meu testemunho por todo o carinho, compreensão, perseverança, apoio, paciência e, tudo o mais que vai ficar por dizer, pois as palavras nunca serão suficientes para expressar tal gratidão.

Ao Bruno, por tudo e mais alguma coisa! Nestas alturas surgem sempre as maiores dificuldades para escrever algo tão especial para uma pessoa tão maravilhosa e compreensível. Soube sempre estar ao meu lado, mesmo aquando dos meus longos períodos de ausência, com um positivismo inigualável. Por todo o apoio e compreensão incondicional que me tem oferecido ao longo destes anos. No entanto, o "Galo" já canta!! Atingimos mais um grande objectivo das nossas vidas, mas ainda faltam muitos outros...

À minha restante família, nomedamente aos meus sobrinhos, Diogo e Frederico. Vocês foram muitas vezes, e sem se aperceberem, a maior fonte de energia interior para eu conseguir rumar a bom porto esta dissertação. Vocês são únicos!

A todos os meus amigos. Um agradecimento especial à Ditinha, ao Adolfo e à Margarida pela sólida amizade que nos une e que tanto me faz sentir segura. Vocês mereciam mais do meu tempo, mas souberam sempre desculpar e compreender os períodos da minha ausência.

A todos muito obrigada.

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ACh: Acetylcholine
ADA: American Diabetes Association
ADP: Adenosine monophosphate
Akt: Protein kinase B (Akt/PKB)
APS: Adaptor protein with PH and SH2 domains
AS160: Akt substrate of 160 kDa
ATP: Adenosine triphosphate
AUC: Area under the curve
BAT: Brown adipose tissue
BMI: Body mass index
BSO: L-buthionine-[S,R]-sulfoximine
bw: Body weight
cAMP: 3',5'-cyclic adenosine 5'-monophosphate
CAP: Cbl associated protein
Cbl: Casitas b-lineage lymphoma
CCAC: Canadian Council on Animal Care
CCK: Cholecystokinin
CHE: Cholesterol esterase
CHO: Cholesterol oxidase
CoA: Coenzyme A
Da: Dalton
DAG: Diacylglycerol
DBcAMP: N^6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate
EASD: European Association for the Study of Diabetes
ECG: Electrocardiogram
ED_{50}: Concentration of agonist that provokes a response halfway between the baseline and maximum response
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-Linked Immuno-Sorbent Assay
F-DAOS: N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxy-4-fluoroaniline
FFA: Free fatty acids
FPG: Fasting plasma glucose

FSIVGTT: Frequently sample intravenous glucose tolerance test

G: Gauge

G-6-Pase: Glucose-6-phosphatase

GIP: Glucose-dependent insulinotropic peptide

GIR: Glucose infusion rate

GK: Glycerol kinase

GLP-1: Glucagon-like peptide 1

GLUT: Facilitative glucose transporter

GOx: Glucose oxidase

GPO: Glycerol phosphate oxidase

GSH: Reduced glutathione

H_2O_2: Hydrogen peroxide

H_2SO_4: Sulfuric acid

HbA_{1c}: Glycosylated hemoglobin

HCl: Chloride acid

H-DAOS: N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

HDIR: HISS-dependent insulin resistance

HDL: High density lipoprotein

HGP: Hepatic glucose production

HIEC: Hyperinsulinemic-Euglycemic Glucose Clamp

HISS: Hepatic Insulin Sensitizing Substance

HMIT: H^+-coupled myo-inositol transporter

IFG: Impaired fasting glucose

IGF: Insulin-like growth factors

IGT: Impaired glucose tolerance

ip: Intraperitoneal

IP_3: Inositol 1,4,5-triphosphate

ipv: Intraportal

IRMA: Radioimmunoradiometric assay

IRS: Insulin receptor substrate

ITT: Insulin tolerance test

iv: Intravenous

kg: Kilogram

kJ: Kilojoule

LDL: Low density lipoprotein

L-NAME: N^G-nitro-L arginine methyl ester

L-NMMA: N-monomethyl-L-arginine

M: Amount of glucose metabolized

Mab: Monoclonal antibody

MAP: Mean arterial pressure

MIS: Meal-induced Insulin Sensitization

NaCl: Sodium chloride

n.d.: not determined

NGT: Normal glucose tolerance

NO: Nitric oxide

NOS: Nitric oxide synthase

OGTT: Oral Glucose Tolerance Test

PDK1: Phosphoinositide-dependent protein kinase-1

PEPCK: Phosphoenolpyruvate carboxykinase

PGC-1: Peroxisome proliferator-activated receptor-γ coactivator-1

PH: Pleckstrin homology

PI3K: Phosphoinositide-3 kinase

PIP_3: Phosphatidylinositol 3,4,5-trisphosphate

PKA: Protein kinase A

PKB: Protein kinase B (Akt/PKB)

PKC: Protein kinase C

PLC: Phospholipase C

POD: Peroxidase

PPG: Postprandial plasma glucose

RBP-4: Retinol binding protein-4

RIST: Rapid Insulin Sensitivity Test

S_G: Glucose effectiveness index

S_I: Insulin sensitivity index

SD: Sprague-Dawley

SGLT: Sodium glucose co-transporter

SH2: Src homology 2

SI_{Clamp}: Insulin sensitivity index derived from clamp

SIN-1: 3-morpholinosydnonimine hydrochloride

SSPG: Steady-state plasma glucose

SSPI: Steady-state plasma insulin

T2D: Type 2 diabetes

TAG: Triglycerides

TCA: Trichloroacetic acid or tricarboxylic acid

TMB: 3,3’,5,5’-tetramethylbenzidine

TNF-α: Tumor necrosis factor-alpha

TRIS: 2-Amino-2-(hydroxymethyl)-1,3-propanediol

VLDL: Very low density lipoprotein

WAT: White adipose tissue

ABSTRACT

Insulin action is associated with the release of the Hepatic Insulin Sensitizing Substance (HISS), which enhances peripheral glucose uptake. In the fed state, HISS release is maximal, decreasing with the duration of fasting. The prandial control of HISS action is mediated through hepatic parasympathetic - derived nitric oxide (NO), and hepatic glutathione (GSH). The current methods used to evaluate insulin sensitivity are only performed in the fasted state. The present thesis focus on the hypothesis that HISS-dependent mechanism exists in humans and can be manipulated.

In humans, a new powerful tool to characterize HISS-dependent insulin action not only in the fasted, but also in the fed state, the Rapid Insulin Sensitivity Test (RIST), was developed. The RIST can be performed with reproducible results, having no intra and inter-variability.

The decreased insulin sensitivity observed in the fasted state is potentiated following a meal, and intravenous atropine administration suppresses this effect. The partial blockade of meal-induced insulin sensitization (MIS), is consistent with the hypothesis that a hepatic parasympathetic "feeding signal" is necessary for hepatic HISS release.

When lean and overweight subjects were submitted to a 24h fasting period, the insulin action *per se*, was similar in both groups studied. However, when one performed the insulin sensitivity in the fed state, the results present within this thesis have shown that, the lower MIS observed in the overweight subjects is associated with an impairment of the HISS-dependent component. These results indicate the importance of postprandial insulin sensitivity evaluation, and also suggested that the prediabetic state can only be detected in the fed state.

Hyperglucagonemia is associated with type 2 diabetes. It is already known that glucagon leads to a decrease in GSH synthesis and hepatic GSH is crucial for HISS release, therefore the effect of glucagon on HISS-pathway was evaluated. In animals, both a cAMP analog, and glucagon produce a decrease of insulin sensitivity in a dose-dependent manner. The HISS-dependent insulin resistance produced by a NO synthase inhibitor was not aggravated by glucagons. These results suggest that glucagon, leads to reduced insulin sensitivity through a decrease of HISS action, and not via some other action. The two observations together are in support that glucagon acts via cAMP pathway to decrease hepatic GSH levels, leading to an impairment of HISS release. This decrease might be responsible for an earlier stage of insulin release.

RESUMO

A acção da insulina está associada à libertação da substância hepática sensibilizadora da insulina (HISS), que aumenta o aporte de glucose periférico. No estado pós-prandial, a libertação da HISS é máxima, diminuindo com o período de jejum. O controlo prandial da acção da HISS é mediado pelo sistema parassimpático hepático/óxido nítrico (NO) e pelo glutationo (GSH) hepático. Os actuais métodos utilizados para avaliar a sensibilidade à insulina são realizados no estado de jejum. A presente dissertação destaca a hipótese de que o mecanismo dependente da HISS existe em humanos, e pode ser manipulado.

Em humanos, uma robusta ferramenta para caracterizar a acção da insulina dependente da HISS, não só no estado de jejum, mas também após uma refeição, o teste rápido de sensibilidade à insulina (RIST), foi desenvolvido. O RIST pode ser realizado com reproductibilidade, e sem intra e inter-variabilidade.

A diminuição da sensibilidade à insulina observada no jejum é potenciada após uma refeição, e a administração de atropina, suprime este efeito. A inibição parcial da sensibilidade à insulina induzida pela refeição, é consistente com a hipótese de que um "sinal prandial" dependente do sistema parassimpático hepático é necessário para a libertação hepática da HISS.

Quando voluntários magros e com excesso de peso foram submetidos a um período de 24h de jejum, a acção da insulina *per se*, foi similar em ambos os grupos estudados. Contudo, quando avaliados no estado pós-prandial, os resultados apresentados nesta dissertação mostraram que, a potenciação induzida pela refeição era inferior nos voluntários com excesso de peso, estando esta associada a uma alteração da componente da acção da insulina dependente da HISS. Estes resultados indicam a importância da avaliação da sensibilidade à insulina no estado pós-prandial, e sugerem também que este estado de pré-diabetes apenas pode ser detectado após a ingestão de uma refeição.

Níveis elevados de glucagina estão associados à diabetes tipo 2. Sabendo que a glucagina leva a uma diminuição da síntese de GSH, e que o GSH é fundamental para a libertação da HISS, foi avaliado o efeito da glucagina na via da HISS. Em animais, tanto a administração de um análogo do cAMP, como de glucagina, produziram um decréscimo da sensibilidade à insulina, sendo este efeito dependente da dose. A resistência à insulina dependente da HISS observada aquando da administração de um inibidor do sintetase do NO, não se agravou com a posterior administração de glucagina. Estes resultados sugerem então que a glucagina induz um decréscimo da sensibilidade à insulina, sendo este dependente da via da HISS, e não por acção de outra via. Estas duas observações indicam que a glucagina, através da via de sinalização do cAMP, leva à diminuição dos níveis hepáticos de GSH e, consequentemente, a uma alteração da via da HISS. Esta alteração poderá ser a responsável por um estado precoce de resistência à insulina.

PREFACE

Diabetes is reaching pandemic proportions across the globe. Estimates from 2006 indicate that 5.9% of the world's population (246 million) have diabetes, and 7.1% (380 million) will have the condition by 2025, with type 2 diabetes responsible for nine in every 10 diabetes patients (Ceriello *et al.*, 2008a).

The majority of studies and diagnosis of type 2 diabetes depend almost entirely upon determinations of fasting plasma levels of glucose and, less frequently, insulin. This approach also leads to a severe underestimation of the metabolic dysfunction that is typical of the type 2 diabetic.

It is known that well before the deterioration of fasting glucose, as glucose intolerance progresses, also the control of postprandial glucose control is lost.

A distinction has been created, to describe an intermediate stage between completely normal glucose homeostasis and the clinical entity of type 2 diabetes, which has become known as prediabetes. The prediabetic state encompasses both impaired fasting glucose and impaired glucose tolerance. The prediabetic state of insulin resistance can be diagnosed by determining the response to a test meal. Early identification and treatment of subjects with prediabetes has the potential to reduce or delay the progression to the disease, and the related complications (Woerle *et al.*, 2004).

Some studies developed by Lautt and Macedo groups, has shown that postprandial regulation of acute metabolic response to insulin is mediated by the hepatic parasympathetic nerves (Lautt *et al.*, 2001). Activation of this mechanism leads to the release of an hormone from the liver, referred to as hepatic insulin sensitizing substance (HISS), which acts to stimulate glucose uptake in skeletal muscle, heart and kidney (Fernandes *et al.*, 2009; Lautt, 1999).

In the absence of the ability to release HISS in response to a meal, the metabolic effect of insulin is reduced, which leads to an increase of postprandial glucose excursions. Hence, the absence of HISS in the postprandial state might be the first metabolic defect that occurs in the prediabetic state. In this setting HISS dysfunction may contribute decisively to the progression of metabolic defects towards type 2 diabetes.

If the HISS quantification will be evaluated in an earlier stage, one can avert the development from prediabetes to diabetes, and taking this in account, HISS quantification could be a very important tool to diagnose diabetes.

Unfortunately, the information available about the acute effect of feeding on insulin sensitivity is scarce.

The basis of this PhD thesis is associated to the implementation, in human subjects, of a new insulin sensitivity method, to evaluate insulin sensitivity, in both fasted and fed states, trying to fill the gap over postprandial insulin sensitivity measurements; and with the involvement of HISS pathway in humans on the increased insulin sensitization induced by a meal.

In insulin resistant animal models, the HISS pathway showed a different behavior from the physiological situation. This dissertation also regards studies that will allow us to understand what ensues in the absence of HISS release, in response to a meal, which takes place when the HISS pathway is pharmacologically blocked or in any state of insulin resistance.

The insulin antagonist hormone, glucagon, have been shown to be altered by a dysfunction of the autonomic nervous system. As the HISS pathway can also be influenced by the autonomic nervous system, it will be very interesting to evaluate the role of glucagon in prediabetes, and on the HISS pathway.

Additionally, other studies revealed the increased levels of cAMP (through the activation of adenylate cyclase, by glucagon) are vigorously related to the decreased hepatic GSH content. Since hepatic GSH is fundamental to HISS synthesis/release, it is relevant to approach the relationship between glucagon and HISS pathway.

This dissertation also considers studies related to the axis prediabetes, glucagon, HISS secretion/action and insulin resistance, in healthy animals.

It is expected that this dissertation will lead to a progression of the knowledge of prediabetes, and will give novel approaches to better understand and reverse the prediabetic state. Also, if the HISS-resistant population are identified, one can prevent and delay the progression from prediabetes towards diabetes, and reverse the insulin resistant state observed in these subjects.

1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. GLUCOSE HOMEOSTASIS

Plasma glucose concentration is a function of the rate of glucose entering the circulation (glucose appearance) balanced by the rate of glucose removal from the circulation (glucose disappearance).

1.1.1. Physiological glucose metabolism

Circulating glucose levels are derived from three sources: intestinal absorption during the fed state, glycogenolysis, and gluconeogenesis. The major determinant of how quickly glucose appears in the circulation during the fed state is the rate of gastric emptying. Other sources of circulating glucose are derived chiefly from hepatic processes: glycogenolysis, the breakdown of glycogen, the polymerized storage form of glucose; and gluconeogenesis, the formation of glucose primarily from lactate and amino acids during the fasting state (Gerich, 1993).

The main tissues that regulate blood glucose levels are the liver (by glucose production and uptake) and skeletal muscle (glucose uptake).

As hepatic glycogen decreases, blood glucose levels fall, as does insulin secretion. A decrease in plasma insulin leads to an increase in glucagon secretion, mobilization of gluconeogenic precursors and alternative fuels, such as, free fatty acids (FFA), and an increase in hepatic gluconeogenesis. The first of these reactions includes conversion of pyruvate to oxaloacetate to phosphoenolpyruvate. The second of these reactions, is the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate, which represents the rate-limiting reaction for gluconeogenesis. The final reaction is the conversion of glucose-6-phosphate to free glucose, which is necessary for glucose to enter the plasma, and to the hepatocytes (Figure 1.1).

The rate-limiting step of glycolysis is the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, requiring an ATP and the enzyme phosphofructokinase-1. This step is considered irreversible. The other two irreversible steps are the conversion of glucose to glucose-6-phosphate by hexokinase/glucokinase and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (Figure 1.1). These three reactions are significant because they are the major obstacles that must be overcome in the formation of glucose during gluconeogenic reactions.

When there is an adequate supply of oxygen to tissues, the overall reaction for the glycolytic pathway ends in pyruvate, which is then converted to acetyl-CoA, which enters

the tricarboxylic acid (TCA) cycle. Under anaerobic conditions the cells rely on glycolysis for ATP, and pyruvate is converted to lactate. Conversion of pyruvate to lactate regenerates cofactors needed for glycolysis (Pickup *et al.*, 2003).

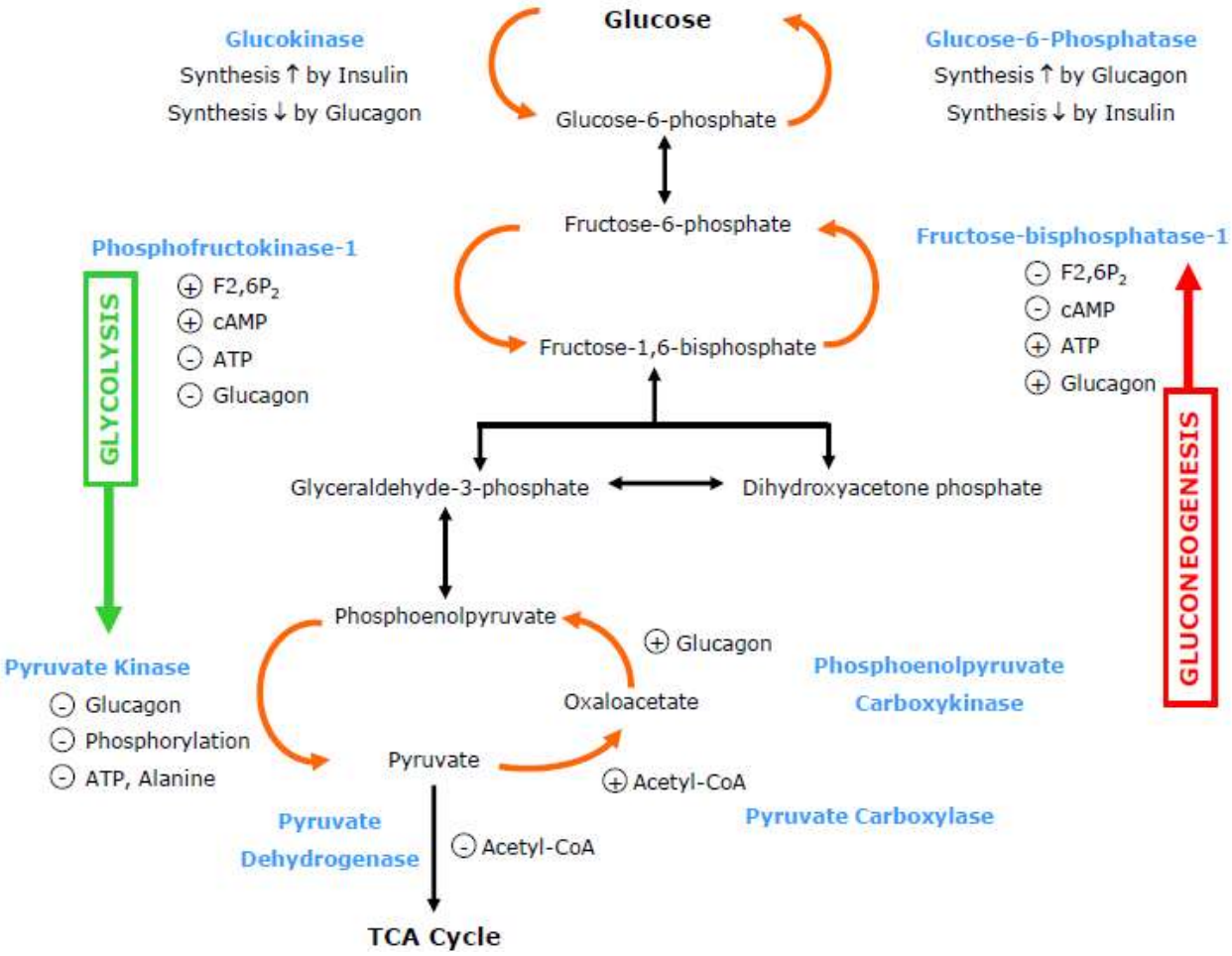


Figure 1.1 - Overview of glycolysis and gluconeogenesis. The level of fructose-2,6-bisphosphate ($F2,6P_2$) is the most important allosteric effector for controlling the rates of gluconeogenesis and glycolysis. CoA: coenzyme A; TCA: tricarboxylic acid; ATP: adenosine triphosphate; cAMP: 3',5'-cyclic adenosine 5'-monophosphate; ↑: increase; ↓: decrease; ⊕: activation; ⊖: inhibition.

1.1.2. Hormonal regulation of glucose metabolism

The glucoregulatory hormones of the body are designed to maintain circulating glucose concentrations in a relatively narrow range.

In the fasting state, glucose leaves the circulation at a constant rate, which indicates that rates of glucose production and utilization are equal. After a meal, glucose absorption results in rates of exogenous glucose delivery into the circulation that can be more than twice the rate of postabsorptive endogenous glucose production, depending on the carbohydrate content of the meal and the rate and degree of glucose absorption. As glucose is absorbed, endogenous glucose production is suppressed, and glucose utilization by liver, muscle, and fat accelerates (Radziuk *et al.*, 2001). With fasting, as plasma glucose decreases, endogenous glucose production increases. The main source of endogenous glucose production is the liver. Renal gluconeogenesis contributes substantially to the systemic glucose pool, only during periods of extreme starvation. Although most tissues have the ability to hydrolyze glycogen, only the liver and kidneys contain glucose-6-phosphatase, the enzyme necessary for the release of glucose into the circulation.

In the following paragraphs, the main key players in the regulation of glucose homeostasis will be approached. Glucoregulatory hormones include insulin, glucagon, somatostatin, amylin, cholecystokinin (CCK), pancreatic polypeptide, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), ghrelin, catecholamines (epinephrine and norepinephrine), cortisol, and growth hormone. Of these, insulin and amylin are derived from the pancreatic β-cells, glucagon from the pancreatic α-cells, somatostatin from the pancreatic δ-cells and pancreatic polypeptide from the PP cells of the pancreas; GLP-1 from the L-cells, GIP from the K-cells and CCK from the I-cells of the intestine.

1.1.2.1. Pancreatic hormones

Insulin

Insulin is the dominant glucoregulatory hormone. Insulin is a key anabolic hormone that is secreted in response to increased blood glucose levels. While glucose is the most potent stimulus of insulin, other factors stimulate insulin secretion. These additional stimuli include increased plasma concentrations of some aminoacids, especially arginine, leucine, and lysine; GLP-1 and GIP released from the gut following a meal; and parasympathetic stimulation via the vagus nerve (Balks et al., 1997; Drucker, 1998).

Like many hormones, insulin exerts its actions through binding to specific receptors present on many cells of the body, including fat, liver, and muscle cells. The primary action of insulin is to stimulate glucose disappearance. Insulin helps control postprandial glucose in three ways. Initially, insulin signals the cells of insulin-sensitive peripheral tissues, mainly skeletal muscle, to increase plasma glucose uptake (Gerich et al., 1976). Secondly, insulin acts on the liver to promote glycogenesis. Finally, insulin simultaneously inhibits glucagon secretion from pancreatic α-cells, thus signalling the liver to stop producing glucose via glycogenolysis and gluconeogenesis (Figure 1.1). All of these actions reduce blood glucose. Other actions of insulin include the stimulation of fat synthesis, promotion of triglyceride storage in fat cells, promotion of protein synthesis in the liver and muscle, and proliferation of cell growth (Pickup et al., 2003).

Insulin action is carefully regulated in response to circulating glucose concentrations. Insulin is not secreted if the blood glucose concentration is ≤60mg/dl, but is secreted in increasing amounts, as glucose concentrations increase beyond this threshold (Gerich, 1993).

Insulin decreases gluconeogenesis by decreasing intracellular 3',5'-cyclic adenosine 5'-monophosphate (cAMP) levels, which inhibits phosphorylation of enzymes. Insulin also inhibits hepatic glucose production indirectly by restraining lipolysis and proteolysis systemically, which reduces delivery of glucose precursors to the liver (DeFronzo, 1988; DeFronzo, 2004).

Since the peripheral action of insulin is a central aspect of this thesis, it will be therefore discussed in more detail in the following sections (Section 1.2).

Glucagon

Glucagon is a key catabolic hormone secreted from pancreatic α-cells. Described by Unger in the 1950's, glucagon was characterized as opposing the effects of insulin (Unger, 1971). Glucagon plays a major role in sustaining plasma glucose during fasting conditions by stimulating hepatic glucose production. Hepatic glucose production, which is primarily regulated by glucagon, maintains basal blood glucose concentrations within a normal range during the fasting state. When plasma glucose falls below the normal range, glucagon secretion increases, resulting in hepatic glucose production and return of plasma glucose to the normal range (Jiang et al., 2003). This endogenous source of glucose is not needed during and immediately following a meal and glucagon secretion is suppressed (Unger, 1985). When coupled with insulin's direct effect on the liver, glucagon suppression results in a near-total suppression of hepatic glucose output (Unger, 1971).

Glucagon has a potent effect on hepatic glucose production by stimulating both glycogenolysis and gluconeogenesis, and it increases glucose output within minutes (Figure 1.1). Glucagon's effects on gluconeogenesis are persistent, whereas its effects on glycogenolysis are short-lived (Gerich et al., 1988). Glucagon stimulates gluconeogenesis by increasing intracellular cAMP levels. Increased hepatic glucose production, in turn, results in suppression of glucagon secretion (Gerich et al., 1976).

Since one of the chapters of this thesis is related with the glucagon's effect on insulin action, it will be therefore discussed in more detail in the following sections (Section 1.3).

Somatostatin

Somatostatin (somatotropin release-inhibiting factor or SRIF) is expressed by neurons in the hypothalamus and other central nervous system sites, and in numerous other tissues, including the pancreatic δ-cells and D-cells of the gastrointestinal tract. Somatostatin secretion is regulated by a combination of nutrients (glucose, aminoacids, fatty acids, ketone bodies), endocrine factors (insulin, glucagon, GIP, GLP-1, CCK) and neural factors (ACh, vasoactive intestinal polypeptide, gastrin-releasing polypeptide) (Pickup et al., 2003). Within the pancreatic islets, somatostatin released from δ-cells is a potencially important paracrine inhibitor of insulin and glucagon secretion from adjacent β- and α-cells (Samols et al., 1988).

Amylin

Amylin (islet amyloid polypeptide or IAPP), a 37 aminoacid peptide, is a neuroendocrine hormone co-expressed and co-secreted with insulin by pancreatic β-cells in response to nutrient stimuli (Koda et al., 1992). Studies in humans have demonstrated that the secretory and plasma concentration profiles of insulin and amylin are similar with low fasting concentrations and increases in response to nutrient intake (Young, 2005). Preclinical findings indicate that amylin works with insulin to help coordinate the rate of glucose appearance and disappearance in the circulation, thereby preventing an abnormal rise in glucose concentrations.

Amylin complements the effects of insulin on circulating glucose concentrations via two main mechanisms. Amylin suppresses postprandial glucagon secretion, thereby decreasing glucagon-stimulated hepatic glucose output following nutrient ingestion (Gedulin et al., 1997). This amylin-dependent suppression of postprandial glucagon secretion is postulated to be centrally mediated via efferent vagal signals. Importantly, amylin does not suppress glucagon secretion during insulin-induced hypoglycemia (Heise et al., 2004; Weyer et al., 2001). Amylin also slows the rate of gastric emptying and,

thus, the rate at which nutrients are delivered from the stomach to the small intestine for absorption (Samsom et al., 2000). In addition to its effects on glucagon secretion and the rate of gastric emptying, amylin dose-dependently reduces food intake and body weight in animal models (Geary, 1999; Rushing et al., 2001).

In summary, amylin works to regulate the rate of glucose appearance from both endogenous (liver-derived) and exogenous (meal-derived) sources, and insulin regulates the rate of glucose disappearance (Buse et al., 2002; Young, 2005).

Pancreatic polypeptide

The pancreatic polypeptide is a 36 aminoacid peptide produced by the PP cells that lie peripherally in islets from the head of the pancreas, and are also sparsely scattered throughout the exocrine parenchyma. Secretion of pancreatic polypeptide is mainly under autonomic control; it is released following feeding or during hypoglycemia, mainly via cholinergic parasympathetic stimulation (Pickup et al., 2003).

The only physiological effects that are recognized in humans are the inhibition of gall bladder contraction, and pancreatic enzyme secretion. Its effect is biphasic in that pancreatic polypeptide initially enhances secretion, and then inhibits secretion. It increases gastric emptying and gut motility, and also relaxes pyloric and ileocecocolic sphincters, colon, and gallbladder. PP levels increase after ingestion of food and remain elevated from 4–8h (Batterham et al., 2003).

1.1.2.2. Gastrointestinal hormones

Cholecystokinin (CCK)

Cholecystokinin (CCK) is a member of the "gut–brain" family of peptide hormones. It is secreted by enteroendocrine cells (I-cells) located in the mucosa of the duodenum, jejunum, and proximal ileum, as well as by specialized neurons in the myenteric plexus and brain. CCK performs numerous regulatory functions in the gut and the brain.

In the gastrointestinal system, CCK inhibits gastric motility and emptying via a capsaicin sensitive vagal pathway. The effects on emptying are via its action on the proximal stomach and pylorus. CCK is also involved in the regulation of food intake. It is released in the gut in response to a meal and acts via vagal afferents to induce satiety. CCK has been implicated in gallbladder contraction, pancreatic secretion, and intestinal motility (Chua et al., 2006).

In the central nervous system, CCK plays a role in learning and memory, anxiogenesis, nociception and satiation. The physiological significance of CCK as a neural

or hormonal regulator of enteroinsular activity remains uncertain, although high concentrations of the peptide clearly stimulate insulin secretion (Liddle, 1995).

Glucose-dependent insulinotropic peptide (GIP)

The intricacies of glucose homeostasis become clearer when considering the role of gut peptides. By the late 1960s, some authors demonstrated that ingested food caused a more potent release of insulin than glucose infused intravenously (Perley et al., 1967). This effect, termed the "incretin effect," suggested that signals from the gut are important in the hormonal regulation of glucose disappearance.

Several incretin hormones have been characterized, and the dominant ones for glucose homeostasis are glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1 (GLP-1).

GIP is a 42 aminoacid peptide produced predominantly in duodenal K-cells in the proximal small intestine. GIP has also been localized in the central nervous system, where it may play a role in the control of cell survival. The predominant stimulus for GIP secretion is nutrient intake; circulating levels of GIP are low in the fasted state, and rise within minutes of food ingestion. GIP stimulates insulin secretion and regulates fat metabolism, but does not inhibit glucagon secretion or gastric emptying (Drucker, 2007).

Glucagon-like peptide 1 (GLP-1)

GLP-1 is produced in enteroendocrine cells in the distal small bowel and colon. Plasma levels of GLP-1 also rise rapidly within minutes of food intake; hence it seems likely that both neural and/or endocrine factors promote GLP-1 secretion from distal L-cells, well before digested nutrients cross the small bowel to make direct contact with enteroendocrine L-cells. GLP-1 stimulates glucose-dependent insulin secretion, but is significantly reduced postprandially in people with type 2 diabetes or impaired glucose tolerance (Vilsboll et al., 2004). GLP-1 stimulates insulin secretion when plasma glucose concentrations are high, but not when plasma glucose concentrations approach or fall. Circulating GLP-1 concentrations are low in the fasting state. However, both GIP and GLP-1 are effectively stimulated by ingestion of a mixed meal or meals enriched with fats and carbohydrates (Herrmann et al., 1995). In contrast to GIP, GLP-1 inhibits glucagon secretion and slows gastric emptying (Drucker, 1998). In the pancreas, GLP-1 stimulates insulin secretion in a glucose-dependent manner while inhibiting glucagon secretion. The postprandial effect of GLP-1 is partly due to inhibition of glucagon secretion. Yet while GLP-1 inhibits glucagon secretion in the fed state, it does not appear to blunt glucagon's response to hypoglycemia (Drucker, 2007). GLP-1 helps to regulate gastric emptying and

gastric acid secretion, perhaps by signaling GLP-1 receptors in the brain, and thereby stimulating efferent tracts of the vagus nerve (Nauck et al., 2002). As gastric emptying slows, the postprandial glucose excursion is reduced (Nauck et al., 1997).

Ghrelin

Ghrelin is a 28 aminoacid peptide secreted mainly by neuroendocrine cells in the stomach mucosa, and it is a powerful stimulator of growth hormone release (Kojima et al., 2005). The secretion of ghrelin increases under conditions of negative energy balance, such as starvation, whereas its expression decreases under conditions of positive energy balance, such as feeding, hyperglycemia, and obesity (Hosoda et al., 2006). In addition, ghrelin has been reported to affect both insulin and glucagon secretion in vitro and in vivo (Dezaki et al., 2008; Salehi et al., 2004).

1.1.2.3. Adipocyte-derived hormones

Leptin

The ob gene was identified in genetically obese insulin resistant mice (Zhang *et al.*, 1994), with a human homologue gene also being described. ob/ob mice were shown to have a mutation in the ob gene which resulted in absence of a functional protein-leptin. Later db/db mice were described which had the protein, but were resistant to its actions. The protein produced was named leptin. In these genetically obese ob(-/-) mice, mutations in the ob gene results in a total lack of leptin production leading to severe obesity. When leptin is administered to these mice, they decrease their food intake, their metabolic rate increases, and they lose significant amounts of weight suggesting that leptin may facilitate negative energy balance.

Leptin is an hormone predominantly produced, and secreted from the adipose tissue, but it is also expressed in low levels in the gastric epithelium, muscle placenta and CNS (Margetic et al., 2002). 20% of circulating leptin is bound to plasma proteins (Houseknecht et al., 1996), with lean humans having higher levels of the free form (Sinha et al., 1996). Bound leptin does not seem to be bioactive, although immunoassays detect both bound and free forms.

Leptin receptors are widely distributed in peripheral organs ensuring that leptin's actions are more widespread than merely on the central nervous system. Leptin receptors have been demonstrated on pancreatic β- and δ-cells, but do not seem to be present on α-cells (Kieffer et al., 1997).

Adiponectin

Adiponectin expression occurs from an intermediate stage of adipogenesis onwards (Hu et al., 1996), and represents the most abundant protein secreted by adipose tissue. Unlike most other adipokines, plasma adiponectin levels were reduced in animal models of obesity and insulin resistance (Hotta et al., 2001; Hu et al., 1996). Administration of recombinant adiponectin to rodents resulted in increased glucose uptake and fat oxidation in muscle, reduced hepatic glucose production, and improved whole-body insulin sensitivity (Yamauchi et al., 2001).

Adiponectin transgenic mice showed partial amelioration of insulin resistance and diabetes and suppression of endogenous glucose production (Combs et al., 2004). In contrast, adiponectin-deficient mice exhibited insulin resistance and glucose intolerance (Maeda et al., 2002; Nawrocki et al., 2006). In addition to its insulin-sensitizing effects, adiponectin may alter glucose metabolism through stimulation of pancreatic insulin secretion in vivo (Okamoto et al., 2008).

Apart from its peripheral actions, adiponectin was shown to modulate food intake and energy expenditure during fasting (increased food intake and reduced energy expenditure) and re-feeding (opposite effects), through its effects in the central nervous system (Kubota et al., 2007). In humans, plasma adiponectin levels were correlated negatively with adiposity (Gavrila et al., 2003), insulin resistance (Abbasi *et al.*, 2004; Yang *et al.*, 2004), type 2 diabetes (Heidemann et al., 2008), and metabolic syndrome (Arner, 2005a; Gavrila et al., 2003).

Resistin

Resistin is an adipocyte hormone expressed in visceral and subcutaneous adipose tissue. Resistin causes insulin resistance and glucose intolerance in mice. Serum resistin levels are elevated in obese rodents (Steppan et al., 2001), although studies have reported variable results (Banerjee et al., 2003).

Administration of resistin to normal mice impairs glucose tolerance and insulin action. Resistin impairs insulin stimulated glucose uptake in culture adipocytes, while administration of anti-resistin antibodies to animals prevents development of insulin resistance and hyperglycemia associated with high fat intake (Steppan et al., 2001). Infusion of resistin under euglycemic hyperinsulinemic conditions produces hepatic insulin resistance. Mice lacking resistin did not show any difference in body weight compared with wild type mice when challenged with a high fat diet, but demonstrated improved glucose tolerance even on high fat diet with preserved insulin sensitivity (Banerjee et al., 2004).

Resistin is expressed at very low levels in human adipocytes (Arner, 2005b; Banerjee et al., 2003). Insulin and TNF-α have been shown to inhibit resistin expression, which might explain the low resistin expression in the obese state (Rabe et al., 2008).

Retinol-binding protein 4 (RBP-4)

Retinol Binding Protein-4 (RBP-4) is elevated in insulin resistant adipose specific GLUT4 knockout mice, and humans with obesity and type 2 diabetes. Transgenic overexpression of human RBP-4 in wildtype mice or administration of recombinant RBP-4 to wildtype mice, was shown to cause insulin resistance through induction of hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase, and impairment of skeletal muscle insulin signaling. In contrast, genetic deletion of RBP4 enhanced insulin sensitivity (Yang et al., 2005).

Serum RBP-4 concentrations were elevated in insulin resistant humans with obesity, impaired glucose tolerance and type 2 diabetes, and even in lean normoglycemic subjects with a strong family history of type 2 diabetes (Graham et al., 2006; Yang et al., 2005). Previous studies shown that serum RBP4 levels are increased in many insulin-resistant states induced by genetic and dietary factors (Graham et al., 2006; Yang et al., 2005).

Visfatin

Visfatin is a newly discovered adipocyte hormone with a direct relationship between plasma visfatin levels and type 2 diabetes mellitus. Visfatin binds to the insulin receptor at a site distinct from that of insulin, and causes hypoglycemia by reducing glucose release from liver cells, and stimulating glucose utilization in adipocytes and myocytes (Beltowski, 2006; Rasouli et al., 2008).

Visfatin is upregulated by hypoxia, inflammation and hyperglycemia and downregulated by insulin, somatostatin and statins. This hormone is found in the cytoplasm, as well as, the nucleus of cells, and has been identified in many tissues and organs including the brain, kidney, lung, spleen and testis but, preferentially expressed in visceral adipose tissue (from which the name visfatin was derived), and upregulated in some animal models of obesity. Visfatin is an endocrine, autocrine, as well as, paracrine peptide with many functions, including enhancement of cell proliferation and hypoglycemic effect (Adeghate, 2008; Rabe et al., 2008). Injection of visfatin in mice lowered blood glucose, and mice with a mutation in visfatin had higher glucose levels (Rasouli et al., 2008).

Chemerin

Chemerin is a recently discovered chemokine highly expressed in liver and white adipose tissue (Bozaoglu et al., 2007; Goralski et al., 2007). It exerts potent anti-inflammatory effects on activated macrophages expressing the chemerin receptor CMKLR1 (chemokine-like receptor-1), in a cysteine protease-dependent manner. Furthermore, chemerin is crucial for normal adipocyte differentiation, and modulates the expression of adipocyte genes involved in glucose and lipid homeostasis, such as GLUT4, fatty acid synthase, and adiponectin via its own receptor (Bozaoglu *et al.*, 2007; Goralski *et al.*, 2007; Roh *et al.*, 2007).

In 3T3-L1 adipocytes, chemerin was reported to enhance insulin-stimulated glucose uptake and insulin receptor substrate-1 tyrosine phosphorylation, suggesting that, chemerin may increase insulin sensitivity in adipose tissue (Takahashi et al., 2008). In humans, chemerin levels did not differ significantly between subjects with type 2 diabetes and normal controls. However, in normal glucose tolerant subjects, chemerin levels were associated significantly with BMI, triglycerides, and blood pressure (Bozaoglu et al., 2007).

Vaspin

Vaspin (Visceral Adipose tissue-derived Serine Protease Inhibitor) was identified in visceral adipose tissue of fatty rats, at an age when body weight and hyperinsulinemia peaked (Hida *et al.*, 2005). Vaspin expression was shown to decrease with worsening of diabetes and body weight loss. It was shown to be regulated in a fat-depot specific manner, and to be associated with obesity and parameters of insulin resistance (Kloting *et al.*, 2006). Likewise, elevated vaspin serum concentrations were correlated with obesity and impaired insulin sensitivity, whereas type 2 diabetes seemed to abrogate this correlation (Youn et al., 2008).

Apelin

Apelin is the endogenous ligand for the G-protein-coupled receptor; is expressed in the gastrointestinal tract, predominantly in the stomach with lower levels in the intestine, and none in the pancreas (Higuchi *et al.*, 2007; Tatemoto *et al.*, 1998; Wang *et al.*, 2004).

Apelin has recently been identified to be secreted by rodent and human adipocytes, suggesting its potential role as an adipokine (Boucher et al., 2005; Dray et al., 2008). Fasting decreases and re-feeding increases apelin levels in fat cells, and initial data seem

to suggest that insulin may control adipocyte apelin gene expression (Boucher et al., 2005).

Apelin levels in fat and plasma seem to correlate with the hyperinsulinism of obesity with higher expression in adipocytes during the stage of differentiation; and stimulates glucose utilization in lean and obese insulin-resistant mice (Beltowski, 2006; Dray et al., 2008). Obesity thus seems to up-regulate apelin expression in adipose tissue. Initial studies do not suggest an appetite modifying action for apelin with central administration (Taheri *et al.*, 2002).

Omentin

Omentin is a recognized gene highly localized to the omental tissue (visceral adipose tissue). Omentin is present in the stromal vascular cells in the adipose tissue, rather than in the adipocytes; and is predominantly expressed in the visceral adipose tissue than the subcutaneous tissue, with the omentin mRNA being 150 times higher in the visceral adipose tissue. Omentin seems to increase Akt phosphorylation independently of insulin presence (Yang et al., 2006). Its role in glucose metabolism and obesity remains to be described; however, an insulin-sensitizing action is possible. Lean subjects have higher plasma omentin than do obese and overweight subjects (de Souza Batista et al., 2007). Plasma omentin is inversely correlated with body mass index, waist circumference, leptin levels and insulin resistance (measured by HOMA), and positively correlated with adiponectin and HDL-cholesterol levels. Accordingly, omentin gene expression is decreased with obesity (de Souza Batista et al., 2007; Wurm et al., 2007).

1.1.2.4. Counterregulatory hormones

Counterregulatory hormones such as cortisol, growth hormone, and catecholamines prevent hypoglycemia by stimulating hepatic glucose production. Epinephrine and norepinephrine promotes glycogenolysis, whereas cortisol promotes gluconeogenesis. The actions of norepinephrine are mediated by the intracellular signals of phosphatidylinositol and Ca^{2+} to promote glycogenolysis (Pickup *et al.*, 2003). Cortisol and epinephrine also limit pyruvate use, allowing it to be converted to free glucose, in the liver and kidneys. Epinephrine rises before growth hormone and cortisol; its increase coincides with the increase in hepatic glucose production (Pickup *et al.*, 2003).

1.2. INSULIN AND GLUCOSE METABOLISM

Insulin plays a central role in the control of the body's metabolism. One of its primary functions is the stimulation of glucose uptake from the systemic circulation, as well as, the suppression of hepatic gluconeogenesis, thereby serving a primary role in glucose homeostasis and preventing any metabolic disorder.

1.2.1. The insulin molecule

The insulin molecule (5.5kDa) is a 51 aminoacid anabolic peptide-hormone that is secreted by the β-cells in the pancreatic islets of Langerhans. Insulin is a two chain heterodimer consisting of a 21 aminoacid A-chain linked to a 30 residue B-chain by two disulfide bonds derived from cysteine residues (A7-B7 and A20-B19). An intrachain disulfide bond also exists in the A-chain (A6-A11) (Figure 1.2).

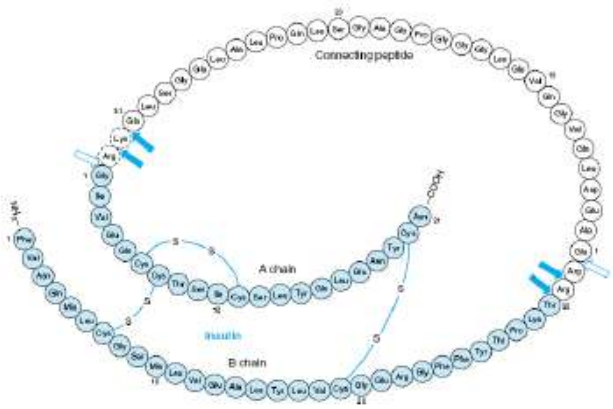


Figure 1.2 - **Structure of human insulin.** Insulin and C-peptide molecules are connected at two sites by dipeptide links. An initial cleavage by a trypsin-like enzyme (open arrows) followed by several cleavages by a carboxypeptidase-like enzyme (solid arrows) results in the production of the heterodimeric (AB) insulin molecule (light blue) and the C-peptide (white). Adapted from (Pickup *et al.*, 2003).

1.2.2. Insulin biosynthesis

Although the primary structure provided valuable information on the aminoacid composition and size of the insulin molecule, questions concerning the processes of insulin synthesis and secretion were not resolved until the late 1960's with the discovery of proinsulin - the precursor of insulin. Proinsulin, a 9kDa protein, contains both the A- and B-chain of insulin in a continuous single chain joined through an intervening region called, C-peptide. C-peptide is a variable length peptide segment, consisting of 26-31 residues, which links the carboxy terminus of the B-chain to the amino terminus of the A-chain via two dibasic residue links (Arg-Arg and Lys-Arg) (Figure 1.2).

C-peptide is stored together with insulin in the mature granules within the β-cell. It is secreted intraportally with insulin, on an equimolar basis, and shows the characteristic biphasic shape in response to iv challenges (Caumo *et al.*, 2004). All of the released C-peptide reaches the peripheral circulation without appreciable extraction by the liver. For this reason, C-peptide has proven to be a fundamental tool for accurate quantification of β-cell secretion. In fact, with the aid of kinetic modelling and deconvolution, C-peptide levels can be used to provide a reliable measure of the insulin secretory rate (Eaton *et al.*, 1980; Polonsky *et al.*, 1986).

The half-life of C-peptide has been shown to be relatively slow (≈35min), and glomerular filtration is the major route of its removal. The slow metabolism of C-peptide is consistent with the hypothesis that, C-peptide diffuses into the extracellular space, but is not integrated into cell membranes. In contrast, insulin is rapidly removed from the circulation (half-life, ≈5min) via insulin receptor-mediated uptake followed by lysosomal degradation. C-peptide acts through the activation of G proteins (Maestroni *et al.*, 2005; Rigler *et al.*, 1999), but the C-peptide-specific G-coupled receptor has not yet been localized. C-peptide has an insulinomimetic effect and, that its action would be mediated through the modulation of the (tyrosine kinase-coupled) receptor of insulin (Grunberger *et al.*, 2001).

Proinsulin is cleaved at those dibasic links by a trypsin-like enzyme to release two chain insulin and free C-peptide. Chan *et al.* subsequently discovered that there was an additional precursor of insulin, preproinsulin. Preproinsulin is a 12kDa single chain polypeptide, which consists of proinsulin extended at the amino terminus by a 24 aminoacid signal peptide region of hydrophobic residues. This signal sequence is characteristic of proteins that enter the secretory pathway (Chan *et al.*, 1976).

Insulin exists primarily as a monomer at low concentrations (~10-6M), and forms dimers at higher concentrations at neutral pH. At high concentrations and in the presence of zinc ions, insulin aggregates further to form hexameric complexes (Goldman *et al.*, 1974).

1.2.3. Insulin secretion

Insulin is stored in large dense core vesicles and is released via exocytosis. This is a multistep process that consists on the transport of the secretory vesicles to the plasma membrane, then docking, priming, and finally fusion of the vesicle with the plasma membrane. It is well known that this process is regulated cooperatively by nutrients, other hormones, and neurotransmitters to cause the electrical depolarization of the β-cell and the release of insulin. However, only a small portion of the insulin stored in vesicles in the β-cell is released even under maximum stimulation. This suggests that the

systemic insulin levels are therefore regulated by secretion instead of synthesis or storage pools (Eliasson *et al.*, 2008).

The best characterized mechanism of coupling glucose metabolism to insulin secretion resides in the electrical excitability of the β-cell (Ashcroft *et al.*, 1989).

Glucose-stimulated insulin secretion *in vivo* typically follows a biphasic time course (Rorsman *et al.*, 2000). After elevation of the glucose concentration, a transient stimulation of insulin secretion is observed, referred to as first phase secretion (short duration, 5-10min), which at later times is followed by a gradually developing secondary stimulation, second phase secretion. Only fuel secretagogues are capable of eliciting the second phase, and, when insulin secretion is evoked by non-metabolizable stimuli, only the first phase is observed. This suggests that second phase insulin secretion is an energy-dependent process (Porksen, 2002; Porksen *et al.*, 2002; Rorsman *et al.*, 2003). The numerous islets composing the endocrine pancreas could be functionally heterogeneous, some of them being responsible for the first phase and others for the second phase (Henquin *et al.*, 2002).

During the biphasic time course of insulin secretion, insulin, like many other hormones, is secreted in a pulsatile fashion, resulting in oscillatory concentrations in peripheral blood. Oscillations include rapid pulses (recurring every 5-15min) superimposed on slower, ultradian oscillations (recurring every 80-150min) that are closely related to fluctuations in glucose concentration (Caumo *et al.*, 2004; Porksen, 2002).

It is important to make a clear distinction between the insulin response following an iv glucose challenge and that following oral glucose or food ingestion. The iv administration of glucose triggers a biphasic insulin response (featuring a rapid increase with a peak, an interpeak nadir, and a subsequent slower increasing phase), only when glucose concentration increases rapidly, as after a glucose bolus or a glucose infusion determining a square wave of hyperglycemia. A "slow-ramp" iv glucose input induces gradually larger secretion without a well-defined first phase. More importantly, a well-defined first phase is lacking under physiological conditions, i.e., when glucose is given orally (Caumo *et al.*, 2004).

As mentioned before, endogenous insulin release is pulsatile in both humans (Lang *et al.*, 1979) and animals (Chou *et al.*, 1991; Goodner *et al.*, 1977). Additionally, studies that compared pulsatile and continuous insulin delivery, showed that exogenous insulin was more effective when administered in a pulsatile manner, particularly with regard to the inhibition of glucagon secretion (Ward *et al.*, 1989), blockade of hepatic glucose production (Paolisso *et al.*, 1991) and its hypoglycemic action, both in the fasting and fed states (Grubert *et al.*, 2005; Matthews *et al.*, 1983; Matveyenko *et al.*, 2008; Meier *et al.*, 2005; Reid *et al.*, 2004; Ward *et al.*, 1989).

Regulation of insulin secretion

Several hormones and neurotransmitters regulate insulin secretion in addition to the voltage sensitive pathways. Molecules such as epinephrine, somatostatin, ACh, and GLP-1 all help regulate insulin secretion, by binding to their own receptors. CCK and Ach potentiate insulin secretion via phosphoinositide catabolism with the subsequent mobilization of intracellular Ca^{2+} (Barg *et al.*, 2004; Lang, 1999). These ligands bind to G-protein coupled receptors that can activate phospholipase C (PLC). PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate produces inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). Two families of Ca^{2+} channels are present on the endoplasmic reticulum: IP_3 receptors and ryanodine receptors. Both of these are capable of causing the release of Ca^{2+} stored in the endoplasmic reticulum. DAG concomitantly causes the activation of protein kinase (PKC) (Barg *et al.*, 2004; Bergsten, 2000). Other potentiators of insulin secretion, such as GLP-1, and GIP, bind to their respective heterotrimeric G-protein coupled receptors to activate adenylate cyclase and increase intracellular cAMP, and subsequently cause the activation of protein kinase A (PKA). Stimulation of either PKC or PKA alters second messenger systems in the β-cell, and can chemically modify ion channels to cause a direct influence on insulin secretion (Lang, 1999).

1.2.4. Insulin signaling and regulation of glucose transport

The concerted regulation of glucose uptake, utilization, and storage by tissues is critical to maintaining blood glucose homeostasis. The first step by which insulin increases energy storage or utilization, involves the regulated transport of glucose into the cell, mediated by the glucose transporters.

Insulin is essential to regulate carbohydrate, lipid, and protein metabolism (Saltiel, 2001). Insulin elicits a diverse array of biological responses by binding to its specific receptor (Goldfine, 1987; White *et al.*, 1994). Decreased cellular responses to insulin or perturbation of the insulin signaling pathways are associated with a number of pathological states (Taylor, 1992).

1.2.4.1. Glucose transporters

Glucose derived from the diet is transferred from the lumen of small intestine into the blood. Both dietary glucose and glucose synthesized within the body have to be transported from the circulation into target cells. These processes involve the transfer of glucose across plasma membranes and this occurs via integral transport proteins.

Glucose transporters are membrane proteins, which exist on the cell surface and incorporate glucose into cells. These transporters comprise two structurally and functionally distinct groups, namely: (i) the Na^+-dependent glucose co-transporters (SGLT, members of a larger family of Na^+-dependent transporters) (Wright, 2001); (ii) the facilitative Na^+-independent sugar transporters (GLUT family) (Asano *et al.*, 2004; Joost *et al.*, 2002; Mueckler, 1994), as depicted in Table I.

1.2.4.1.1. Sodium-dependent glucose transporters (STGL)

The SGLT transports glucose (and galactose), with different affinities, via a secondary active transport mechanism. The Na^+-electrochemical gradient provided by the Na^+–K^+ ATPase pump is utilized to transport glucose into cells against its concentration gradient. This form of glucose transport takes place across the lumenal membrane of cells lining the small intestine and the proximal tubules of the kidneys (Wood *et al.*, 2007).

SGLT1 has a limited tissue expression and is found essentially on the apical membranes of small-intestinal absorptive cells (enterocytes) and renal proximal straight tubules (S3 cells).

A second Na^+-glucose transporter, SGLT2, is of low affinity for glucose and is predominantly expressed on the apical membrane of renal convoluted proximal.

Current studies with the human analogue of SGLT3 suggest that its function may require a re-evaluation (Wright, 2001). Signals have been detected for SGLT3 in the small intestine of pigs (Wells *et al.*, 1992) and in human subjects (Wood IS, unpublished data). These findings conflict with collective data indicating that SGLT1 is the only Na^+–glucose co-transporter expressed in the small intestine.

1.2.4.1.2. Facilitative glucose transporters (GLUT)

The facilitative glucose transporters (GLUT) utilize the diffusion gradient of glucose (and other sugars) across plasma membranes and exhibit different substrate specificities, kinetic properties and tissue expression profiles. This class of glucose transporters can be divided into three subclasses.

Class I is comprised of the extensively characterized glucose transporters GLUT1 to GLUT4, which can be distinguished on the basis of their distinct tissue distributions (GLUT1[1], erythrocytes, brain microvessels; GLUT2[2], liver, pancreatic β-cells, kidneys, intestine; GLUT3, neuronal cells; GLUT4[3], heart, brain, skeletal muscle, adipose tissue) and their hormonal regulation (e.g., insulin sensitivity of GLUT4).

Class II is comprised of the fructose-specific transporter GLUT5 (GLUT 5, small intestine, testis and kidney) and three related proteins, GLUT7, GLUT9, and GLUT11[4] (GLUT 7, intestine, testis; GLUT 9, liver, kidneys; GLUT 11, heart, muscle).

Class III facilitative transporters comprise five members: GLUT6, GLUT8, GLUT10, GLUT12 and H^+-coupled myo-inositol transporter (HMIT, (Uldry *et al.*, 2001)). This class of facilitative transporters is characterized by the lack of a glycosylation site in the first extracellular linker domain, and by the presence of such a site in loop 9. Glucose transport activity has been demonstrated for GLUT6 and GLUT8 (GLUT 6, spleen, leucocytes, brain; GLUT 8, testis, brain, adipose tissue) (Doege *et al.*, 2000; Wood *et al.*, 2007). GLUT10 is expressed in the insulin-sensitive tissues of skeletal muscle and heart and has also been reported as being expressed in the liver and pancreas (Dawson *et al.*, 2001; McVie-Wylie *et al.*, 2001). HMIT has been shown to be an H^+-coupled myo-inositol transporter, expressed predominantly in the brain (Uldry *et al.*, 2001). GLUT 12 has not the primary substrate identity characterized, but is expressed in heart, small intestine, prostate and insulin-sensitive tissues (Rogers *et al.*, 2002).

It should be emphasized, however, that the designation of the family does not necessarily reflect the substrate specificity of its members, which may transport sugars or polyols other than glucose (e.g., GLUT5, fructose; HMIT, H^+-coupled myo-inositol) (Joost *et al.*, 2002).

[1] Moderate levels of GLUT 1 expression are also observed in adipose tissue, muscle and the liver (Mueckler, 1994).
[2] GLUT2 is thought to play a role in the glucose-sensing mechanism, while in the liver it is expressed on the sinusoidal membrane of hepatocytes, and allows for the bi-directional transport of glucose under hormonal control. GLUT2 is also found on the basolateral surface of proximal renal tubules, and enterocytes, where it forms part of the transcellular pathway for glucose and fructose transport (Asano *et al.*, 2004; Joost *et al.*, 2002).
[3] Insulin acts by stimulating the translocation of specific GLUT4-containing vesicles from intracellular stores to the plasma membrane resulting in an immediate 10–20-fold increase in glucose transport (Shepherd *et al.*, 1999).
[4] Low-affinity glucose transport is demonstrated by the short form of GLUT11, and is competed for by fructose, with the transporter being expressed predominantly in heart and skeletal muscle (Doege *et al.*, 2001). The long form of GLUT11, which is not expressed in heart or skeletal muscle but is detected in liver, lung, trachea and brain, was shown to increase fructose transport (Wu *et al.*, 2002).

Table I - Summary of the properties of facilitative glucose transporter (GLUT) and Na^+/Glucose co-transporter family members (SGLT). Adapted from (Joost *et al.*, 2002; Wood *et al.*, 2007; Zhao *et al.*, 2007).

Isoform	Tissue distribution	Transported molecule	Proposed function	Insulin sensitive?
Facilitative glucose transporter (GLUT)				
GLUT 1	Ubiquitous distribution in tissues	Glucose	Basal glucose uptake; transport across blood tissue barriers	No
GLUT 2	Liver, pancreas, kidney, small intestine	Glucose (low affinity); fructose	High-capacity low-affinity transport	No
GLUT 3	Brain, nerves cells	Glucose (high affinity)	Neuronal transport	No
GLUT 4	Muscle, WAT, BAT, heart, brain	Glucose (high affinity)	Insulin-regulated transport in muscle and fat	Yes
GLUT 5	Intestine, kidney, testis	Fructose; glucose (very low affinity)	Transport of fructose	No
GLUT 6	Spleen, leukocytes, brain	Glucose		No
GLUT 7	Small intestine, colon, testis	n.d.	Transport of fructose	n.d.
GLUT 8	Testis, blastocyst, brain, muscle, adipocytes	Glucose	Fuel supply of mature spermatozoa; Insulin-responsive transport in blastocyst	No (Yes, in blastocytes)
GLUT 9	Liver, kidney	n.d.		n.d.
GLUT 10	Liver, pancreas	Glucose		No
GLUT 11	Heart, muscle	Glucose (low affinity); fructose (long form)	Muscle-specific; fructose transporter	Yes
GLUT 12	Heart, prostate, muscle, small intestine, WAT	n.d.	H^+/myo-inositol co-transporter	Yes
HMIT	Brain	H^+-myo-inositol		n.d.
Na^+/Glucose co-transporter (SGLT)				
SGLT1	Kidney, small intestine	Glucose (high affinity); galactose	Glucose reabsorption in intestine and kidney	
SGLT2	Kidney	Glucose (low affinity)	Low affinity and high selectivity for glucose	
SGLT3	Small intestine, skeletal muscle		Glucose activated Na^+ channel	

GLUT - Facilitative glucose transporter; SGLT - Sodium glucose co-transporter; HMIT - H^+-coupled myo-inositol transporter; n.d. – not determined; WAT - White adipose tissue; BAT - Brown adipose tissue.

1.2.4.2. The insulin receptor: structure and function

The insulin receptor is a heterotetrameric protein consisting of two extracellular α-subunits (135kDa), and two transmembrane β-subunits (95kDa), which are linked together by disulphide bonds (Figure 1.3). Insulin binds to α-subunits, inducing a conformational change which results in autophosphorylation of seven tyrosine residues present in the β-subunit (Boni-Schnetzler *et al.*, 1986; Schenker *et al.*, 1991; White *et al.*, 1994). This tyrosine phosphorylation stimulates the tyrosine kinase activity intrinsic to the β-subunit of the receptor (Van Obberghen *et al.*, 2001).

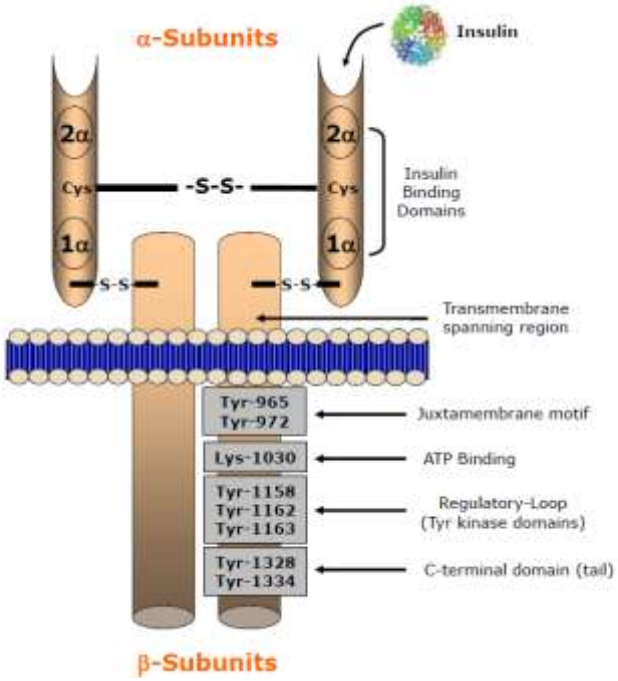


Figure 1.3 - **Schematic diagram of the insulin receptor tetramer.** Insulin binding sites are shown in the α-subunit, and the autophosphorylation sites are listed in the β-subunits. The insulin receptor α-subunits are linked together and to a β-subunit, by disulfide bonds. The relative positions of the insulin binding domain, the cysteine-rich region and the transmembrane spanning region are showed. α, α-subunit; Cys, cysteine residue; -S-S-, disulfide bond; Tyr, tyrosine residue; Lys, lysine residue. Adapted from (Kido *et al.*, 2001; White, 1997; White *et al.*, 1994).

The ability of the receptor to autophosphorylate and phosphorylate intracellular substrates is essential for its mediation of the complex cellular responses to insulin (Ellis *et al.*, 1987; Kasuga *et al.*, 1982; Rosen *et al.*, 1983; Yu *et al.*, 1984). Structure biology studies reveal that the two α-subunits jointly participate in insulin binding, and the kinase domains in the two β-subunits are in a juxtaposition that permits autophosphorylation of tyrosine residues, the first step of insulin receptor activation (Luo *et al.*, 1999;

Ottensmeyer *et al.*, 2000). The kinase domain undergoes conformational change upon autophosphorylation, providing a basis for activation of the kinase, and binding of downstream signaling molecules (Hubbard, 1997; Hubbard *et al.*, 1994).

1.2.4.3. Insulin signaling pathway and glucose uptake

Over the last few years, it has been established that two pathways are necessary for insulin-stimulated glucose transport; a phosphatidylinositol-3 kinase (PI3K)-dependent pathway and a PI3K-independent pathway (Saltiel *et al.*, 2002).

In muscle and adipose tissue, the PI3K-dependent pathway is induced when activation of the insulin receptor by insulin, results in tyrosine phosphorylation of several substrates, including the insulin receptor substrate 1 (IRS-1), IRS-2 and IRS-3 (in the adipose tissue) (Taniguchi *et al.*, 2006). Following tyrosine phosphorylation, IRS proteins bind and activate the PI3K enzyme. Once activated, PI3K mediates the increase in serine phosphorylation of protein kinase B (Akt/PKB), which, in turn, stimulates glucose transport and lipogenesis (Saltiel *et al.*, 2002; Taniguchi *et al.*, 2006)[5] (Figure 1.4).

Recently, the proto-oncogene, Casitas b-lineage lymphoma (Cbl), has been suggested to play a role in insulin action, independently of the PI3K/Akt pathway, that is localized in lipid raft microdomains (Baumann *et al.*, 2000; Saltiel *et al.*, 2003; Saltiel *et al.*, 2002). At least some of the insulin receptor has been shown to reside in these microdomains, perhaps through its interaction with the raft protein caveolin (Kimura *et al.*, 2002). In 3T3-L1 adipocytes, Cbl forms a complex with 2 adaptor proteins, adapter protein with pleckstrin homology and Src homology 2 domains (APS) and Cbl associated protein (CAP). Upon insulin stimulation, this complex binds to the insulin receptor via an interaction between an SH2 domain in APS and the tyrosylphosphorylated receptor tail (Liu *et al.*, 2002). Cbl then undergoes insulin-dependent tyrosine phosphorylation, resulting in the recruitment of the CAP/Cbl complex to lipid rafts, via an interaction

[5] Upon tyrosine phosphorylation, IRS proteins interact with the p85 regulatory subunit and with the p110 catalytic subunit of PI3K, leading to the activation of the enzyme and, its targeting to the plasma membrane. The enzyme generates the lipid product phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which regulates the localization and activity of numerous proteins (Khan *et al.*, 2002). Insulin-stimulated increases in PIP_3 result in the recruitment and/or activation of pleckstrin homology (PH) domain–containing proteins, including various enzymes, their substrates, adapter molecules, and cytoskeletal proteins. Among these is the phosphoinositide-dependent protein kinase-1 (PDK1), which phosphorylates and activates several downstream kinases, including Akt/PKB and the atypical protein kinase C (PKCζ and PKCλ) (Pessin *et al.*, 2000). The active Akt/PKB phosphorylates AS160 protein (Akt substrate of 160 kDa) (Kane *et al.*, 2002; Miinea *et al.*, 2005), which is constitutively associated with GLUT4 vesicles (Brozinick *et al.*, 2007) and in particular Rab protein (small G-proteins involved in vesicle movement and fusion) (Zerial *et al.*, 2001). AS160 phosphorylation by Akt/PKB leads to the activation of Rab proteins (Brozinick *et al.*, 2007; Sano *et al.*, 2003), which results in an increased rate of GLUT4 translocation (Jordens *et al.*, 2005).

between the CAP and the lipid raft protein flotillin (Baumann *et al.*, 2000). Within the lipid raft, tyrosyl-phosphorylated Cbl recruits the CrkII/C3G heterodimer via an interaction between an SH2 domain in CrkII and a tyrosine phosphorylation site in Cbl (Chiang *et al.*, 2001; Ribon *et al.*, 1996). C3G, a guanine nucleotide exchange factor for small molecular weight GTPases, activates the small G protein TC10 (Chiang *et al.*, 2001; Watson *et al.*, 2001). The activation of TC10 provides a second signal to the GLUT4 translocation and, may function in parallel with the activation of the PI3K pathway (Chiang *et al.*, 2001)[6] (Figure 1.4).

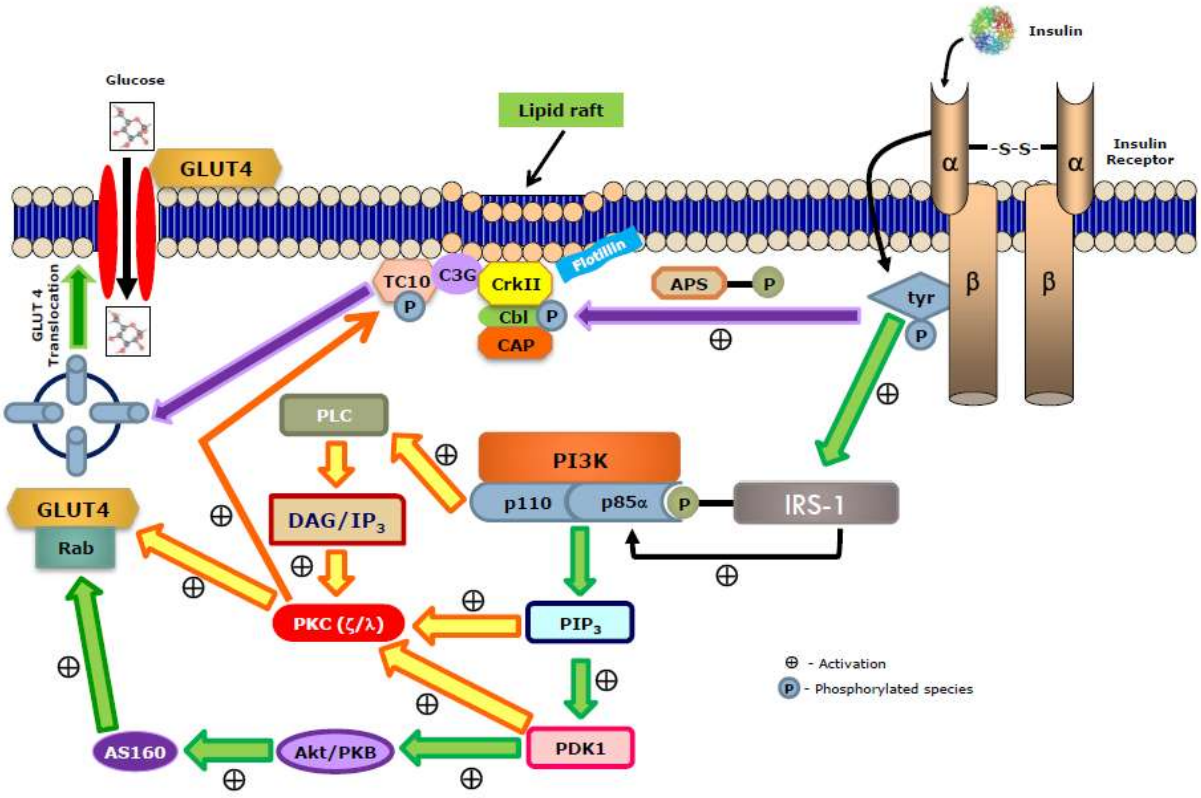


Figure 1.4 – Simplified representation of molecular mechanism involved in insulin signaling pathway that regulates glucose transporter (GLUT4) translocation to cell membrane. Some of the details like, other isoforms of insulin receptor substrate and multiphosphorylation of insulin receptor substrate are not shown here. Letter 'P' indicates phosphorylated species.GLUT4, Glucose transporter isoform 4; IRS-1: Insulin receptor substrate-1; tyr, Tyrosine residue; PI3K: Phosphatidylinositol-3-kinase; Akt/PKB: Protein kinase B; p85, regulatory subunit of PI3K; p110, catalytic subunit of PI3K; APS, Adaptor protein associated with pleckstrin homology (PH) and SH2 domains; AS160, Akt substrate of 160 kDa; Cbl, Casitas b-lineage lymphoma; CAP, Cbl-associated protein; CrkII, SH2/SH3 adapter protein; C3G, Crk SH3-binding guanine nucleotide-releasing factor; PDK1, Phosphoinositide-dependent kinase 1; PKC (ζ/λ), Atypical protein kinase C; PLC, Phospholipase C; DAG, Diacylglycerol; IP_3, Inositol-1,4,5-triphosphate; PIP_3, Phosphatidylinositol-3-phosphate.

[6] The TC10 protein promotes the translocation and docking of GLUT4 in the membrane (Saito *et al.*, 2008; Watson *et al.*, 2001), possibly through the regulation of actin microfilaments (Brozinick *et al.*, 2007). Although the TC10 pathway can be seen as independent of the PI3K pathway, some studies have suggested that atypical protein kinase C (PKCζ and PKCλ) also promote TC10 activation (Kanzaki *et al.*, 2004; Saito *et al.*, 2008).

1.3. GLUCAGON AND GLUCOSE HOMEOSTASIS

Glucagon is critical in regulating hepatic glucose production, and induces about two-thirds of the glucose released in the post-absorptive and fasted states. Physiological increments in glucagon levels stimulate both glycogenolysis and gluconeogenesis (Unger, 1971). The inappropriate raised plasma glucagon levels found in many patients with poorly controlled type 2 diabetes, may play a role in the enhanced gluconeogenesis, and excessive glucose release from the liver that contribute to their fasting hyperglycemia (Dunning *et al.*, 2007).

1.3.1. The glucagon molecule: structure and synthesis

Glucagon is a single straight-chain peptide hormone of 29 aminoacid (3.5kDa) which was discovered as a "contaminant" hyperglycemic factor in pancreatic extracts. Glucagon is synthesized in and secreted from α-cells of pancreatic islets as a 160 aminoacid prohormone (proglucagon)[7]. Proglucagon ultimately undergoes cleavage into four peptides. L-cells of the small intestine synthesize an identical proglucagon molecule, but different processing results in the formation of different polypeptides, of which glucagon-like peptide 1 (GLP-1) and 2 (GLP-2) are probably of most physiologic importance. Glucagon is stored in dense granules and is released by exocytosis. This process is inhibited if the levels of Ca^{2+} in the α-cell are decreased (Jaspan *et al.*, 1977).

1.3.2. Glucagon secretion

Glucagon, stored within α-cells in distinctive granules, is secreted by a process called emiocytosis, which involves migration of secretory granules to the periphery of cells, fusion of granules with the plasma membrane, and extrusion of granule contents into the extracellular space (Gerich *et al.*, 1976). Secretion of glucagon involves α-cell substrate metabolism, and consequent signals which affect cellular K^+ and Ca^{2+} channels, and cAMP levels, as well as, PKA and PKC (Figure 1.5A and Figure 1.5B).

[7] In humans, the circulating half-life of immunoreactive glucagon is estimated to be between 5-6min (Alford *et al.*, 1976; Hinke *et al.*, 2000).

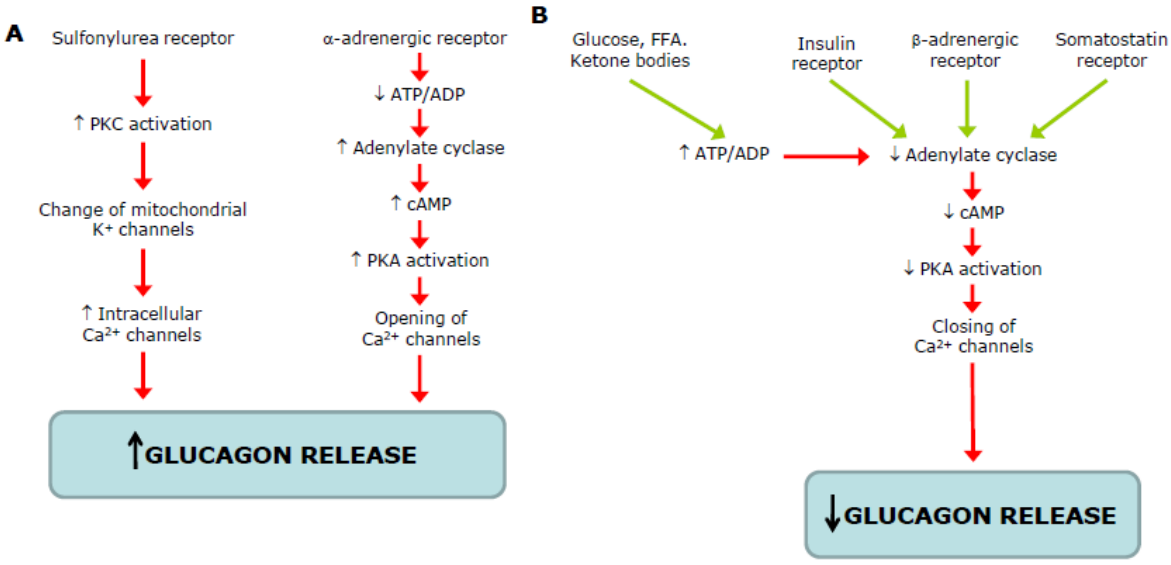


Figure 1.5 - Schematic representation of stimulators (A) and inhibitors (B) of glucagon of glucagon secretion. PKA, Protein kinase A; PKC, Protein kinase C; FFA, Free fatty acids, Ca^{2+}, calcium.

Most substrates (glucose, free fatty acids and ketone bodies), except certain aminoacids, suppress glucagon secretion (Gerich *et al.*, 1976). Inhibition of the metabolism of these substrates prevents the inhibition of glucagon secretion, suggesting that, in contrast to insulin secretion by β-cells, production of ATP inhibits glucagon secretion. While this is consistent with the reciprocal roles of insulin and glucagon in glucose homeostasis, a definitive explanation for this difference remains to be elucidated.

α-cells contain ATP-sensitive K^+ channels as well as sulfonylurea, adrenergic, insulin and somatostatin receptors (Bokvist *et al.*, 1999). Sulfonylurea receptors and ATP-sensitive K^+ channels are associated with both plasma membranes and secretory granule membranes. Sulfonylureas stimulate glucagon release under appropriate conditions (Grodsky *et al.*, 1977). This effect is dependent on PKC, mimicked by inhibitors of mitochondrial ATP-sensitive K^+ channels, and inhibited by K^+ channel openers (diazoxide) (Hoy *et al.*, 2000) (Figure 1.5A). However, it has been proposed that a decrease in the intra α-cell ATP/ADP ratio activates adenylate cyclase, and the resultant increase in cAMP stimulates PKA, which causes opening of Ca^{2+} channels, and an increase in intra-α-cell Ca^{2+}, which will trigger glucagon release (Figure 1.5B)[8].

[8] Plasma glucagon concentration depends on the balance between rates of secretion and degradation, and also on the sampling site (e.g. peripheral venous *vs* portal venous). Basal (non-stimulated) secretion rates of glucagon can be estimated from data on portal venous-arterial differences and portal venous plasma flow rates. From what is known of the pancreatic content of glucagon and secretory rates of glucagon, it can be estimated that at least 25%, and probably more, of the pancreatic content of glucagon is secreted each day (Fisher *et al.*, 1976).

Regulation of glucagon secretion

Glucose is the most important physiologic regular of glucagon secretion. Hyperglycemia decreases and hypoglycemia increases glucagon secretion (Gerich *et al.*, 1976). *In vitro* studies, indicate that the α-cell is as exquisitely sensitive to changes in the ambient extracellular glucose concentration as is the β-cell (Gerich *et al.*, 1974a). To some extent the inhibition of glucagon secretion is dependent on concomitant stimulation of insulin release (Mitrakou *et al.*, 1991). Other substrates also influence glucagon secretion. Various aminoacids stimulate α-cell release of glucagon (Rocha *et al.*, 1972), while free fatty acids (Gerich *et al.*, 1974b) and ketone bodies (Goberna *et al.*, 1974) suppress glucagon secretion (Quesada *et al.*, 2008).

The islets of Langerhans are richly innervated. Like insulin release, glucagon secretion is influenced by both sympathetic and parasympathetic nervous systems; epinephrine, norepinephrine, ACh, electrical stimulation of mixed pancreatic, splanchnic, and vagus nerves augment glucagon release. Both α- and β-cell secretion are influenced in the same direction by parasympathetic (i.e. increase), β-adrenergic (i.e. increase), and α-adrenergic (i.e. decrease) mechanisms (Gerich *et al.*, 1976; Gromada *et al.*, 1997). The observation that glucagon secretion is increased by epinephrine while insulin release is simultaneously decreased, can best be explained by postulating that, α-cell contains a preponderance of β-adrenergic receptors, while the β-cell contains a preponderance of α-adrenergic receptors. Neural input to the α-cell is probably important in modulating the increases in plasma glucagon, observed during stress and, perhaps also after mixed meals.

Hypoglycemia stimulates glucagon secretion through both intraislet and central nervous system mediated autonomic signals. Within the islets low glucose concentrations increase α-cell glucagon secretion directly and, by reducing β-cell secretion, decrease tonic α-cell inhibition by insulin. Autonomic adrenergic (i.e. norepinephrine), cholinergic, and peptidergic neural and adrenomedullary hormonal (epinephrine) signals, triggered by hypoglycemia, may also contribute (Lefebvre, 1995).

Another important feature to consider, is the pancreatic intraislet communication between α-, β- and δ-cell, considering the anatomy of the pancreatic islets[9] (Samols *et al.*, 1988; Stagner *et al.*, 1988). The regulation of pancreatic islet hormone secretion may occur by one, or both of two major routes of intraislet communication: the paracrine route, utilizing hormone diffusion through interstitial spaces, is thought to operate only over short distances, requiring cells to be contiguous or close to one other; and a

[9] β-cells are located in the core of the islet, α-cells are situated at the periphery of the islet and δ-cells are located between the α- and β-cells (Unger *et al.*, 1977).

vascular route, which may deliver endogenous hormones to islet cells located downstream, allows cellular interactions to occur at greater distances, i.e., within the confines of the islet (Samols *et al.*, 1988). The vascular cellular perfusion showed that β-cells inhibit α-cell secretion and α-cells stimulate δ-cell secretion. α-cells appear not to significantly stimulate β-cells, nor do δ-cells appear to inhibit α-cells in terms of microvascular communication (Stagner *et al.*, 1988). Therefore, the intraislet vascular compartment is very important in islet regulatory events and, that intraislet interactions are dependent upon the direction of flow; and the cellular order of perfusion ($\beta \rightarrow \alpha \rightarrow \delta$) through the islet microvasculature (Samols *et al.*, 1988; Stagner *et al.*, 1988).

1.3.3. Molecular mechanism for glucagon-mediated glucose regulation

Glucagon signals through its receptor on the cell surface (Figure 1.6). The binding of glucagon to the extracellular loops of the glucagon receptor results in conformational changes of the latter, leading to subsequent activation of the coupled G proteins. At least two classes of G proteins are known to be associated with and involved in the signal transduction of the glucagon receptor, namely $G_s\alpha$ and G_q. The activation of $G_s\alpha$ leads to activation of adenylate cyclase, increase in intracellular cAMP levels, and subsequent activation of PKA. The activation of G_q leads to the activation of phospholipase C, production of inositol 1,4,5-triphosphate, and subsequent release of intracellular Ca^{2+} (Authier *et al.*, 2008; Burcelin *et al.*, 1996; Christophe, 1996). Activation of glucagon receptors has been shown to increase intracellular Ca^{2+} in pancreatic α-cells and hepatocytes (Gromada *et al.*, 2007; Mayo *et al.*, 2003).

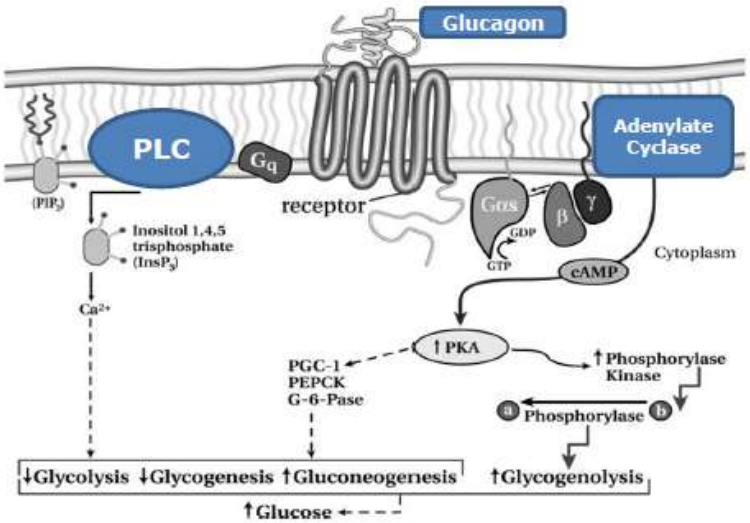


Figure 1.6 – Glucagon signaling pathway. PLC, Phospholipase C; PIP_2, phosphatidylinositol 4,5-biphosphate; PGC-1, peroxisome proliferator-activated receptor-γ coactivator-1; PEPCK, phosphoenolpyruvate carboxykinase; G-6-Pase, glucose-6-phosphatase (Jiang *et al.*, 2003).

Stimulation of PKA leads to increased glycogenolysis and gluconeogenesis via activation of glycogen phosphorylase, the rate-limiting step in glycogen breakdown, and increased expression of genes including phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting step in gluconeogenesis. Activated PKA also contributes to phosphorylation and inactivation of glycogen synthase, reducing the incorporation of glucose into glycogen. Further, PKA phosphorylates and inhibits pyruvate kinase, reducing glycolysis. These actions lead to mobilization of glucose as needed for normal fuel availability and maintenance of euglycemia (Figure 1.6) (Pickup *et al.*, 2003).

1.3.4. Role of glucagon in glucose homeostasis

Glucagon is released into the bloodstream when circulating glucose is low. The main physiological role of glucagon is to stimulate hepatic glucose output, thereby leading to increases in glycemia. This provides the major counterregulatory mechanism for insulin in maintaining glucose homeostasis *in vivo* (Unger, 1971; Unger, 1985).

Numerous *ex vivo* and *in vitro* studies have directly demonstrated that glucagon stimulates glucose output from intact perfused rat livers (Beuers *et al.*, 1990; Doi *et al.*, 2001; Ikeda *et al.*, 1989), resulting from increases in both glycogenolysis and gluconeogenesis. Similarly, glucagon also stimulates glucose output from primary hepatocytes in culture (Weigle *et al.*, 1986; Weigle *et al.*, 1984).

Several lines of evidence indicate that glucagon is a sensitive and timely regulator of glucose homeostasis *in vivo*. Small doses of glucagon are sufficient to induce significant glucose elevations (Lins *et al.*, 1983; Myers *et al.*, 1991). The effect of glucagon can occur within minutes and dissipate rapidly (Dobbins *et al.*, 1998). Glucagon is secreted from islets in a pulsatile fashion (Opara *et al.*, 1988), and such pulsatile deliveries of glucagon are more effective in inducing hepatic glucose output (Komjati *et al.*, 1986; Paolisso *et al.*, 1989; Weigle *et al.*, 1986).

Insulin counters glucagon action on hepatic glucose output by activating phosphodiesterases[10] that decrease cAMP levels (Furman *et al.*, 2006; Furman *et al.*, 2004). Insulin action results in phosphorylation and inactivation of glycogen synthase kinase-3, allowing activation of glycogen synthase, and suppression of gluconeogenesis. Together, this leads to a decreased expression of gluconeogenic enzymes, including PEPCK and glucose-6-phosphatase (Figure 1.7).

[10] The phosphodiesterases (PDEs) are a superfamily of enzymes which catalyse the hydrolysis of the cyclic nucleotides cAMP and cGMP to their corresponding inactive 5-monophosphate counterparts.
Presently, at least 11 different families of PDE isoenzymes are known. Some of them are characterized by substrate specificity (cAMP or cGMP), different kinetic properties and different tissue distribution (Pyne *et al.*, 2003).

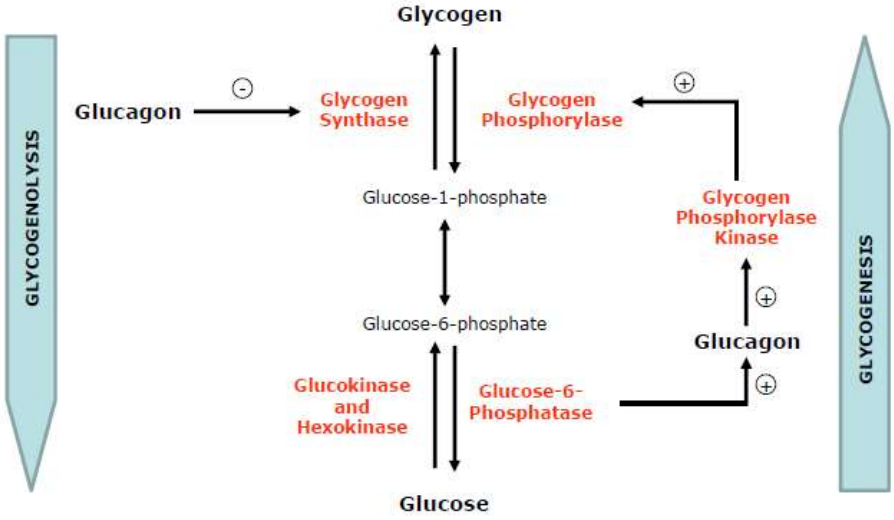


Figure 1.7 – Regulation of glycogen metabolism by glucagon in the liver. Diagram outlines the effects of glucagon on glycogenolysis and glycogenesis in the liver. ⊕: promoted by glucagon; ⊖: inhibited by glucagon.

1.3.5. Glucagon and hepatic GSH content

Hormones, such as glucagon, that increase intracellular cAMP concentration and activate PKA, decrease the activity of γ-glutamylcysteine synthase and, as a consequence, decrease glutathione (GSH) production (Lu *et al.*, 1991).

GSH is synthesized from precursor aminoacids in virtually all cells. The liver plays a central role in the interorgan homeostasis of GSH and, is the only organ in two aspects of GSH biosynthesis. First, it has the unique ability to convert methionine to cysteine through transsulfuration. Secondly, it is the major source of plasma GSH, exporting GSH into plasma and bile at a rate that matches nearly all of its biosynthesis (Meister *et al.*, 1983).

The synthesis of GSH from its constituent aminoacids, glutamate, cysteine, and glycine, involves two ATP-requiring enzymatic steps: (1) γ-glutamylcysteine synthase catalyzes the formation of an amide link between cysteine and glutamate, and (2) the product of this reaction, γ-glutamylcysteine, is linked to glycine by GSH synthase. The first step is rate-limiting and catalyzed by γ-glutamylcysteine synthase, regulated physiologically by (a) feedback competitive inhibition by GSH, and (b) the availability of its precursor, cysteine (DeLeve *et al.*, 1990; Kaplowitz *et al.*, 1985; Meister *et al.*, 1983).

Hormones that increase cytosolic Ca^{2+} and DAG, and activate PKC and calmodulin-dependent kinases also decrease activity of hepatic γ-glutamylcysteine synthase (Lu *et al.*, 1991). This short-term inhibitory control of GSH synthesis is thought to result from hormone-stimulated phosphorylation of γ-glutamylcysteine synthase (Lu *et al.*, 1991). Activation of PKA and PKC also stimulates GSH efflux from the liver (Lu *et al.*, 1990).

1.3.6. Role of glucagon and insulin in deregulation of glucose homeostasis

There is ample evidence suggesting that glucagon plays an important role in initiating and maintaining hyperglycemic conditions in diabetic animals and humans. Insulin and glucagon are the key regulatory hormones for glucose homeostasis. The absolute levels and, even more so, the ratios of the two hormones are tightly regulated *in vivo*, depending on nutritional status (Cherrington, 1999; Unger, 1971). It has been reported that the absolute levels of glucagon or the ratios of glucagon to insulin are often elevated in various forms of diabetes, in both animal and human subjects (Burcelin *et al.*, 1996; Unger, 1985; Unger, 1978). Chronic hyperglucagonemia is correlated with and, is at least partially responsible for increased hepatic glucose output and hyperglycemia in type 2 diabetes (Consoli, 1992; Rizza *et al.*, 1979a).

It is controversial whether the number of glucagon receptors is altered in diabetic states. Most studies, however, appear to suggest that the number of glucagon receptors is reduced in diabetic subjects. Interestingly, even in the presence of fewer glucagon receptors, the ability of glucagon to stimulate cAMP production may remain unchanged or even be elevated (Birnbaumer *et al.*, 1972; Burcelin *et al.*, 1996).

In healthy animals and human subjects, the levels of insulin increase immediately after a meal, whereas the levels of glucagon decrease. In type 2 diabetic subjects, however, the postprandial secretion of insulin is delayed and depressed, whereas that of glucagon is not suppressed or is even elevated (Basu *et al.*, 2004; Butler *et al.*, 1991; Larsson *et al.*, 2000b; Mitrakou *et al.*, 1990). Such abnormality in insulin and glucagon secretion is associated with and predictive of glucose intolerance in type 2 diabetic human subjects (Ahrén *et al.*, 2001; Larsson *et al.*, 2000a). The cause-and-effect relationship between hyperglucagonemia and hyperglycemia is strongly implied in studies showing that, suppression of postprandial hyperglucagonemia corrects postprandial hyperglycemia in type 2 diabetic subjects (Shah *et al.*, 2000). Although, hyperglucagonemia results in glucose intolerance in diabetic subjects with impaired insulin secretion or in normal subjects whose insulin secretion is experimentally blocked, it does not produce the same effects when insulin secretion is intact (i.e., in normal healthy subjects) (Shah *et al.*, 1999; Sherwin *et al.*, 1976; Toft *et al.*, 2002). Taken as a whole, the discussion above indicates that hyperglucagonemia plays an important role in initiating and maintaining hyperglycemia when combined with delayed or deficient insulin secretion, as in some cases of type 2 diabetes.

1.4. RELEVANCE OF THE PARASYMPATHETIC NERVOUS SYSTEM IN REGULATION OF GLUCOSE HOMEOSTASIS

Since the observation by Claude Bernard in the 1850s that puncture of the floor of the fourth ventricle in dogs results in the appearance of transient glucosuria, the central nervous system has been implicated in the control of certain metabolic processes of energy homeostasis, mainly through neuroendocrine systems (Nonogaki, 2000).

The autonomic nervous system is the primary neural mediator of physiological responses to internal and external stimuli. It is composed of 2 branches: the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). Sympathetic fibers are carried primarily in the splanchnic nerve, while parasympathetic fibers travel in the vagus nerve.

In respect to the gastrointestinal tract, the SNS mediates physiological responses to insults from external stimuli through activation of the splanchnic nerve, which releases norepinephrine from nerve terminals and epinephrine from the adrenal medulla. In contrast, through activation of the vagus nerve, the PNS mediates anabolic responses to internal stimuli from the viscera, and external stimuli originating from the sensory components of food (Teff, 2000).

The majority of peripheral tissues are innervated by both PNS and SNS fibers. Typically, the 2 branches of the autonomic nervous system act in a reciprocal fashion to maximize the efficacy of tissue response, with an increase in activity in one branch coincident to a decrease in activity of the other (Puschel, 2004). The dual innervation and reciprocity of action enhance neural control over tissue function. However, under some physiological conditions, both branches of the autonomic system may be activated or inhibited at the same time (Teff, 2008)[11].

Activation of PNS occurs at the onset of and during meal ingestion. It is initiated when the sensory components of food stimulate receptors in the oropharyngeal region, stimulating fibers in the nucleus of the solitary tract, which then, send descending fibers to the dorsal motor nucleus of the vagus; and ultimately activating vagal efferent nerves (Berthoud *et al.*, 1990). These fibers in turn, elicit the release of hormones from the endocrine pancreas (Laughton *et al.*, 1987).

Hormones released in response to oral sensory stimulation are considered cephalic phase reflexes as their release is dependent on neural stimulation rather than nutrient-induced stimulation (Powley, 1977).

[11] Neural control of the endocrine pancreas is exemplary of both reciprocity and coactivation of autonomic activity (Ahrén *et al.*, 1986). Insulin release from the pancreatic β-cell is enhanced by vagal activation, but inhibited by norepinephrine released by the splanchnic nerve. In contrast, both PNS and SNS activation stimulate the release of glucagon from the α-cell of the pancreatic islet during conditions of hypoglycemia (Teff, 2008).

The vagus nerve innervates almost all tissues involved in the digestion, absorption, and metabolism of nutrients, and therefore has the potential to profoundly influence the metabolic processing of food (LeBlanc, 2000).

The central nervous system modulates hepatic glucose production via parasympathetic and sympathetic fibers to the liver (Lautt *et al.*, 1978b; Puschel, 2004; Shimazu, 1987). Hypoglycemia stimulates hepatic glucose production via glucose sensors in the hypothalamic region of the brain, which activate hepatic glycogenolysis via sympathetic fibers to the liver (DeFronzo *et al.*, 1987; DeFronzo *et al.*, 1983; Shimazu *et al.*, 1965).

The vagus nerve also plays an important role in the regulation of blood glucose levels through innervation of the liver and the pancreas, two organs critical for glucose homeostasis. At the level of the liver, vagal activation inhibits enzymes involved in gluconeogenesis and activates enzymes promoting glycogen synthesis (Shimazu, 1971), and the central nervous system-dependent decrease of hepatic glucose production is also mediated by vagal efferent fibers (Lautt *et al.*, 1978b; Pocai *et al.*, 2005). In humans, administration of the muscarinic agonist bethanechol decreases hepatic glucose production (Boyle *et al.*, 1988).

The vagus nerve also plays an important role in both acute insulin responses to a meal, and compensatory insulin responses during prolonged stimulation of the β-cell. On a meal-to-meal level, vagal activation is involved in the regulation of early insulin release (Teff *et al.*, 1993; Woods *et al.*, 1977), enhances peak insulin responses (Teff *et al.*, 1996), and contributes to the rate at which insulin returns to baseline levels through hepatic insulin degradation (Chap *et al.*, 1985).

In fact, the liver is the most likely target site as it would be the tissue receiving the highest concentration of insulin over a brief period. As cephalic phase insulin accounts for such a small percentage of total insulin release, it is improbable that a response of insulin of this magnitude would significantly alter peripheral glucose uptake. However, the concentration in the portal vein would be significantly greater since approximately 50% of the insulin released from the pancreas undergoes hepatic degradation (Teff, 2000). Thus, at the level of the liver, neurally-mediated insulin release may be sufficiently large to initiate changes in hepatic glucose or insulin metabolism.

The importance of portal nutrient delivery in the regulation of hepatic glucose uptake was first suggested by studies showing differences in splanchnic glucose uptake between oral and iv glucose administration (DeFronzo *et al.*, 1978; Ferrannini *et al.*, 1980). To account for these differences, an "intraportal signal" which activates net hepatic glucose uptake has been hypothesized (Adkins *et al.*, 1987; Mittelman *et al.*, 1997), and other neural factors, particularly ACh have been proposed as key regulators of hepatic glucose metabolism (Adkins-Marshall *et al.*, 1992; Gardemann *et al.*, 1986; Stumpel *et al.*, 1997).

Thus, an intact parasympathetic neural system is important for maintaining normal glucose metabolism in the liver, under postabsorptive and postprandial conditions, and the physiological significance of neurally-mediated insulin release is part of the portal signal, which contributes to the regulation of glucose metabolism and that this signal seems to be essential to occur prior to nutrient absorption.

Additionally, a role for hepatic Ach in peripheral glucose metabolism was suggested by experiments demonstrating that sectioning the anterior hepatic nerve plexes or the administration of ipv atropine leads to insulin resistance with a decrease in glucose tolerance (Xie *et al.*, 1995a; Xie *et al.*, 1996b).

1.5. THE HEPATIC INSULIN SENSITIZING SUBSTANCE (HISS) HYPOTHESIS

After a meal, the body's dynamic response to insulin is approximately doubled in comparison to the fasted state. This has been mainly attributed to the increase in insulin secretion in response to a meal. However, a novel neurohumoral regulatory mechanism was described by which postprandial insulin action is potentiated and, when absent, results in severe postprandial insulin resistance. This novel mechanism involves the liver, as a key regulator in the modulation of peripheral tissue insulin sensitivity, and the release by the liver of the hepatic insulin sensitizing substance (HISS). The study of the HISS pathway is the main focus of our research group and is the basis of this PhD dissertation.

Based on the HISS hypothesis, in the fed state a hepatic parasympathetic reflex is triggered leading to the release of acetylcholine (ACh) which activates M_1 muscarinic receptors in the liver, leading to the generation of nitric oxide (NO) and, subsequently, to the release of a hormone referred to as HISS (Lautt, 1999). HISS then travels through the bloodstream to enhance skeletal muscle insulin-stimulated glucose uptake, consequently accounting, in rats, for 50-60% of the whole-body glucose uptake (Lautt *et al.*, 2001). The hypoglycemic action of insulin in the postprandial state can therefore be divided into two components. The first component is insulin action *per se*, corresponding to the direct action of insulin on target cells, and is referred to as HISS-independent component. The second component of insulin action is dependent on the HISS pathway, which potentiates insulin action, and is referred to as HISS-dependent component. The response to insulin consists of a quite constant HISS-independent component and a larger, but highly regulated, HISS-dependent component that is greatest in the immediate postprandial state and least in the fasted state, when hypoglycemia is normally neither required nor desirable. Interruption of this reflex release of HISS by

surgical or pharmacological ablation leads to immediate severe (Latour *et al.*, 2002b) insulin resistance (Xie *et al.*, 1996b).

Insulin resistance was first demonstrated in response to hepatic denervation in 1993 (Xie *et al.*, 1993). Surgical denervation of the anterior hepatic plexus results in insulin resistance, which is not further worsened by denervation of the posterior nerve plexus or bilateral vagotomy (Xie *et al.*, 1993). Surgical denervation of the anterior plexus involves elimination of some of the sympathetic and afferent nerves, as well as, parasympathetic nerves[12](Latour *et al.*, 2002b). Administration of atropine results in insulin resistance of a magnitude similar to that produced by surgical denervation; the response to denervation is not further aggravated by administration of atropine, nor is the response to atropine different by addition of hepatic denervation (Xie *et al.*, 1995a; Xie *et al.*, 1996b). Denervation-induced insulin resistance can be reversed by ipv ACh administration (Xie *et al.*, 1996a).

1.5.1. The role of nitric oxide and glutathione on HISS release

Because many of the biological effects of ACh appear to be mediated by NO release, Lautt's group tested the hypothesis that the hepatic parasympathetic reflex release of HISS in the liver was also mediated through the generation of NO. Indeed, the comparison between iv and ipv administrations of the nitric oxide synthase (NOS) antagonists, revealed that hepatic NO synthesis is required for full postprandial insulin action[13] (Sadri *et al.*, 1999; Sadri *et al.*, 1997). Additionally, the nitric oxide donor 3-morpholinosydnonimine hydrochloride (SIN-1) was able to reverse insulin resistance produced by the NOS antagonist N-monomethyl-L-argine (L-NMMA)[14], when administered into the portal vein, but not systemically. Moreover, SIN-1 is also able to reverse insulin resistance produced by hepatic surgical denervation (Sadri *et al.*, 1999). Thus, these experiments support the hypothesis that the hepatic parasympathetic nerves act through the release of NO within the liver.

Although compelling data demonstrate that ACh and NO are necessary for HISS secretion, it was not known whether, in the liver, activation of NOS is a downstream consequence of hepatic parasympathetic nerve stimulation or, in contrast, leads to ACh

[12] The presence of parasympathetic nerves in this nerve bundle was shown by the rapid decrease in hepatic glucose output, produced by electrical stimulation after elimination of the sympathetic nerve effects, by administration of both α- and β-adrenergic antagonists (Gardemann *et al.*, 1986) or pre-treatment with 6-hydroxydopamine, to destroy the sympathetic nerve terminals (Lautt *et al.*, 1978a).
[13] The approach of using comparative responses to iv versus ipv drug administration allows assessment of the site of action as hepatic or extrahepatic. If the liver is the target organ, ipv administration will have a greater effect than the same dose administered iv; if extrahepatic tissues are the target, ipv administration will have a lesser effect.
[14] Administration of the NOS antagonist N^G-nitro-L arginine methyl ester (L-NAME), produced the same hepatic insulin resistance as observed after L-NMMA administration.

release and ultimately to HISS secretion. It is known that binding of ACh to M_1 muscarinic receptors leads to NOS activation and NO synthesis (Lopez-Jaramillo *et al.*, 1990); however, NO can also stimulate the release of ACh in a number of physiological systems, like the central nervous system, or the enteric system (Leonard *et al.*, 1997; Smith *et al.*, 1998). Previous studies, revealed that ipv administration of ACh is not able to reverse the state of insulin resistance induced by hepatic NOS inhibition, whereas ipv administration of the NO donor, SIN-1, reverses the insulin resistance induced by either NOS inhibition or muscarinic antagonism (Guarino *et al.*, 2004). Hepatic, but not the systemic, NO is necessary for the HISS-dependent insulin action. These observations suggest that HISS synthesis involves the binding of ACh to hepatic M_1 muscarinic receptors, leading to activation of NOS and consequently the synthesis of hepatic NO (Figure 1.8).

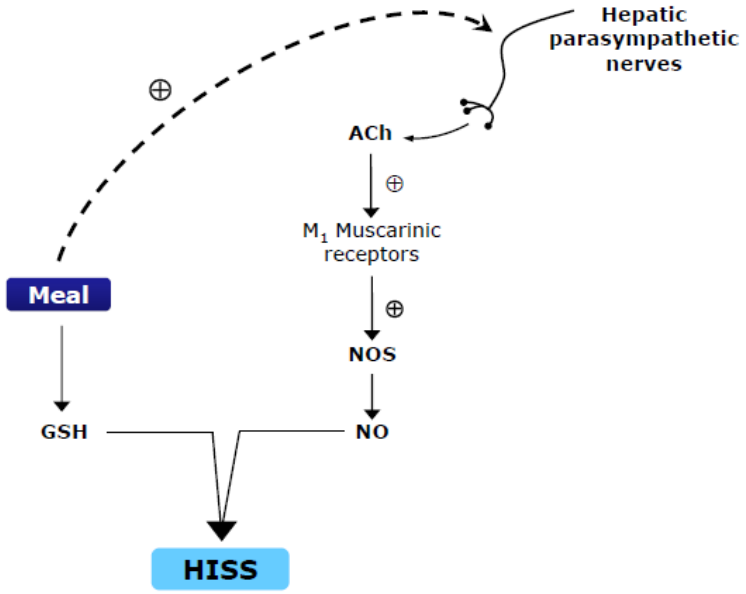


Figure 1.8 – Overview of the hepatic insulin sensitizing substance (HISS) synthesis/secretion pathway. Following a meal, hepatic parasympathetic nerves are stimulated and lead to the release of acetylcholine (ACh) that acts on hepatic M_1 muscarinic receptors, activating the enzyme hepatic nitric oxide synthase (NOS), resulting in nitric oxide (NO) production, which together with reduced glutathione (GSH) result in the synthesis and/or release of the HISS from the liver. ⊕, Activation

Several authors suggest that the impaired glucose tolerance observed after reduced glutathione (GSH) depletion is a direct effect of the lack of GSH in the liver (Khamaisi *et al.*, 2000). Studies performed by Guarino *et al.* showed that hepatic GSH depletion, produced by administration of the γ-glutamylcysteine synthase inhibitor, leads to a state of insulin resistance similar to the one obtained after hepatic NOS blockade; and hepatic GSH administration after NOS blockade did not change the insulin resistance state observed. These results indicated that hepatic GSH depletion and NOS blockade affect

the HISS pathway at different steps, inhibiting the insulin-sensitizing signal in the liver (Guarino *et al.*, 2003).

Moreover, exogenous NO (by administration of SIN-1) was not able to restore insulin action in GSH-depleted rats, which suggests that both GSH and NO are required in the liver to allow full peripheral insulin action (Guarino *et al.*, 2003).

Further studies were performed to deepen the previous observations and was demonstrated that, while administration of GSH or NO by itself did not increase insulin sensitivity, co-administration of GSH with NO to the liver enhanced insulin action to values obtained only when HISS was present (Guarino *et al.*, 2006). With these results one can consider that two different hepatic signals are needed for HISS synthesis/secretion: elevated NO levels and elevated GSH levels.

In summary, after a meal, and after absorption of the meal nutrients, three signals seem to occur. The first corresponds to the increased of insulin levels to further increase glucose uptake at insulin-stimulated glucose tissues. A second signal is concerning to an increase of hepatic GSH levels. The third signal involves the hepatic parasympathetic activation. Thereby, the three signals together are responsible and fundamental for the HISS synthesis/release, which afterwards will increase glucose uptake at HISS sensitive tissues (Figure 1.9).

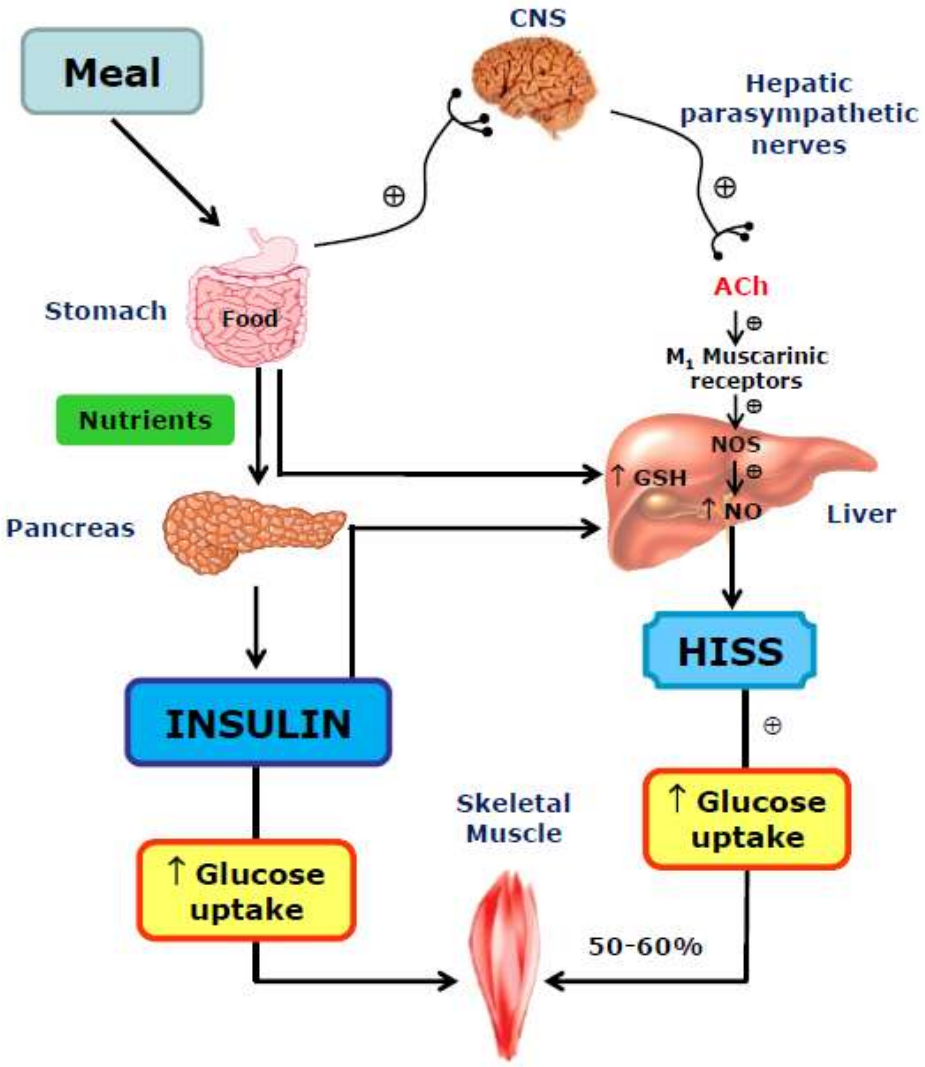


Figure 1.9 – The Hepatic insulin sensitizing substance (HISS) hypothesis. Following a meal, nutrients absorption and pancreatic insulin release will occur, and hepatic parasympathetic nerves are stimulated and lead to the release of acetylcholine (ACh) that acts on hepatic M_1 muscarinic receptors, activating the enzyme hepatic nitric oxide synthase (NOS), resulting in nitric oxide (NO) production, which together with reduced glutathione (GSH) result in the synthesis and/or release of the HISS from the liver. HISS acts on skeletal muscle and accounts for 50-60% of the glucose disposal. Lack of HISS release results in insulin resistance. Adapted from (Lautt, 2004).

1.5.2. HISS action and the target organ of insulin resistance

The studies described in previous sections are consistent with the hypothesis that hepatic parasympathetic nerves regulate the response to insulin, but do not specify on which tissues insulin sensitivity is regulated. To approach this problem, arterial–venous concentrations of glucose were measured after administration of atropine or denervation, and after the combination of both procedures. The intestines showed no response to insulin either before or after induction of insulin resistance. Similarly, the hepatic response to insulin was not significantly altered by induction of insulin resistance. In contrast, the glucose uptake across the hindlimbs was impaired by interruption of the hepatic parasympathetic reflex (Xie *et al.*, 1996b). This suggests that, although the liver is the organ where HISS is synthesized, insulin resistance occurs in peripheral tissues. At this time Lautt's group could not conclude which specific tissues are involved, but the large muscle mass of the hindlimbs has led the authors to tentatively conclude that skeletal muscle is at least one of the tissues where glucose uptake is regulated by HISS (Xie *et al.*, 1996b).

Recent studies were performed in order to evaluate the major site of HISS action. In the *in vivo* incorporation of [^{3}H]2-deoxy-glucose uptake into individual tissues (liver, adipose tissue, pancreas, skeletal muscle, heart, kidney), the authors observed that the skeletal muscle of denervated animals was affected, mainly the soleus and the EDL muscles. Surprisingly, this surgical procedure also affected the kidney and heart. However, the adipose tissue and the liver did not have any significant change on [3H]2-deoxy-glucose incorporation, indicating that these tissues are not affected by HISS action (Fernandes *et al.*, 2009).

1.5.3. Prandial control of HISS release

Insulin is a complex hormone that carries out a number of other growth-related functions and pulsatile insulin release occurs in conditions throughout the day. It would be biologically inappropriate for insulin released in the fasting state to produce the same magnitude of glucose uptake from blood, as is required in the absorptive postprandial state.

In fasted rats, HISS is not released, thus assuring that insulin sensitivity is extremely low at a time when an insulin effect is neither useful nor desirable (Lautt et al., 2001). It was also demonstrated, in rats, the ability of insulin to release HISS from the liver, at a maximal level immediately after eating (2h post-feeding), and declining progressively with the duration of fasting until it was minor or insignificant after approximately a 24h fasting in both anesthetized (Lautt *et al.*, 2001; Lautt *et al.*, 1998a), and conscious animals (Latour *et al.*, 2002c). In contrast, an 16-18h fast in either cats (Xie *et al.*,

1995a; Xie *et al.*, 1995b; Xie *et al.*, 1993) or dogs (Moore *et al.*, 2002), still leaves 25-35% of the glucose disposal action of insulin being accounted by HISS release; and 24h fasting induces a 45% reduction in insulin action in mice (Latour *et al.*, 2002a).

Animals tested after feeding period (2h post-feeding) showed a high responsiveness to insulin, and a high HISS dependence, whereas increasing periods of fasting led to a decrease in insulin action, accounted by a decrease in the HISS-dependent component. The HISS-independent component was similar in rats that had been recently fed, or that had been subjected to a 6, 18, or 24h fast (Lautt *et al.*, 2001).

The glucose disposal effect of a bolus of insulin administered in the postprandial state is double that produced by administration of the same dose of insulin in the fasted state, or when the hepatic parasympathetic nerves have been blocked (Xie *et al.*, 1996b).

Regulation of HISS release by insulin is thus an effective means to sensitize the body to insulin released following a meal, but not in the fasted state. This suggests a physiological regulation by the hepatic parasympathetic nerves of the ability of insulin to release HISS. In this way, the parasympathetic nerves are said to play a permissive regulatory role in HISS release. Thus, in the fasted sate, a natural and regulated state of HISS-dependent insulin resistance (HDIR) exists (Lautt *et al.*, 2001). The regulated production of HDIR in the fasted state would confer a protective role, minimizing the hypoglycemic effect of insulin in the absence of ingested glucose.

Insulin secretion shows pulsatile or rhythmic secretion in fasted rats independent of arterial glucose (Bizot-Espiard *et al.*, 1998), and only about one-half of the daily insulin secretion is meal related (Beyer *et al.*, 1990). Conditioned reflex secretion of insulin is also able to reach early peaks at similar levels to those produced by a meal, even in the absence of a meal (Steffens *et al.*, 1994). In such situations, it is not useful for insulin to exhibit full action since the result could be hypoglycemia. HISS, therefore, provides a useful physiological mechanism to selectively partition glucose to skeletal muscle, when glucose absorption is high, but would minimize uptake by muscle in the fasted state or, in any other state of HDIR (Lautt *et al.*, 2001).

HDIR is a state produced by fasting, and can be produced by any means that interrupts the parasympathetic signaling process, including hepatic surgical denervation (Xie *et al.*, 1996b; Xie *et al.*, 1993), blockade of hepatic muscarinic cholinergic receptors (Xie *et al.*, 1996a), hepatic NOS blockade (Sadri *et al.*, 1999), hepatic cyclooxygenase blockade (Sadri *et al.*, 2000b) or hepatic guanylyl cyclase blockade (Correia *et al.*, 2002; Guarino *et al.*, 2004), or by depletion of hepatic glutathione levels (Guarino *et al.*, 2003). Thereby, the HISS pathway (described in Figure 1.9) could be inhibited by any of the procedures described above.

1.5.4. Role of HISS-dependent insulin resistance (HDIR) in pathology

Circumstantial evidence was provided suggesting that insulin resistance seen in several disease states might be accounted for, at least in part, by HDIR. These disease states include aging (Ribeiro *et al.*, 2008), the spontaneously hypertensive rat (Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2001b), sucrose fed rats (Ribeiro *et al.*, 2001a; Ribeiro *et al.*, 2005), high-fat diet-induced obese rat (Afonso *et al.*, 2007a; Afonso *et al.*, 2010), genetically obese Zucker *fa/fa* rat (Afonso *et al.*, 2007a; Afonso *et al.*, 2007b; Ribeiro *et al.*, 2001c), animals with liver disease induced by chronic bile duct ligation (Lautt *et al.*, 1998b) and adult offspring of fetal alcohol exposure (Minuk *et al.*, 1998; Sadri *et al.*, 2005; Ting *et al.*, 2006).

Aging leads to insulin resistance that is attributable to reduced HISS-dependent insulin action. It was shown that HISS action decreased gradually with age in rats; however, while the HISS-independent component decreased until approximately 9 weeks of age, the HISS-dependent component remained stable until 9 weeks of age and decreased steadily with further aging (Ribeiro *et al.*, 2008).

Spontaneously hypertensive rats have been shown to develop progressive HDIR but the HISS-independent component of insulin action significantly increases so that, at some time points, the total insulin action may not be greatly impaired as a result of the increased HISS-independent insulin action. This is the only condition so far identified that might suggest that the HISS-independent component of insulin action could increase in a compensatory manner in the presence of HDIR (Ribeiro *et al.*, 2007; Ribeiro *et al.*, 2001b).

Sucrose feeding results in HDIR. After 9 weeks of feeding with normal rat chow and access to normal water or a 35% sucrose solution, we observed that the 35% sucrose solution produced insulin resistance that had HDIR characteristics. Comparing the sucrose fed rats with the standard diet group, the HISS-independent component remained unchanged, while the HISS-dependent component of insulin action was dramatically decreased, showing that the insulin resistance observed in the sucrose rats was due entirely to an impairment of HISS action (Ribeiro *et al.*, 2001a; Ribeiro *et al.*, 2005).

The insulin resistance noticed in the high-fat diet-induced obese rat model is quite different from the genetically Zucker *fa/fa* rats. In fact, the high-fat diet-induced obese rats are specifically HISS-dependent insulin resistant, but not HISS-independent insulin resistant; and diet enriched with fat leads to a progressive increase in adiposity, which correlates with the development of postprandial insulin resistance. Furthermore, since the high-fat diet-induction of obesity is more representative of obesity in humans, it

seems to be a better animal model to study the obesity-related pathophysiology (Afonso *et al.*, 2007a; Afonso *et al.*, 2010).

In the genetically obese Zucker *fa/fa* rats, the insulin resistance seen is equally attributed to decreases in HISS-dependent and HISS-independent components of insulin action, thus suggesting that blockade of HISS release is not a primary phenomenon in this animal model but could be a defect common to both components (Afonso *et al.*, 2007a; Afonso *et al.*, 2007b; Ribeiro *et al.*, 2001c).

Chronic bile duct ligation in rats leads, to complete HDIR by 10 days. These animals showed severe insulin resistance that was not made worse by the administration of atropine, thereby suggesting that the insulin resistance was HDIR; ipv administration of ACh resulted in restoration of insulin action (Lautt *et al.*, 1998b).

Exposure of the developing fetus to alcohol through the maternal drinking water has been shown to result in alcohol dose-related HDIR in the adult offspring and the HISS-independent component of insulin action was not altered in the adult offspring. Drinking water containing 20% alcohol resulted in severe but not complete HDIR in the male offspring, but full HDIR in females (Minuk *et al.*, 1998; Sadri *et al.*, 2005).

HDIR is suggested to occur in association with a wide range of chronic inflammatory states, and to contribute negatively as a strong risk factor for development of diabetes, obesity, cardiac and vascular disease. If the "HISS hypothesis" is correct, it represents a new paradigm impacting diagnosis and therapy of a broad spectrum of metabolic disorders.

1.6. PATHOLOGIES ASSOCIATED WITH INSULIN RESISTANCE

Although insulin has a number of metabolic effects, insulin resistance is normally defined in terms of the blood glucose lowering effect of insulin. It is a state in which greater than normal insulin levels are required to elicit a quantitatively normal glucose response in the whole body, a tissue or at the cellular level. The term is often used interchangeably with diminished insulin action or decreased insulin sensitivity. The following sections will address the diseases most commonly associated with progression of insulin resistance, including type 2 diabetes (T2D) and obesity.

1.6.1. Insulin resistance and Obesity

Insulin resistance is strongly associated with obesity for which several mechanisms have been invoked. A number of circulating hormones, cytokines, and metabolic fuels, such as free fatty acids (FFA) that originate in the adipocyte, modulate insulin action.

An increased mass of stored triglyceride, especially in visceral or deep subcutaneous adipose depots, leads to large adipocytes that are themselves resistant to the ability of insulin to suppress lipolysis. This results in increased release and circulating levels of FFA and glycerol, both of which aggravate insulin resistance in skeletal muscle and liver (Boden, 1997). Excessive fat storage, not only in adipocytes, but "ectopically" in non-adipose cells, also has an important role (Danforth, 2000). For example, increased intramyocellular lipids are associated with skeletal muscle insulin resistance under some circumstances (Machann *et al.*, 2004).

Both in the basal state and after a hyperglycemic stimulus, obese people display a level of hyperinsulinemia that correlates with the degree of insulin resistance, in order to maintain normal glucose tolerance (Bonadonna *et al.*, 1990). A number of factors present in obesity (elevated FFA levels, decreased adiponectin and increased adipocytokine levels) are responsible for induction of insulin resistance. However, as in the development of T2D, progressive deterioration of the metabolic state results in eventual failure of endogenous hyperinsulinemia to compensate fully for the insulin resistance, and impaired glucose tolerance develops (DeFronzo, 1988; DeFronzo *et al.*, 1992).

Studies have highlighted the inflammatory nature of adipose tissue in the setting of weight gain and obesity and thus have provided another mechanism to the development of insulin resistance (Hundal *et al.*, 2002). A high body mass index (BMI), increased visceral fat and insulin resistance are associated with increased circulating inflammatory markers such as the acute phase protein, pro-inflammatory cytokines and soluble cell adhesion molecules (Ghanim *et al.*, 2004; Maachi *et al.*, 2004). In rodent models fed a

high fat "western diet", the occurrence of hyperinsulinemia corresponded to an infiltration of activated macrophages into fat tissue. Moreover, in obese, insulin-resistant individuals circulating mononuclear cells (precursor cells of tissue macrophages) were reported to be in a pro-inflammatory state (Esposito *et al.*, 2003). Such observations have led to the hypothesis that low-grade inflammation generated by adipose tissue and FFA is a key player in the development of insulin resistance and endothelial dysfunction, leading ultimately to the manifestation of diabetes and atherosclerosis. Several studies have noted elevated mean circulating cytokine levels (TNF-α, IL-6), as well as, plasma FFA that, in a paracrine approach, inhibit insulin signaling pathways in peripheral tissues, and result in decreased whole body insulin-mediated glucose disposal (Maachi *et al.*, 2004).

Studies performed in our lab, showed that a diet enriched with fat (HFD) leads to a progressive increase in adiposity, which correlates with the development of postprandial insulin resistance. The major contribution to HFD-induced insulin resistance derives from the reduced contribution of the HPN pathway to overall postprandial insulin sensitivity. HFD induced overall postprandial insulin resistance, already present after 1 week (HFD-1), and was further aggravated by 4 weeks on HFD (HFD-4). HISS-independent insulin action was unaltered during the HFD-1 and only slightly decreased in the HFD-4 group. On the other hand, the reduction in HISS-dependent insulin action paralleled the reduction in total postprandial insulin sensitivity: an impairment of about 65 % was observed after 1 week on the HFD (compared with control group) and, by 4 weeks on HFD, full HISS pathway-dependent insulin resistance had developed. The HISS pathway-dependent insulin resistance associated with HFD, seems to be primarily responsible for the early effect that precedes the development of diabetes, as indicated by the observation that 1 week on HFD was not sufficient to affect HISS-independent insulin action, but was sufficient to impair the insulin-sensitizing action of the HPN pathway dramatically (Afonso *et al.*, 2010).

The insulin resistance of HFD-induced obesity model, is mechanistically different from that observed in other animal models of obesity, such as the genetically obese Zucker (*fa/fa*) rat, in which both components of insulin action were impaired in the same proportion (Afonso *et al.*, 2007b), probably due to post-insulin receptor abnormalities at the level of the skeletal muscle (Brozinick *et al.*, 1994; King *et al.*, 1992).

Visceral adiposity and insulin resistance

While the relationship between insulin resistance and overall obesity is well established in cross-sectional and longitudinal studies, debate continues about which of the fat depots, visceral adipose tissue (VAT) or subcutaneous adipose tissue (SAT), is of greater importance in this relationship (Virtanen *et al.*, 2005).

Several studies have been performed to address this question and some of them found a strong independent relationship between both visceral and subcutaneous adiposity and insulin resistance. According to Wagenknecht *et al.*, VAT was a more potent predictor of insulin resistance than SAT (Wagenknecht *et al.*, 2003). In another study, Gastaldelli and colleagues determined that visceral fat accumulation decreased peripheral insulin sensitivity and enhanced hepatic gluconeogenesis (Gastaldelli *et al.*, 2002). It appears, however, that the deeper layer of SAT has an association with insulin resistance that bears resemblance to the pattern for VAT, whereas the superficial layer has a weaker association (Kelley *et al.*, 2000). It also seems that accumulation of fat within the liver and muscle leads to insulin resistance in these tissues, resulting in elevated fasting plasma glucose levels (due to accelerated hepatic glucose production) and postprandial hyperglycemia (due to decreased insulin-mediated glucose uptake by muscle) (Bays *et al.*, 2004; Bonadonna *et al.*, 1990; Roden *et al.*, 1996).

Furthermore, it is recognized that plasma FFA are a central component of the insulin resistance syndrome in accordance with the "overflow hypothesis"[15], in that when adipocyte storage capacity is exceeded, there is an overflow and accumulation of FFA in the form of toxic ceramide, long-chain fatty acyl CoA and sphingolipid in muscle, liver, pancreas and arteries, resulting in physiological features of insulin resistance (Miranda *et al.*, 2005). Plasma FFA also contributes to activation of several inflammatory pathways, which hamper insulin signal transduction pathways in muscle tissue.

Afonso *et al.* have shown that the decrease in insulin sensitivity observed in the HFD animals was correlated with the increase in adipose mass; the effect of adiposity is predominantly mediated by effects upon the HISS pathway-dependent component of insulin action. Indeed, there was a significant polynomial correlation between whole-body fat mass and HISS-dependent insulin action, which was similar to the correlation between whole-body fat mass and total insulin action, whereas the HISS-independent insulin action did not correlate with the whole-body fat mass (Afonso *et al.*, 2010). On the other hand, the abdominal (regional) fat mass presents a linear correlation with both components of insulin action, although this correlation was stronger for total and for HISS-dependent than for the HISS-independent insulin action, as given by the adjusted-R^2 and linear regression slopes. These data suggests that both the HISS-dependent and -independent components of insulin action decrease with adiposity; however the HISS-independent insulin action tends to decrease at a slower rate.

[15] The **overflow hypothesis** proposes that as the size of an adipocyte increases, it will eventually reach a limit and be unable to store further lipid. Excess fatty acids then 'overflow' to ectopic sites, including muscle and liver, leading to peripheral and hepatic insulin resistance, respectively (Sniderman *et al.*, 2007; Unger, 2003).

The increase in abdominal fat pad and whole-body fat masses were correlated, suggesting that HFD does not induce fat accumulation in any specific site, but rather a general increase in adiposity, *i.e.*, whole-body fat. Therefore, the development of HISS-dependent insulin resistance does not seem to be associated with fat deposition in any particular depot (Afonso *et al.*, 2010).

1.6.2. Insulin resistance and Type 2 diabetes

Diabetes is a metabolic disorder characterized by resistance to the action of insulin, insufficient insulin secretion, or both (The expert committee on the diagnosis and classification of diabetes mellitus, 2002). The major clinical manifestation of the diabetic state is hyperglycemia, resulting from defects in insulin secretion, insulin action, or both. Insulin deficiency and/or insulin resistance are also associated with disturbances in lipid, and protein metabolism (DeFronzo *et al.*, 1992).

Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of the pancreatic β-cells, with consequent insulin deficiency to abnormalities that result in resistance to insulin action. The basis of the abnormalities in carbohydrate, fat, and protein metabolism in diabetes is deficient action of insulin on target tissues. Deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin at one or more points in the complex pathways of hormone action. Impairment of insulin secretion and defects in insulin action frequently coexist in the same patient, and it is often unclear which abnormality, if either alone, is the primary cause of the hyperglycemia.

The degree of hyperglycemia may change over time, depending on the extent of the underlying disease process. A disease process may be present but may not have progressed far enough to cause hyperglycemia. The disease process can cause impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG), without fulfilling the criteria for the diagnosis of diabetes (American Diabetes Association, 2010).

Diagnosis and classification of diabetes and prediabetes

The diagnosis of diabetes requires the identification of a glycemic cut-off point which discriminates normal individuals from those with diabetes. The present cut-off points reflect the level of glucose above which microvascular complications have been shown to increase (American Diabetes Association, 2010).

American Diabetes Association (ADA) has not previously recommended the use of glycosylated hemoglobin (HbA1c) for diagnosing diabetes, in part due to lack of standardization of the assay. However, HbA_{1c} assays are now highly standardized, and their results can be uniformly applied both temporally and across populations. In a recent report (The expert committee on the diagnosis and classification of diabetes mellitus, 2009), after an extensive review of both established and emerging epidemiological evidence, an international expert committee recommended the use of the HbA1c test to diagnose diabetes with a threshold of ≥6.5%, and ADA affirms this decision (Table II) (American Diabetes Association, 2010). The diagnostic test should be performed using a method certified by the National Glycohemoglobin Standardization Program and standardized or traceable to the Diabetes Control and Complications Trial reference assay.

Table II - American Diabetes Association (ADA) criteria for the diagnosis of type 2 diabetes mellitus (T2D), impaired fasting glucose (IFG) and impaired glucose tolerance (IGT).
FPG = fasting plasma glucose; 2h PG = 2h plasma glucose concentration during a standard oral glucose (75g) tolerance test.

	FPG	2h PG	HbA1c
Normal	< 100mg/dl (< 5.6mmol/l)	< 140mg/dl (< 7.8mmol/l)	5.7-6.4%
IGT	-------	140-199mg/dl (7.8-11.0mmol/l)	5.7-6.4%
IFG	100-125mg/dl (5.6-6.9mmol/l)	-------	5.7-6.4%
T2D*	≥ 126mg/dl (≥ 7.0mmol/l)	≥ 200mg/dl (≥ 11.1mmol/l)	≥ 6.5%

When both tests are performed, IGT or IFG should be diagnosed only if diabetes is not diagnosed by the other test. *A diagnosis of diabetes needs to be confirmed on a separate day.

To minimize the discrepancy between the fasting plasma glucose and 2h plasma glucose concentration measured during the oral glucose tolerance test (OGTT)[16], cut-off values of ≥126mg/dl and ≥200mg/dl, respectively, were chosen. Although the ADA recommended use of the fasting plasma glucose concentration as the principal tool for the diagnosis of diabetes, the published results of the Diabetes Prevention Program (Diabetes Prevention Program Research Group, 2002) have given renewed emphasis to the OGTT, since diet/exercise, as well as, drug therapy were shown to slow/prevent the

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

progression of impaired glucose tolerance (IGT) to overt diabetes. It was hypothesized that the progression from normal glucose tolerance to IGT and then to T2D began with insulin resistance (Edelman, 1998), leading to a compensatory increase in insulin secretion and, in turn, to eventual pancreatic β-cell exhaustion. Moreover, there is also some evidence that impaired insulin secretion is the initial, and main predisposing factor in diabetes (Gerich, 1998).

It is presently considered that T2D is established if blood glucose level after an overnight fast is equal or over 126mg/dl, and/or the blood glucose level obtained 2h after an OGTT is equal or over 200 mg/dl, and/or the $HbA_{1c} \geq 6.5\%$. It was established that the diagnosis of IGT only can be made from the 2h plasma glucose concentration (≥140 to 199mg/dl) during the OGTT. The Expert Committee recognized an intermediate group of subjects whose glucose levels, although not meeting criteria for diabetes, are nevertheless too high to be considered normal (The expert committee on the diagnosis and classification of diabetes mellitus, 2009). Impaired fasting glucose (IFG) is defined by a fasting plasma glucose ≥100mg/dl, but less than 125mg/dl (American Diabetes Association, 2010).

Patients with IGT and/or IFG are referred to as having prediabetes, indicating the relatively high risk for development of diabetes in these patients. IGT and IFG states are not clinical entities in their own right, but rather risk factors for future diabetes, as well as, cardiovascular disease (Abdul-Ghani *et al.*, 2009). IGT and IFG are associated with the metabolic syndrome, which includes obesity (especially abdominal or visceral obesity), dyslipidemia, and hypertension. It is worth mentioning that nutrition therapy aimed at producing 5-10% loss of body weight, exercise, and certain pharmacological agents have been often demonstrated to prevent or delay the development of diabetes in people with IGT. Many individuals with IGT are normoglycemic in their daily lives, and individuals with IFG or IGT may have normal or near normal HbA1c levels[17] (American Diabetes Association, 2010).

The fasting and postprandial glucose levels do not measure the same physiologic processes and, not surprisingly, they do not identify the same individuals as having diabetes. The fasting plasma glucose concentration is, in large part, determined by basal rate of hepatic glucose production (DeFronzo, 1988; DeFronzo *et al.*, 1992). Thus, IFG primarily reflects hepatic resistance to the action of insulin. Under postabsorptive conditions, the majority of glucose is taken up by insulin-independent tissues (brain and liver) (DeFronzo, 1988); considering that tissue (muscle) glucose clearance is reduced in the postabsorptive state, in absolute terms, the muscle is responsible for only a small amount of glucose uptake in the basal state, in individuals with IFG (DeFronzo, 1988).

[17] It is reasonable to consider an HbA1c range of 5.7 to 6.4% as identifying individuals with high risk for future diabetes, and to whom the term prediabetes may be applied.

Moreover, basal insulin secretion is well preserved, even in individuals with overt T2D (DeFronzo, 1988; Reaven, 1988), and, therefore, cannot be explained the rise in fasting plasma glucose concentration in individuals with IFG. In contrast, the postprandial plasma glucose concentration primarily depends on insulin sensitivity in muscle and liver, as well as on insulin secretion by the pancreatic β-cells (DeFronzo, 1988), and defects in both tissue (muscle) sensitivity to insulin and impaired insulin secretion are responsible for IGT. Although IFG and IGT are equally strong predictors of the development of future T2D (DECODE Study Group, 1999), the prevalence of IFG in the general population is significantly less than the prevalence of IGT (Gabir *et al.*, 2000). The use of the fasting plasma glucose concentration (≥126mg/dl), as opposed to the 2h plasma glucose concentration during the OGTT (≥200mg/dl), also significantly underestimates the prevalence of diabetes in the general population (DECODE Study Group, 1999; Gabir *et al.*, 2000).

Figure **1.10** illustrates the curvilinear relation between normal β-cell function and insulin sensitivity (Bergman, 1989). Deviation from this hyperbola, such as in the patients with IGT and T2D, occurs when β-cell function is inadequately low for a specific degree of insulin sensitivity. Thus, β-cell dysfunction is a critical component in the pathogenesis of T2D. However, not only deviation from the hyperbola, but also progression along the hyperbola affects glycemia. When insulin action decreases (as with increasing obesity), the system usually compensates by increasing β-cell function. However, at the same time, concentrations of blood glucose at fasting and 2h after glucose load will increase mildly (Stumvoll *et al.*, 2003). This increase may well be small, but over time becomes damaging because of glucose toxicity, and in itself a cause for β-cell dysfunction. Thus, even with (theoretically) unlimited β-cell reserve, insulin resistance paves the way for hyperglycemia and T2D (Stumvoll *et al.*, 2005).

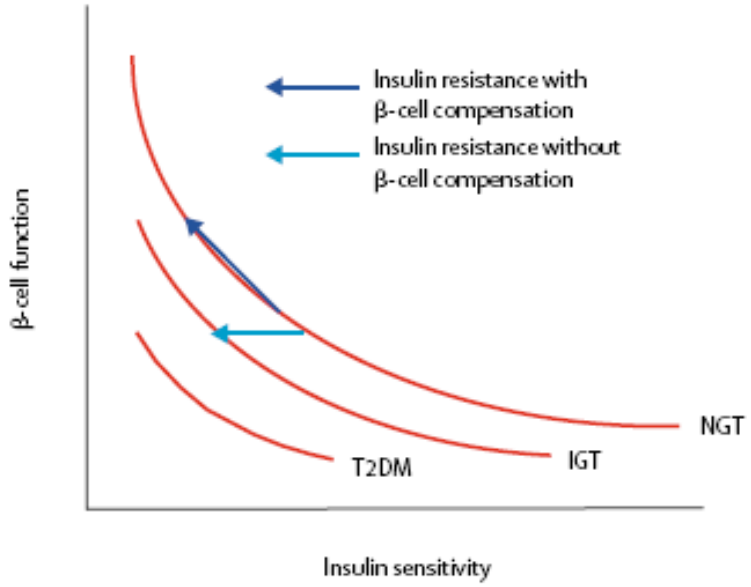


Figure 1.10 - Hyperbolic relation between β-cell function and insulin sensitivity. In people with normal glucose tolerance (NGT) a quasi-hyperbolic relation exists between β-cell function and insulin sensitivity. With deviation from this hyperbola, deterioration of glucose tolerance (impaired glucose tolerance [IGT], and type 2 diabetes (T2DM)) occurs. Adapted from (Weyer *et al.*, 1999a).

Glucose dysfunction in type 2 diabetes

Type 2 diabetic subjects manifest multiple disturbances in glucose homeostasis, including: (1) impaired insulin secretion; (2) insulin resistance in muscle, liver, and adipocytes; and (3) abnormalities in splanchnic glucose uptake (Cerasi, 1995; DeFronzo, 1988).

Early in the natural history of type 2 diabetes, insulin resistance is well established, but glucose tolerance remains normal because of a compensatory increase in insulin secretion.

In type 2 diabetics, the fasting plasma insulin concentration is normal or increased, and basal insulin secretion (measured from C-peptide kinetics) is elevated. The relationship between the fasting plasma glucose (FPG) and insulin concentrations resembles an inverted U shape and it has been referred to as Starling's curve of the pancreas (Figure 1.11) (DeFronzo, 1988). As the fasting glucose rises from 80 to 120mg/dl, the fasting plasma insulin concentration increases progressively. The progressive rise in fasting plasma insulin level can be viewed as an adaptive response of the pancreas to offset the progressive deterioration in glucose homeostasis. When the FPG exceeds 120mg/dl, the β-cell is unable to maintain its elevated rate of insulin secretion, and the fasting insulin concentration declines precipitously. This decrease in fasting insulin level has important physiologic implications, because it is at this point that

hepatic glucose production (the primary determinant of the FPG concentration) begins to rise.

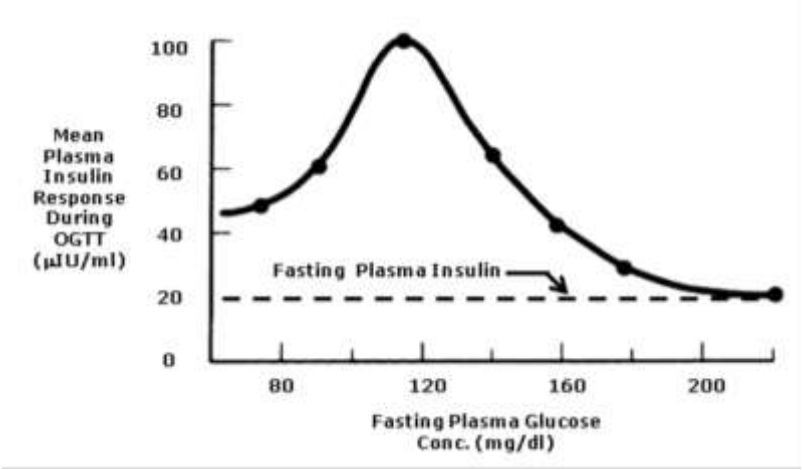


Figure 1.11 - Starling's curve of the pancreas for insulin secretion. In normal-weight patients with IGT and mild diabetes, the plasma insulin response to OGTT increases progressively until the fasting glucose reaches 120mg/dl. Thereafter, further increases in the fasting glucose concentration are associated with a progressive decline in insulin secretion. From (DeFronzo, 1988).

The relationship between the mean plasma insulin response during an OGTT and the FPG concentration also resembles an inverted U-shaped curve (Figure 1.11) (DeFronzo, 1988). The curve, however, is shifted to the left compared with the basal insulin secretory rate, and the glucose-stimulated insulin response begins to decline at a fasting glucose concentration of approximately 120mg/dl. A typical type 2 diabetic subject with a FPG level of 150–160mg/dl secretes an amount of insulin similar to that in a healthy nondiabetic individual; however, a ''normal'' insulin response in the presence of hyperglycemia and underlying insulin resistance, is markedly abnormal. At FPG levels in excess of 150–160mg/dl, the plasma insulin response, when viewed in absolute terms, becomes insulinopenic. Finally, when the fasting glucose exceeds 200–220mg/dl, the plasma insulin response to a glucose challenge is markedly blunted. Nonetheless, the fasting hyperinsulinemia persists despite FPG concentrations as high as 250–300mg/dl, and 24h integrated plasma insulin and C-peptide profiles in lean type 2 diabetic patients remain normal. These normal day-long values result from the combination of elevated fasting and decreased postprandial insulin and C-peptide secretory rates (DeFronzo, 2004).

It should be emphasized that, even though the plasma insulin response is increased in absolute terms early in the development of T2D (FPG $\leq$120mg/dl), this does not mean that β-cell function is normal. The β-cell responds to an increment in plasma insulin by an increment in plasma glucose, and this response is modulated by the severity of insulin

resistance, that is, the more severe the insulin resistance, the greater the insulin response (DeFronzo *et al.*, 1992).

In summary, several studies are consistent in demonstrating that hyperinsulinemia precedes the development of T2D, and hyperinsulinemia is a strong predictor of the development of IGT and T2D. It should be emphasized, however, that overt diabetes (fasting glucose ≥126mg/dl) does not develop in the absence of a significant defect in β-cell function.

1.7. METHODS AND INDEXES OF INSULIN SENSITIVITY ASSESSMENT

Measurements of insulin sensitivity provide clinicians and researchers with excellent instruments to objectively evaluate the efficiency of both current and potentially useful interventional tools.

It is of great importance to develop tools for quantifying insulin sensitivity/resistance in humans, which may be used to appropriately investigate the epidemiology, pathophysiological mechanisms, outcomes of therapeutic interventions, and clinical course of patients with insulin resistance. In this section, a new and some currently used methods for assessing insulin sensitivity, their applications, merits, and limitations will be discussed.

1.7.1. Methods of insulin sensitivity/resistance assessment

1.7.1.1. Hyperinsulinemic Euglycemic Glucose Clamp (HIEC)

The hyperinsulinemic euglycemic clamp (HIEC), originally developed by DeFronzo is widely accepted as the "gold standard" for directly determining metabolic insulin sensitivity in humans (DeFronzo *et al.*, 1979). After an overnight fast, insulin is infused intravenously at a constant rate that may range from 5 to 120mU/m^2/min (dose per body surface area per minute, during 180min). This constant insulin infusion results in a new steady-state insulin level that is above the fasting level (hyperinsulinemic). Consequently, glucose disposal in skeletal muscle and adipose tissue is increased while hepatic glucose production (HGP) is suppressed. Under these conditions, a glucose analyzer is used to frequently monitor blood glucose levels at 5-10min intervals, while 20% dextrose is given intravenously at a variable rate in order to "clamp" blood glucose concentrations in the normal range (euglycemic). After several hours of constant insulin infusion, steady-state conditions are typically achieved for plasma insulin, blood glucose, and the glucose infusion rate (GIR). Assuming that the hyperinsulinemic state is

sufficient to completely suppress HGP, and since there is no net change in blood glucose concentrations under steady-state clamp conditions, the GIR must be equal to the glucose disposal rate (M). Thus, whole body glucose disposal at a given level of hyperinsulinemia can be directly determined. M is typically normalized to body weight or fat-free mass to generate an estimate of insulin sensitivity. Alternatively, an insulin sensitivity index (S_I) derived from clamp data can be defined as $SI_{Clamp} = \frac{M}{G \times \Delta I}$, where M is normalized for G (steady-state blood glucose concentration) and ΔI (difference between fasting and steady-state plasma insulin concentrations) (Katz *et al.*, 2000).

The validity of glucose clamp measurements of insulin sensitivity depends on achieving steady-state conditions. "Steady-state" is often defined as a period greater than 30min (at least 1h after initiation of insulin infusion) during which the coefficient of variation for blood glucose, plasma insulin, and GIR are less than 5% (Chen *et al.*, 2003; Katz *et al.*, 2000). It is possible to use radiolabeled glucose tracer under clamp conditions to estimate hepatic glucose production, so that appropriate corrections can be made to M in the event HGP is not completely suppressed (Finegood *et al.*, 1987; McMahon *et al.*, 1989; Radziuk *et al.*, 2002; Rizza *et al.*, 1981). An alternative approach is to use an insulin infusion rate sufficiently high to completely suppress HGP according to the insulin sensitivity/resistance of the population to be studied.

M is routinely obtained at only a single insulin infusion rate, and therefore comparisons between M or SI_{Clamp} among different subjects is valid only if the same insulin infusion rate is used for all subjects.

The principal advantage of the glucose clamp in humans is that, it directly measures whole body glucose disposal at a given level of insulinemia under steady-state conditions. Conceptually, the approach is straightforward and there are a limited number of assumptions that are clearly defined. In research settings where assessing insulin sensitivity/resistance is of primary interest and feasibility is not an issue, it is appropriate to use the glucose clamp technique.

The main limitations of the HIEC approach are that it is time-consuming, labor intensive, expensive, and requires an experienced operator to manage technical difficulties. Another limitation is that the clamp utilizes steady-state insulin levels that may be supraphysiological. This results in a reversal of the normal portal to peripheral insulin gradient. Thus, the glucose clamp may not accurately reflect insulin action and glucose dynamics under physiological conditions that a dynamic test, such as, an oral meal or oral glucose load may determine. Further, in the HIEC insulin sensitivity is measured only under a steady-state condition, and therefore, the test does not realistically portray dynamic conditions such as those occurring after normal meals.

Because HIEC is dependent on steady-state conditions, insulin infusion is continuous for ≈3h, and the subjects are in the fasted state. The results of the HIEC may be limited by these restraints, because insulin release is pulsatile (Hansen *et al.*, 1982; Lang *et al.*, 1982; Lang *et al.*, 1979; Porksen *et al.*, 1996), and insulin action is sensitized in the postprandial state (Lautt *et al.*, 2001).

1.7.1.2. Insulin Tolerance Test (ITT)

The insulin tolerance test (ITT) was one of the first methods developed to assess insulin sensitivity *in vivo* (Horgaard *et al.*, 1929). In this method, a fixed bolus of regular insulin (0.1IU/kg bw) is given iv after an 8 to 10h fast. Blood samples are collected at 15 and 5min before and 3, 6, 9, 12, 15, 20 and 30min after insulin injection, and the plasma glucose decrement is then measured. Glucose is injected at 30min to stop the fall in plasma glucose (Bonora *et al.*, 1989; Inchiostro, 2005; Young *et al.*, 1996). The faster the decline in glucose concentration, the more insulin sensitive the subject is. The slope of the linear decline in plasma glucose (K_{ITT}) can be calculated by dividing 0.693 by the plasma glucose half-time (50% from baseline):

$$K_{ITT} = \frac{0.693}{t^{1/2}} \times 100$$

where $t^{1/2}$ represents the half-life of plasma glucose decrease, and is calculated from the slope of least square analysis of the plasma glucose concentrations from 3 to 15min after iv insulin injection, when the plasma glucose concentration declined linearly. Normal K_{ITT} is >2.0%/min and values <1.5%/min are considered abnormal. This method gives an indirect estimate of overall insulin sensitivity. It has been to correlate with the HIEC in several studies (Akinmokun *et al.*, 1992; Bonora *et al.*, 1989). However, arterialization of blood was essential in the ITT, as data from standard venous blood measurements showed no significant relationship with HIEC-derived glucose disposal (Akinmokun *et al.*, 1992).

Some of the drawbacks of this method include the supraphysiological insulin dose used, and also the fact that the test does not differentiate peripheral *vs* hepatic insulin resistance (Hirst *et al.*, 1993). A major limitation of this test is the risk of hypoglycemia. Moreover, hypoglycemia triggers counterregulatory hormonal responses, which may interfere with insulin sensitivity and in turn slows the disappearance rate of glucose from plasma (Garber *et al.*, 1976; Reaven, 1983). In this view, the fall in plasma glucose concentration would be a function of the interplay between insulin, on the one hand, and glucagon, catecholamines, growth hormone and cortisol, on the other. However, the counterregulatory response occurs at least 15 to 20min after insulin injection. Thus, the

glucose fall occurring in the first 15min after iv insulin administration is probably a function of insulin-stimulated glucose uptake by tissues, as well as, insulin-inhibited glucose output by the liver (Rizza *et al.*, 1979b).

A lower insulin dose method of 0.05IU/kg, or shortening the test to 15min was suggested as an attempt to decrease the risk of hypoglycemia (Chen *et al.*, 1998; Hirst *et al.*, 1993). The shorter version (Akinmokun *et al.*, 1992; Bonora *et al.*, 1989) derived from the notion that the counterregulatory hormone response occurs only after 20min of the insulin infusion (Monzillo *et al.*, 2003; Rizza *et al.*, 1979b).

The advantages of the ITT include its simplicity, rapidity, use of a bolus injection of insulin and ability to measure insulin sensitivity in the fed or fasting state. The bolus injection of insulin mimics the physiological pulsatile release of insulin (Hansen *et al.*, 1982; Porksen *et al.*, 1996). Furthermore, because glucose tolerance after a meal is dependent on insulin sensitivity, measuring insulin sensitivity in the prandial state is physiologically relevant.

In conclusion, the ITT should be used with great caution in insulin sensitive individuals because of the increased risk of hypoglycemia, even when the smaller dose version of the test is used. The shorter ITT is a valid test in large-scale studies, especially when the site of resistance is not of importance.

1.7.1.3. Insulin Suppression Test (IST)

The insulin-suppression test (IST), another method that directly measures metabolic insulin sensitivity/resistance, was introduced by Shen *et al.* in 1970 and subsequently modified by Harano *et al.* (Harano *et al.*, 1978; Shen *et al.*, 1970). After an overnight fast, somatostatin (250μg/h) or the somatostatin analogue octreotide (25μg bolus, followed by 0.5μg/min) (Pei *et al.*, 1994) is intravenously infused, to suppress endogenous secretion of insulin and glucagon. Simultaneously, insulin (25mU/m^2/min) and glucose (240mg/m^2/min) are intravenously infused over 3h. Blood samples for glucose and insulin determinations are taken every 30min for 2.5h, and then at 10min intervals from 150 to 180min of the IST. The constant infusions of insulin and glucose determine steady-state plasma insulin (SSPI) and glucose (SSPG) concentrations. The steady-state period is assumed to be from 150 to 180min after initiation of the IST. SSPI concentrations are generally similar among subjects. Therefore, the SSPG concentration will be higher in insulin resistant subjects and lower in insulin sensitive subjects, *i.e.*, SSPG values are inversely related to insulin sensitivity. The IST provides a direct measure (through SSPG) of the ability of exogenous insulin to mediate disposal of an iv glucose load, under steady-state conditions, where endogenous insulin secretion is suppressed (Greenfield *et al.*, 1981; Pickup *et al.*, 2003).

The SSPG is a highly reproducible direct measure of metabolic actions of insulin, that is, less labor intensive and less technically demanding than HIEC. Indeed, since there are no variable infusions with the IST, steady-state conditions are more easily achieved with the IST than with HIEC.

In research settings, the IST can be used for larger populations that may pose difficulties for application of HIEC (Yeni-Komshian *et al.*, 2000). Many of the limitations of the IST are similar to those described for HIEC (with the exception that the IST is less technically demanding). Thus, it is impractical to apply the IST in large epidemiological studies or in the clinical care setting. SSPG under ideal conditions determines primarily skeletal muscle insulin sensitivity, and is not designed to reflect hepatic insulin sensitivity (Greenfield *et al.*, 1981).

1.7.1.4. Continuous Infusion of Glucose with Model Assessment (CIGMA)

The continuous infusion of glucose with model assessment (CIGMA) is a procedure that assesses insulin sensitivity through the evaluation of the near steady-state glucose and insulin concentrations after a continuous infusion of glucose, with model assessment (Hosker *et al.*, 1985). This method mimics postprandial glucose and insulin concentrations. CIGMA not only provides information about glucose tolerance and insulin sensitivity, but also about β-cell function. Using a mathematic model of glucose homeostasis, glucose and insulin values are compared with known physiologic data of glucose, and insulin kinetics in response to glucose infusion, which are derived from healthy lean subjects with no family history of diabetes (Hosker *et al.*, 1985).

The glucose and insulin values used for CIGMA are obtained during the last 15min of the 60min continuous glucose infusion (5mg glucose/kg bw/min). Samples are collected at 5min intervals, to avoid the oscillatory variation in insulin concentration. The average is then compared with predicted values from the computer model. The median value for normal subjects is 1.35, and for diabetic patients with mild hyperglycemia is 4.0 (Hosker *et al.*, 1985).

There are two main advantages of CIGMA over HOMA. First, the insulin values that are measured in CIGMA are much higher than those in HOMA owing to the glucose stimulus and second, higher insulin concentration in CIGMA stimulates peripheral glucose uptake producing a steady-state glucose concentration, which is a better reflection of the peripheral insulin sensitivity (Monzillo *et al.*, 2003; Vague *et al.*, 2002).

Although CIGMA is more practical, cheaper and less invasive than the FSIVGTT and HIEC procedure, the model incorrectly assumes that levels of insulin resistance at the liver and peripheral tissues are equal. Furthermore, in insulin-deficient subjects, where

the insulin response is insufficient to stimulate glucose uptake, the interpretation of CIGMA is difficult (Ferrannini *et al.*, 1998).

1.7.1.5. Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test (OGTT) is a simple test widely used in clinical practice to diagnose glucose intolerance and type 2 diabetes (American Diabetes Association, 2007; Monzillo *et al.*, 2003; Sievenpiper *et al.*, 2000; Soonthornpun *et al.*, 2003). After an overnight fast, blood samples for determinations of glucose and insulin concentrations are taken at 0, 30, 60, and 120min following a standard oral glucose load (75g) (American Diabetes Association, 2007). A diagnosis of diabetes is conferred if an individual has a plasma glucose level ≥200mg/dl as measured 2h after the ingestion of a 75g glucose load. If an individual has a value in the range of 140-199mg/dl 2h post–glucose load, is designated as having impaired glucose tolerance (American Diabetes Association, 2007). Oral glucose tolerance reflects the efficiency manner in which the body handles glucose after an oral glucose load.

The OGTT mimics the glucose and insulin dynamics of physiological conditions more closely than conditions of the HIEC, IST or frequently sample intravenous glucose tolerance test (FSIVGTT) (Berthiaume *et al.*, 2002; Steil *et al.*, 2004). However, it is important to recognize that glucose tolerance and insulin sensitivity are not equivalent concepts. In addition to metabolic actions of insulin, insulin secretion, incretin effects, and other factors contribute importantly to glucose tolerance. Thus, the OGTT provide useful information about glucose tolerance but not insulin sensitivity/resistance *per se* (Caumo *et al.*, 2000; Steil *et al.*, 2004).

The OGTT is technically quite simple to perform and certainly lower in cost than HIEC or FSIVGTT. These considerations have made the OGTT the glucose challenge test of choice in clinical situations (American Diabetes Association, 2007; American Diabetes Association, 2008). However, there are some problems with the OGTT that make it less desirable for use in research situations. First, there is variability in the rate of gastric emptying and glucose absorption from the gastrointestinal tract, causing some imprecision from the start. This variability can partially account for poorly reproducible results even within the same individual (American Diabetes Association, 2004). Second, glucose measurements in the standard OGTT do not give adequate information regarding the dynamics of glucose and insulin action (The expert committee on the diagnosis and classification of diabetes mellitus, 2003).

The OGTT is a relatively crude measure of glucose tolerance. It does not measure the components of insulin sensitivity and insulin secretion. In light of this limitation, attempts have been made to obtain indices from OGTT data that might better reflect β-cell function and insulin sensitivity (Avignon *et al.*, 1999; Belfiore *et al.*, 1998; Cederholm *et al.*,

1990; Gutt *et al.*, 2000; Matsuda *et al.*, 1999; McAuley *et al.*, 2001; Stumvoll *et al.*, 2001), which are reviewed below.

1.7.1.6. Minimal model analysis of Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT)

The minimal model, developed by Bergman, Cobelli and colleagues in 1979, provides an indirect measure of metabolic insulin sensitivity/resistance based on glucose and insulin data obtained during a frequently sampled intravenous glucose tolerance test (FSIVGTT) (Bergman *et al.*, 1979). After an overnight fast, an intravenous bolus of glucose (0.3g/kg bw) is infused over 2min starting at time 0.

Currently, a modified FSIVGTT is used, where exogenous insulin (4mU/kg/min) is also infused over 5min beginning 20min after the iv glucose bolus (Finegood *et al.*, 1990; Quon *et al.*, 1994; Saad *et al.*, 1997). Some studies use tolbutamide instead of insulin in the modified FSIVGTT, to stimulate endogenous insulin secretion (Bergman *et al.*, 1987; Saad *et al.*, 1997; Yang *et al.*, 1987).

Blood samples are taken for plasma glucose and insulin measurements at different time points, before and 180min after glucose infusion. The data obtained are then subjected to minimal model analysis using the computer program MINMOD (minimal model approach - MINMOD), to generate an index of insulin sensitivity (S_I).

The MINMOD is defined by two coupled differential equations with four model parameters (Figure 1.12). The first equation describes plasma glucose dynamics in a single compartment. The second equation describes insulin dynamics in a "remote compartment". The structure of the MINMOD allows it to uniquely identify model parameters, which determine a best fit to glucose disappearance during the modified FSIVGTT. S_I is calculated from two of these model parameters, and is defined as fractional glucose disappearance *per* insulin concentration unit (Bergman, 2005).

In addition to S_I, other minimal model parameters may be used to estimate a "glucose effectiveness" index (S_G). S_G is defined as the ability of glucose *per se* to promote its own disposal and inhibit hepatic glucose production (HGP) in the absence of an incremental insulin effect (i.e., when insulin is at basal levels).

$$\frac{dG(t)}{dt} = -\left[p_1 + X(t)\right]G(t) + p_1 G_b \qquad 1^{st} \text{ equation}$$

$$\frac{dX(t)}{dt} = -p_2 X(t) + p_3\left[I(t) - i_b\right] \qquad 2^{nd} \text{ equation}$$

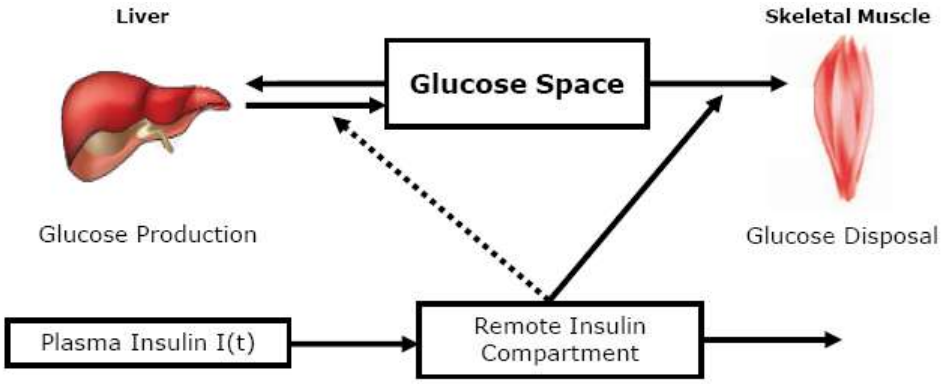


Figure 1.12 - Schematic equations and parameters for the minimal model of glucose metabolism. Differential equations describing glucose dynamics [G(t)] in a monocompartmental "glucose space" and insulin dynamics in a "remote compartment" [X(t)] are shown at the top. Glucose leaves or enters its space at a rate proportional to the difference between plasma glucose level, G(t) and the basal fasting level, G_b. In addition, glucose also disappears from its compartment at a rate proportional to insulin levels in the "remote" compartment [X(t)]. In this model, t - time; G(t) - plasma glucose at time t; I(t) - plasma insulin concentration at time t; X(t) - insulin concentration in "remote" compartment at time t; G_b - basal plasma glucose concentration; I_b - basal plasma insulin concentration; G(0) - G_0 (assuming instantaneous mixing of the iv glucose load); p_1, p_2, p_3 and G_0 - unknown parameters in the model that are uniquely identifiable from FSIVGTT; glucose effectiveness (S_G) - p_1 and insulin sensitivity - p_3/p_2. Adapted from (Bergman, 2005).

Minimal model analysis of the modified FSIVGTT is easier than HIEC method because it is slightly less labor intensive, steady-state conditions are not required, and there are no iv infusions that require constant adjustment. Unlike HIEC or IST, information about insulin sensitivity, glucose effectiveness, and β-cell function can be derived from a single dynamic test. The minimal model generates excellent predictions of glucose disappearance during the FSIVGTT.

In research settings, where assessing insulin sensitivity along with glucose effectiveness and β-cell function is of interest, minimal model analysis of the insulin-modified FSIVGTT may be appropriate. The minimal model approach is simpler than direct methods for determining insulin sensitivity. Nevertheless, it still involves iv infusions with multiple blood sampling over a 3h period, that is, nearly as labor intensive as the HIEC or IST. In addition, many limitations of minimal model analysis stem from the fact that the model oversimplifies the physiology of glucose homeostasis (Muniyappa *et al.*, 2008).

Another oversimplification of the minimal model involves lumping together effects of insulin to promote peripheral glucose utilization and suppress HGP. As insulin

sensitivity/resistance varies, the relative contribution of HGP to S_I may vary significantly. Since the minimal model relies on a dynamic test to evaluate insulin sensitivity, estimates of S_I are much less reliable in individuals with impaired insulin secretion and/or significant insulin resistance (when compared with healthy subjects). Under these conditions, the minimal model may overestimate S_G to accurately predict the disappearance of glucose during the FSIVGTT. Indeed, estimates of S_G are spuriously affected by differences in insulin secretory capacity (Finegood *et al.*, 1996; Quon *et al.*, 1994). Moreover, for similar reasons, minimal model analysis often generates nonsensical negative values for S_I in a substantial proportion of subjects with diabetes, who have minimal insulin secretory capacity and significant insulin resistance (Finegood *et al.*, 1996; Katz *et al.*, 2000). These nonsystematic errors inherent in the minimal model approach are highlighted by calibration model analysis, demonstrating that some simple surrogate indexes of insulin sensitivity have better absolute accuracy for predicting SI_{Clamp} than the minimal model-derived S_I (Chen *et al.*, 2005).

1.7.1.7. Meal Tolerance Test (MTT)

In an attempt to study the ability to regulate blood glucose in a more physiological situation than the OGTT, some authors measure the glycemic profile in response to the ingestion of a mixed meal constituted by carbohydrates, fat and proteins - meal tolerance test (MTT).

The experimental procedure for the MTT is similar to the OGTT, that is, after an overnight fast (10-12h), a mixed meal[18] (liquid or solid) is given and the glycemic profile is measured throughout 2h (Berthiaume *et al.*, 2002; Caumo *et al.*, 2000; Steil *et al.*, 2004); usually the insulin profile is also determined during the same period of time (Steil *et al.*, 2004).

The MTT is a "physiologic" variant of OGTT (Lefebvre *et al.*, 1976) offering several advantages: (a) lack of artifactual postload hypoglycemia, thus making this test suitable for the study of postprandial hypoglycemia (Brun *et al.*, 1995), a situation which is frequently due to high values of insulin sensitivity (Brun *et al.*, 1996; Brun *et al.*, 2000), but also to hyperinsulinism in a context of insulin resistance (Brun *et al.*, 2000); (b) use of a physiologic stimulus triggering a cephalic phase proportional to palatability scores[19]

[18] The dose and the concentrations of the components of the mixed meal used in MTT are not standardized yet.
[19] The palatability was assessed with a 14cm visual analogue scale, ranging from "extremely unpleasant" to "extremely pleasant". Just after eating, subjects were asked to mark a vertical line on the scale. For data analysis, the centre of the scale was considered as the zero value (neutral), and scores of palatability were measured as deviations in cm from the zero point. Thus, the scores ranged from -7 (distaste) to +7 (extreme pleasure) (Raynaud *et al.*, 1999).

(Raynaud *et al.*, 1999); (c) possibility to measure insulin sensitivity with a modified algorithm based on the minimal model (Caumo *et al.*, 2000) as well as glucose effectiveness and insulin secretion (Mari *et al.*, 2002); (d) potential for evaluating the physiologic effects of incretins (Rijkelijkhuizen *et al.*, 2010).

The MTT can represent a simple procedure, less unpleasant for the patient than the standard OGTT, and providing both a physiologic picture of glucoregulation, and a sophisticated and precise analysis of this glucoregulation, in terms of insulin sensitivity, glucose effectiveness, and insulin secretion (Aloulou *et al.*, 2006).

The β-cell response is stronger after a mixed meal than after an OGTT with equal carbohydrate quantity, both for classical and model-based parameters. The higher response was mostly explained by higher β-cell sensitivity during the meal, which may lead to lower glucose excursions (Rijkelijkhuizen *et al.*, 2009).

Several factors may contribute to differences in insulin secretion following an MTT compared with the OGTT. The MTT has a lower glycemic index than the OGTT, which may lead to lower glucose excursions (Wolever *et al.*, 2006). Slower gastric emptying following the MTT due to larger volume (Doran *et al.*, 1998), solid character (Achour *et al.*, 2001), and fat content (Cunningham *et al.*, 1989) will lead to a slower entry of nutrients into the circulation.

The MTT might be considered as an additional tool for the assessment of metabolic abnormalities, in glucose-intolerant and insulin-resistant states (Berthiaume *et al.*, 2002).

Thus, the MTT is a more physiological test than the OGTT, in regard to human diet, and is potentially able to give useful information concerning islet β-cell function in the different categories of glucose intolerance (Marena *et al.*, 1992), but not insulin sensitivity/resistance *per se* (Caumo *et al.*, 2000; Steil *et al.*, 2004).

As any other method that measures glucose tolerance, the MTT does not assess insulin sensitivity directly and may not be repeated in the same subject or animal on the same day.

From a nutritional perspective, meals that are capable of producing meal-induced insulin sensitization (MIS) are advantageous, whereas meals that cannot produce MIS will result in nutrients being processed primarily as fat. Glucose and sucrose clearly are ineffective at serving as an appropriate feeding signal (Sadri *et al.*, 2006). In addition, high-sucrose diets not only are not capable of activating MIS, but actually result in complete HISS-dependent insulin resistance after as short a period of feeding as 2 weeks on a 35% sucrose diet (Ribeiro *et al.*, 2005).

These observations may be directly related to the cause of the epidemic increase in insulin resistance, type 2 diabetes and obesity, in developed countries where refined sugar products make up a very large component of the normal diet. The demonstration

that MIS cannot be generated by these refined sugars, places added emphasis on the need for studies related to nutrient content of meals, especially in high risk groups. Sucrose and glucose are not capable of producing normal feeding signals thereby questioning the value of the oral glucose tolerance test for studies intended to quantitate responses to feeding (Sadri *et al.*, 2006).

1.7.1.8. Rapid Insulin Sensitivity Test (RIST)

From all the methods above described, none of them proves to be a reliable way to assess insulin sensitivity/resistance, since most of them have non-physiological continuous infusion of insulin and/or glucose, which interfere with peripheral insulin sensitivity/resistance; take a long time to be performed; could not avoid counter-regulatory responses to the hypoglycemia that follows an insulin bolus; could not allowed the assessment of insulin sensitivity in different conditions in the same subject, and in the same day; and it only evaluates insulin sensitivity resistance in the fasted state. Based on all of these drawbacks, it was necessary to develop another method for assessing insulin sensitivity/resistance.

A new method for insulin sensitivity quantificarion, called the Rapid Insulin Sensitivity Test (RIST), was described and evaluated for use in rats (Lautt *et al.*, 1998a; Xie *et al.*, 1996a), cats (Xie *et al.*, 1995a; Xie *et al.*, 1996c) and mice (Latour *et al.*, 2002a). Minor modifications and further technical aspects of the RIST have been recently updated and a current operating procedure is described (Lautt *et al.*, 1998a). The standard time line of the RIST, in rats, is shown in Figure 1.13.

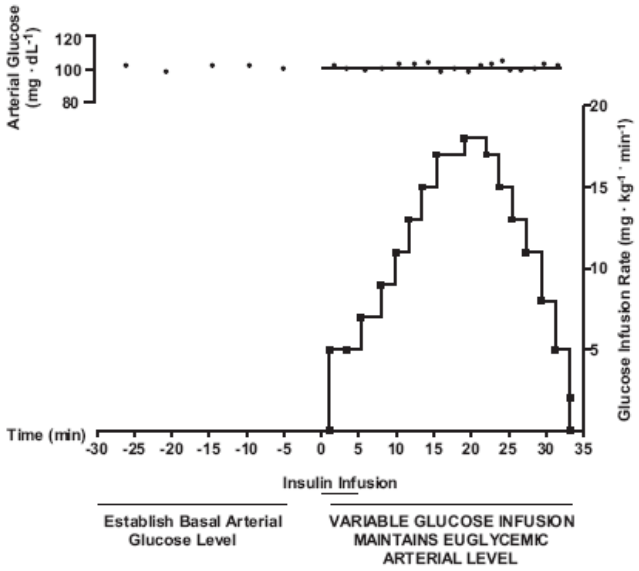


Figure 1.13 - Rapid insulin sensitivity test (RIST) time line. Three stable arterial glucose levels determined at 5min intervals established the ideal euglycemic baseline. Intravenous insulin infusion is administered over 5min with the glucose infusion and first arterial glucose sample beginning after 1min of glucose infusion. A variable iv glucose infusion is adjusted to maintain euglycemia based on arterial samples taken at 2min intervals throughout the test period. The RIST index is the total amount of glucose infused to maintain euglycemia over the test period, which is terminated when no further glucose infusion is required. The test period for the standard insulin dose in rats (50mIU/kg bw) is 30min, but different durations may be needed for higher doses, different conditions, or different species (Lautt *et al.*, 1998a).

Briefly, the RIST procedure, so far described in animals, is an euglycemic test and is carried out after establishing the glycemic baseline, which is done by taking arterial blood samples at 5min intervals until three consecutive measurements are stable. An insulin infusion is commenced (50mIU/kg administered over 5min) and, after 1min, glucose samples are taken at 2min intervals, and glucose is infused intravenously at a variable rate to maintain euglycemia. The test is completed when no more glucose is required. At the standard test dose of insulin of 50mIU/kg, the RIST is complete within 40min. The RIST index, the insulin sensitivity parameter, is simply the amount of glucose that had to be administered in order to maintain euglycemia after the bolus administration of insulin (Lautt *et al.*, 1998a).

One of the objectives of this thesis was to implement the RIST in humans, and the detailed methodology used will be introduced in chapter 3. In the remaining protocols of the PhD work, both in humans and in animals, the RIST was the selected methodology to evaluate insulin sensitivity.

The primary technical difficulty with the RIST methodology is the need for rapid sampling of arterial blood. This was overcome by the use of an arterial-venous vascular shunt. The shunt, in rats, has been tested in various combinations, shunting from the femoral artery to the femoral vein or the carotid artery to the jugular vein. The shunt

drains blood from the artery, passes it through a segment of silicone catheter and, directs it back into the venous compartment. Arterial glucose samples are obtained by needle puncture into the silicone segment of the shunt and direct transfer of the blood sample (25µl) to a glucose analyzer capable of providing a reading within 1–2min. The arterial-venous loop allows iv infusions to be made through the shunt, and the perfusion pressure can be monitored through a side branch. Continuous monitoring of shunt blood pressure provides early warning of either venous or arterial obstruction. The arterial-venous shunt, blood sampling and pressure monitoring method has great applicability for many *in vivo* studies.

Another methodological issue relates to the basal glucose concentration determined before and after the RIST. A number of our previous studies demonstrate clearly that there is no mean change in basal blood glucose levels used as the euglycemic target when, for example, compared before and after denervation of the hepatic plexus or atropine. In addition, we have also determined that there is no correlation between the magnitude of the RIST index and basal glucose levels when compared using a large number of data points (Lautt *et al.*, 1998a). Of more concern is the importance that glucose uptake or output should not change during the RIST. Whatever stimulus is used, including either ablation or stimulation protocols, the stimulus is administered prior to conducting the RIST, and a new stable glycemic baseline must be demonstrated. In addition, at the conclusion of the RIST index, the re-established baseline must not be significantly altered. In the event that such alteration occurs, it suggests that glucose output either increased or decreased during the test. This is usually obvious by comparing the shape of normal RIST curves with that obtained in the presence of the altered baseline. In such situations, the data must be excluded, and the RIST repeated (Lautt *et al.*, 1998a).

The RIST is not affected by anesthesia in rats (Latour *et al.*, 2002b; Lautt *et al.*, 1998a) and is equally effective in anesthetized or conscious rats (Latour *et al.*, 2002c).

Insulin release normally occurs in a pulsatile manner, and hormones released in a pulsatile manner are best studied by pulsatile administration (Lautt, 2005). Based on this assumption, the iv insulin bolus administered at the beginning of the RIST mimics the physiological insulin action. It also avoids the vagal withdrawal and sympathetic activation induced by sustained hyperinsulinemia, during the HIEC (Latour *et al.*, 2002a; Reid *et al.*, 2002) and the hypoglycemia caused by the acute ITT (Reid *et al.*, 2002). It does not alter levels of counter-regulatory hormones, such as catecholamines, somatostatin or glucagon (Xie *et al.*, 1995a). Moreover, both insulinemia and glycemia return to basal levels after each RIST.

The RIST is extremely sensitive and can be shown to generate dose–response relationships to insulin (Lautt *et al.*, 1998a), which makes the RIST the most

advantageous method in the determination of small differences in insulin sensitivity. This method is able to be carried out routinely four sequential times in the same animal with high reproducibility (Lautt *et al.*, 1998a), and is sufficiently versatile to permit paired experimental designs showing, in the same animal and on the same day. Both the accuracy and precision of the test can be assessed from determination of the deviation from the ideal euglycemic target (Lautt *et al.*, 1998a).

The majority of the insulin sensitivity tests are done in the fasted state, when insulin sensitivity would be logically anticipated to be at its lowest level. Studies performed by Lautt *et al.* indicated that the fasted state results in a very low insulin responsiveness. It is reasonable that insulin sensitivity should be under a regulatory mechanism such that in the fasted state insulin effect would be minimized, and inappropriate release of insulin would not, therefore, lead to life-threatening hypoglycemia. The RIST can be carried out in the fed state and it allows to determine full insulin sensitivity (Lautt *et al.*, 2001). Furthermore, the RIST allows insulin sensitivity assessment before and after a meal, making it possible to be test both meal and drug effects on insulin sensitivity (Sadri *et al.*, 2006).

To summarize, the RIST is a quick method to evaluate insulin sensitivity, reproducible until 4 tests in the same animal and on the same day, utilizes a bolus of insulin to mimic pulsatile insulin release, and can be performed in the fed or fasting state. In addition, since the RIST is an euglycemic test, avoids hypoglycemia and prevents the activation of counter-regulatory hormones.

The advantages of the RIST, in humans, in comparison with other insulin sensitivity tests will be discussed in detail in chapter 4.

1.7.2. Simple surrogate indexes for insulin sensitivity/resistance

1.7.2.1. Homeostasis Model Assessment (HOMA)

The homeostasis model assessment (HOMA), developed in 1985, is a model of interactions between glucose and insulin dynamics, that is then used to predict fasting steady-state glucose and insulin concentrations, for a wide range of possible combinations of insulin resistance and β-cell function (Matthews *et al.*, 1985). The model assumes a feedback loop between the liver and β-cell (Levy *et al.*, 1998; Matthews *et al.*, 1985; Wallace *et al.*, 2004); and glucose concentrations are regulated by insulin-dependent hepatic glucose production, while insulin levels depend on the pancreatic β-cell response to glucose concentrations. Thus, deficient β-cell function reflects a diminished response to glucose-stimulated insulin secretion. Likewise, insulin resistance is reflected by diminished suppressive effect of insulin on hepatic glucose production.

HOMA model describes this glucose-insulin homeostasis by a set of empirically derived non-linear equations. The model predicts fasting steady-state levels of plasma glucose and insulin for any given combination of pancreatic β-cell function (HOMA%B) and insulin sensitivity (HOMA%S)[20].

In practical terms, most studies using HOMA employ an approximation described by a simple equation to determine a surrogate index of insulin resistance. This is defined by the product of the fasting glucose and fasting insulin, divided by a constant.

The formula for the HOMA model is:

$$HOMA = \frac{Fasting\ Insulin\ (\mu IU/ml) \times Fasting\ Glucose\ (mmol/l)}{22.5}$$

The denominator of 22.5 is a normalizing factor; *i.e.*, the product of normal fasting plasma insulin of 5μIU/ml and normal fasting plasma glucose of 4.5mmol/l obtained from an "ideal and normal" individual (Matthews *et al.*, 1985). Therefore, for an individual with normal insulin sensitivity, HOMA = 1. It is important to note that, over wide ranges of insulin sensitivity/resistance, log (HOMA) transforms the skewed distribution of fasting insulin values to determine a much stronger linear correlation with HIEC estimates of insulin sensitivity (Katz *et al.*, 2000).

HOMA or log (HOMA) is extensively used in large epidemiological studies, prospective clinical trials, and research studies (Radikova, 2003; Wallace *et al.*, 2004). In research settings where assessing insulin sensitivity/resistance is of secondary interest or feasibility issues preclude the use of direct measures by HIEC, it may be appropriate to use log (HOMA) (Wallace *et al.*, 2004).

1.7.2.2. Quantitative Insulin Sensitivity Check Index (QUICKI)

Quantitative insulin sensitivity check index (QUICKI) is an empirically-derived mathematical transformation that uses fasting blood glucose and plasma insulin concentrations. It provides a reliable, reproducible, and accurate index of insulin sensitivity with excellent positive predictive power (Chen *et al.*, 2005; Chen *et al.*, 2003; Hanley *et al.*, 2003; Katz *et al.*, 2000; Mather *et al.*, 2001). Since fasting insulin levels have a non-normal skewed distribution, log transformation improves its linear correlation with reference standard glucose clamp (SI_{Clamp}). However, as with 1/(fasting insulin) and

[20] An important caveat for HOMA%B (determined from fasting glucose and insulin concentrations) is that it imputes a dynamic β-cell function (*i.e.*, glucose-stimulated insulin secretion) from fasting steady-state data. In the absence of dynamic data, it is difficult, if not impossible, to determine the true dynamic function of β-cell insulin secretion (Matthews *et al.*, 1985).

the glucose/insulin ratio, this correlation is not maintained in diabetic subjects with fasting hyperglycemia and impaired β-cell function, that is, insufficient to maintain euglycemia. To accommodate these clinically important circumstances where fasting glucose is inappropriately high, insulin is inappropriately low, application of logarithm to both fasting glucose, and fasting insulin provides a reasonable correction such that the linear correlation with SI_{Clamp} is maintained, in both diabetic and non-diabetic subjects. The reciprocal of this sum results in further transformation of the data generating an insulin sensitivity index that has a positive correlation with SI_{Clamp}.

Thus, QUICKI is defined by the following formula:

$$QUICKI = \frac{1}{\log(fasting\ insulin, \mu IU/ml) + \log(fasting\ glucose, mg/dl)}$$

QUICKI and HOMA were derived in a completely different conceptual fashion. Nevertheless, these two surrogate indexes are mathematically related, *i.e*, QUICKI is proportional to 1/log (HOMA).

QUICKI is among the most thoroughly evaluated and validated surrogate index for insulin sensitivity. As a simple, useful, inexpensive, and minimally invasive surrogate for HIEC-derived measures of insulin sensitivity, QUICKI is appropriate and effective for use in large epidemiological or clinical research studies, to follow changes after therapeutic interventions, and for use in studies where evaluation of insulin sensitivity is not of primary interest (Abbasi *et al.*, 2002; Katz *et al.*, 2000).

The major advantage of both the QUICKI and HOMA models is that they both require only one blood draw from a fasting patient. They thus do not require extensive technical expertise, and constitute a much lower cost *per* subject when compared with the HIEC or the FSIVGTT, making the QUICKI and HOMA models much more practical for use in large-scale epidemiologic studies, and for clinical situations (Wallace *et al.*, 2004).

However, the major disadvantage is that both of these methods fail to provide information about the stimulated glucose and insulin systems. Essentially, they provide information only about what is occurring with homeostatic mechanisms in the fasting state, largely reflecting insulin's effect on hepatic glucose production and not on peripheral glucose uptake, which is the more relevant aspect concerning insulin action/resistance.

1.7.2.3. Insulin sensitivity indexes based on OGTT

Cederholm and Wibell Index

The insulin sensitivity index proposed by Cederholm and Wibell (Katz *et al.*, 1983) represents mainly peripheral insulin sensitivity and muscular glucose uptake, due to the dominant role of peripheral tissues in glucose disposal after an oral glucose load (Cederholm *et al.*, 1990).

The formula for the Cederholm index is:

$$ISI_{Cederholm} = \frac{75000 + (G_0 - G_{120}) \times 1.15 \times 180 \times 0.19 \times m}{120 \times G_{mean} \times \log(I_{mean})}$$

where, 75000 – oral glucose load in an OGTT in mg, G_0 – fasting plasma glucose concentration (mmol/l), G_{120} – plasma glucose concentration in the 120th min of OGTT (mmol/l), 1.15 – factor transforming whole venous blood glucose to plasma values (not necessary, if glucose concentration is estimated in plasma), 180 – conversion factor to transform plasma glucose concentration from mmol/l into mg/dl, 0.19 – glucose space in liter per kg of body weight, m – body weight (kg), 120 – duration of OGTT (min), I_{mean} – mean plasma insulin concentration during OGTT (mIU/l) and G_{mean} – mean plasma glucose concentration during OGTT (mmol/l).

Values found in normal non-obese individuals were reported to be about 79±14mg.l^2/mmol/mIU/min, lower in obese individuals, in subjects with impaired glucose tolerance and in patients with type 2 diabetes (Cederholm *et al.*, 1990).

Gutt et al. Index

The $ISI_{0,120}$ was adapted from the Cederholm insulin sensitivity index (Gutt *et al.*, 2000), by omitting the constant terms, and using the plasma glucose and insulin concentration from fasting (0min) and 120min samples from the OGTT (Gutt *et al.*, 2000).

The $ISI_{0,120}$ index is defined as:

$$ISI_{0,120} = \frac{75000 + (G_0 - G_{120}) \times 0.19 \times m}{120 \times G_{mean} \times \log(I_{mean})}$$

where, 75000 – oral glucose load in an OGTT in mg, G_0 – fasting plasma glucose concentration (mg/dl), G_{120} – plasma glucose concentration in the 120th min of OGTT (mg/dl), 0.19 – glucose space in l/kg of body weight, m – body weight (kg), 120 – duration of OGTT (min), I_{mean} – mean plasma insulin concentration during OGTT (mIU/l) and G_{mean} – mean plasma glucose concentration during OGTT (mmol/l).

The reference range for lean controls was 89±39, for obese 58±23 and for diabetic patients 23±19mg.l^2/mmol/mIU/min (Monzillo *et al.*, 2003).

Avignon et al. Index

Avignon (Cobelli *et al.*, 1987) proposed 3 insulin sensitivity indices : Sib (derived from fasting plasma insulin and glucose concentrations), Si2h (derived from plasma insulin and glucose concentrations in the 120th min of OGTT) and SiM (derived by averaging Sib and Si2h after balancing Sib by a coefficient of 0.137 to give the same weight to both indices):

$$Sib = \frac{10^8}{I_0 \text{ x } G_0 \text{ x } VD} \qquad Si2h = \frac{10^8}{I_{120} \text{ x } G_{120} \text{ x } VD} \qquad SiM = \frac{(0.137 \text{ x } Sib) + Si2h}{2}$$

where, I and G represent the plasma concentrations of insulin (mIU/l) and glucose (mmol/l), respectively and, VD is the glucose distribution volume calculated using a monocompartmental model: VD=150ml/kg of body weight (Bergman *et al.*, 1987).

Matsuda et al. Index

Originally proposed by Matsuda and DeFronzo (Matsuda *et al.*, 1999), insulin sensitivity index-Matsuda ($ISI_{(Matsuda)}$) is an whole body insulin sensitivity index that reflects a composite estimate of hepatic and muscle insulin sensitivity. This index is calculated from plasma glucose (mg/dl) and insulin (mIU/l) concentrations in fasting state and during OGTT.

The formula for the Matsuda index is:

$$ISI_{(Matsuda)} = \frac{10000}{\sqrt{G_0 \text{ x } I_0 \text{ x } G_{mean} \text{ x } I_{mean}}}$$

where, 10000 – simplifying constant to get numbers from 0 to 12, √ - correction of the nonlinear values distribution, G_0 – fasting plasma glucose concentration (mg/dl), I_0 – fasting plasma insulin concentration (mIU/l), G_{mean} – mean plasma glucose concentration during OGTT (mg/dl), from 0 to 120min and I_{mean} – mean plasma insulin concentration during OGTT (mIU/l), from 0 to 120min.

The insulin secretion/insulin resistance (disposition) index calculated as the product of insulin secretion measured with ($\Delta I_{0-30}/\Delta G_{0-30}$ or $\Delta I_{0-120}/\Delta G_{0-120}$) and $ISI_{(Matsuda)}$ (or modified $ISI_{(Matsuda)}$ using plasma glucose and insulin concentrations at 30min during the OGTT), had excellent power to predict onset of type 2 diabetes (Belfiore *et al.*, 2001; Belfiore *et al.*, 1998).

Belfiore et al. Index

The condition for calculation of the Belfiore formula is the definition of the normal value for basal glucose and insulin concentrations, and for mean normal value for glucose and insulin areas during OGTT (Monzillo *et al.*, 2003). The main point of the Belfiore formula is the comparison of insulin and glucose values measured (fasting, 0-1-2h areas or 0-2h areas) with the defined normal reference values.

The $ISI_{Belfiore}$ index is defined as:

$$ISI_{Belfiore} = \frac{2}{\frac{G_S}{G_N} \times \frac{I_S}{I_N} + 1}$$

where G_s, G_N – plasma glucose concentrations expressed as fasting values or as areas obtained during a standard OGTT at 0 and 2h (0-2h areas are equal to $G_{S,N} = G_0 + G_{120}$) or at 0, 1 and 2h (0-1-2h areas equal to $G_{S,N} = ½G_0 + G_{60} + G_{120}$, I_s, I_N – plasma insulin concentrations expressed as fasting values or as areas obtained during a standard OGTT at 0 and 2h (0-2h areas are equal to $I_{S,N} = I_0 + I_{120}$) or at 0, 1 and 2h (0-1-2h areas equal to $I_{S,N} = ½I_0 + I_{60} + I_{120}$. The subscripts S and N refer to "subjects" and "normal reference values", respectively.

Insulin sensitivity calculated using these formulas can achieve only values between 0 and 2. In subjects with normal insulin sensitivity is it around 1; in overweight subjects, in subjects with impaired glucose tolerance, and with type 2 diabetes this value is below 1 (Stumvoll *et al.*, 2000; Stumvoll *et al.*, 2001).

Stumvoll et al. Index

Stumvoll proposed a series of indices calculated from plasma glucose (mmol/l) and insulin (pmol/l concentrations during OGTT) (Stumvoll *et al.*, 2001). The equations were generated using the multiple linear regression analysis and adapted to the availabilities of sampling times during OGTT, and of demographic parameters (BMI, age).

An example equation could be the index of insulin sensitivity calculated from data obtained in 0, 60 and 120min of OGTT either with or without demographic data:

$$ISI_{Stumvoll} = 0.222 - 0.00333 \times BMI - 0.0000779 \times I_{120} - 0.000422 \times age$$
$$ISI_{Stumvoll} = 0.156 - 0.0000459 \times I_{120} - 0.000321 \times I_0 - 0.00541 \times G_{120}$$

McAuley et al. Index

The authors proposed a formula for predicting insulin resistance in normoglycemic individuals (McAuley *et al.*, 2001). Regression analysis was used to estimate the cut-off points and the importance of various data for insulin resistance (fasting concentrations of insulin, triglycerides, aspartate aminotrasnferase, BMI, waist circumference). A bootstrap procedure[21] was used to find an index most strongly correlating with insulin sensitivity index, corrected for fat-free mass obtained by HIEC $\left(\frac{\text{Mffm}}{\text{I}}\right)$ (DeFronzo *et al.*, 1979).

An insulin sensitivity index obtained from HIEC of ≤ 6.3 (expressed as glucose disposal rate in mg/kg/min divided by average plasma insulin concentration in mIU/l) was seen as a cut-off for individuals with insulin resistance. The combination of fasting insulin (mIU/l) and triglycerides (TAG, mmol/l) showed the best prediction of insulin resistance as follows:

$$\left(\frac{\text{Mffm}}{\text{I}}\right) = e^{2.63 - 0.28 \ln(I_0) - 0.31 \ln(TAG_0)}$$

where, I_0 – fasting plasma insulin concentration (mIU/l) and TAG_0 – fasting plasma triglycerides concentration (mmol/l).

1.7.2.4. Oral Glucose Insulin Sensitivity (OGIS)

The oral glucose insulin sensitivity (OGIS) is a method for the assessment of insulin sensitivity from the OGTT. OGIS provides an index which is analogous to the index of insulin sensitivity obtained from the HIEC.

This method calculates insulin sensitivity with a model-derived equation of the form:

$$\text{OGIS} = f(G_0, G_{90}, G_{120}, I_0, I_{90}, I_{120}, D_O)$$

where G and I are glucose and insulin concentrations (subscripts represent time instant) and D_O is the oral glucose dose (g/m^2 body surface area).

The function *f* is complex, but can be easily programmed on a spreadsheet (see http://www.isib.cnr.it/bioing/ogis/home.html, where a web-based calculator is also available). The expression of *f* contains some parameters, chosen to maximize the

[21] The bootstrap method is a very general re-sampling procedure for estimating the distributions of statistics based on independent observations. The bootstrap method is shown to be successful in many situations, which is being accepted as an alternative to the asymptotic methods.

agreement with the HIEC. Glucose and insulin can be given in either in common or international units (with appropriate parameters, see table 2 in (Mari *et al.*, 2001)).

OGIS is a predictor of the HIEC insulin sensitivity, expressed as glucose clearance M/G, normalized to body surface area. The units of OGIS are thus ml/min/m^2 of body surface area. OGIS has been validated against an 120mU/min/m^2 insulin infusion HIEC (by direct comparison of the glucose clearance values), instead of the more standard 40mU/min/m^2 used in the previous methods[22]. Formulas for a 3h and 2h OGTT are also available (Mari *et al.*, 2001).

OGIS exploits the known quantitative relationships between the observed data and the HIEC insulin sensitivity to attempt a genuine insulin sensitivity prediction. However, this advantage is limited by the necessity to use empirical assumptions, and to calculate parameters from regression (Mari *et al.*, 2001).

1.7.2.5. Rapid Insulin Sensitivity Test (RIST) Index

The RIST index is the parameter used to evaluate insulin sensitivity that represents the total amount of glucose infused during the Rapid Insulin Sensitivity Test (RIST), ir order to maintain euglycemia after the exogenous bolus administration of insulin. It corresponds to the area under the curve of total glucose infused throughout the test (Lautt *et al.*, 1998a).

$$\text{RIST Index} = \text{AUC of Glucose}$$

where AUC is the area under the curve (mg glucose/kg bw) during the RIST.

[22] OGIS has been validated for a 75g OGTT. Its use with other OGTT doses is possible, though not validated. OGIS requires glucose values at 0, 90, 120min and insulin values at 0 and 90min (2-h OGTT) or glucose values at 0, 120, 180 min and insulin values at 0 and 120min (3-h OGTT) (Mari *et al.*, 2001).

2. HYPOTHESES AND OBJECTIVES

2. HYPOTHESES AND OBJECTIVES

The results presented in this PhD thesis concern mainly the establishment of the Rapid Insulin Sensitivity Test (RIST) in humans. Additionally, I aimed to study the Hepatic Insulin Sensitizing Substance (HISS)-dependent insulin action, a novel neurohumoral regulatory mechanism that controls insulin sensitivity, both in lean and overweight subjects. I also evaluated the involvement of the cholinergic system in HISS action, in humans.

The animal studies intended to evaluate the contribution of the 3',5'-cyclic adenosine 5'-monophosphate (cAMP) on HISS synthesis/release, since the relationship between HISS release and hepatic glutathione (GSH) content, and hepatic cAMP and GSH is well known.

The **hypotheses** tested, in humans, were: 1) the RIST is a sensitive and reproducible test to evaluate insulin sensitivity in humans; 2) the activation of the HISS-dependent mechanism in response to the ingestion of a meal is responsible for the increased insulin sensitivity; 3) the HISS pathway is regulated by a cholinergic mechanism; and 4) the HISS-dependent mechanism is compromised in overweight subjects.

In animals, the general hypothesis tested was that glucagon-dependent activation of the intracellular cAMP pathway leads to a decrease in hepatic GSH content resulting in HISS-dependent insulin resistance (HDIR).

The **first objective** was to establish an insulin sensitivity test capable of evaluating insulin action in both the fasted and fed state, in both in lean and overweight humans, and to further characterize the physiology of regulation of insulin action by hepatic parasympathetic nerves, through the HISS pathway.

The **second objective** was to determine if glucagon affects the intracellular cAMP pathway altering HISS action through hepatic GSH content.

2.1. THE HISS IN HUMANS

The **first specific objective** of this work was to establish, for the first time, the Rapid Insulin Sensitivity Test (RIST) as a standard operating procedure in humans. Since the RIST was well established in rats (Lautt *et al.*, 1998a), the human studies developed were based on the following **hypothesis**: in humans, the RIST is a diagnostic tool to quantify whole body insulin action.

In animals, the HISS mechanism and the details of its regulation were discovered to some extent because development of the RIST, which is simply a transient euglycemic clamp to determine the dynamic glucose uptake response to a bolus of insulin.

The usual means of assessing insulin sensitivity allowed testing only in the fasted state. However, in this condition, HISS release is absent. Taking this into account, the establishment of the RIST could provide a powerful research tool to assess insulin sensitivity both in the fasted and fed state.

The **second specific objective** of this work was to test the concept of postprandial HISS-dependent insulin sensitization in healthy and overweight subjects. Since studies done in rats showed the relationship between HISS release and the prandial state, I postulate the following **hypothesis**: in lean healthy humans, feeding results in meal-induced insulin sensitization (MIS) that is HISS-dependent; overweight subjects have impaired MIS due to a compromise of HISS action.

Animal studies have demonstrated that the HISS release in response to insulin is controlled by the prandial status so that, in the immediate postprandial state HISS release is maximal and decreases with the fast interval (Lautt *et al.*, 2001; Sadri *et al.*, 2006). HISS action in response to insulin is enhanced after a meal and is blocked by interfering with the hepatic parasympathetic nerves, ntric oxide levels and hepatic glutathione content (Guarino *et al.*, 2003; Lautt *et al.*, 2001; Sadri *et al.*, 1998). Animal models of obesity were insulin resistant and had an impairment of HISS action (Afonso *et al.*, 2010; Afonso *et al.*, 2007b; Ribeiro *et al.*, 2005).

The **third specific objective** in the human studies was to determine whether HISS is regulated by a cholinergic mechanism in humans. The following **hypothesis** was tested: in

humans, the HISS-dependent component of insulin action is inhibited by a cholinergic antagonist.

Studies in rats showed that atropine induces the same degree of insulin resistance as seen with hepatic parasympathetic surgical denervation, suggesting that atropine is effective in eliminating the hepatic parasympathetic component of peripheral insulin action (Teff *et al.*, 1999b; Teff *et al.*, 2004; Xie *et al.*, 1996a; Xie *et al.*, 1996b; Xie *et al.*, 1994). This insulin resistance state can be reversed by intraportal (ipv) but not by intravenous (iv) infusion of acetylcholine (ACh). However, the HISS-independent component of insulin action, obtained after blockade of muscarinic receptors with atropine administration, was not affected by feeding or fasting (Xie *et al.*, 1994) and this mechanism was only present in healthy rats.

2.2. THE RELEVANCE OF GLUCAGON ON THE HISS PATHWAY

In healthy subjects, glucagon levels increase in the fasted state and decrease in the immediate postprandial state (Ahren, 2006; Dunning *et al.*, 2007; Fanelli *et al.*, 2006; Young, 2005), opposite to the changes of hepatic GSH, which are increased in the fed state and decreased in the fasted (Tateishi *et al.*, 1974). It has been observed that, in diabetic patients, plasma levels of glucagon are often abnormally high and may contribute to impaired glucose tolerance and other metabolic changes (Butler *et al.*, 1991; Unger, 1978).

In the fasted state and during the immediate postabsorptive state, the hyperglycemia observed in diabetic subjects may be caused not only by glucose underutilization, due to a deficiency or lack of insulin, but also by elevated concentrations of glucagon. These changes suggested that cAMP signaling pathway is upregulated, in insulin resistant subjects (Ahren, 2006; Chen *et al.*, 2004; Dunning *et al.*, 2007; Fanelli *et al.*, 2006; Henkel *et al.*, 2005; Raju *et al.*, 2005; Unger, 1978).

Our group have recently proposed that the regulation of insulin action by the prandial status is dependent on hepatic GSH content (Guarino *et al.*, 2003), which is known to be strongly related to the nutritional status (Tateishi *et al.*, 1977; Tateishi *et al.*, 1974; Taylor *et al.*, 1996). Since hepatic GSH is a key factor for HISS action (Guarino *et al.*, 2006), it was imperative to understand the mechanisms involved in the regulation of hepatic GSH levels. One important regulator of hepatic GSH levels is the cAMP pathway. It has been shown that as cAMP levels increase, GSH synthesis is inhibited (Goss *et al.*, 1994; Higashi *et al.*, 1976; Lu *et al.*, 1990; Lu *et al.*, 1991; Rozwadowski *et al.*, 1995). Lu *et al.* reported that hormones

with effects mediated via cAMP/protein kinase A (PKA), such as glucagon, decrease plasma GSH levels due to an inhibition of the enzyme γ-glutamylcysteine synthase, which has a key role in GSH synthesis (Lu *et al.*, 1991).

The **fourth specific objective** in the rat studies was to determine the hepatic effect of a cAMP analogue on HISS-dependent insulin resistance.

Hepatic GSH levels are controlled by the cAMP/PKA pathway and are apparently essential for HISS release. I therefore propose the following **hypothesis**: in fed rats, HISS-dependent insulin sensitivity is inhibited by a cAMP analog.

The **fifth specific objective** in rat studies was to ascertain the role of glucagon on HISS-dependent insulin action.

Due to the relationship between glucagon and cAMP levels, and hepatic GSH, the proposed **hypothesis** is: glucagon acts via cAMP to decrease hepatic GSH levels, leading to an impairment of HISS synthesis/release resulting in an insulin resistance state responsible for the development of hyperglycemia and type 2 diabetes.

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

The human experiments were developed at the Medicine Unit of the Instituto Português de Oncologia (IPO), Lisboa, Portugal and the animal experiments were performed at the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Canada.

3.1. HUMAN STUDIES

3.1.1. Subjects

Lean healthy (aged 28.5±0.9 years, BMI 23.3±0.4 kg/m^2) and overweight male subjects (aged 24.3±0.9 years, BMI 27.8±0.4 kg/m^2) were admitted as outpatients to the Medicine Unit of IPO, Lisboa, between 9:00 and 9:30am.

Clinical guidelines recommended that body mass index (BMI) be used to identify obesity (National Institutes of Health, 1998). The recommended standard definitions are: healthy lean, a BMI between 18.5 and 24.9 kg/m^2; overweight, a BMI between 25 and 29.9 kg/m^2; and obese, a BMI greater than or equal to 30 kg/m^2.

The recruited subjects had normal systolic and diastolic pressure, normal basal values of glycemia, insulin, C-peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides in both fasted and fed state, according to the recommendations of the American Diabetes Association (ADA) and European Association for the Study of Diabetes (EASD) (Authors/Task Force Members *et al.*, 2007; Revisions, 2009).

None of the subjects had any history or signs of Type 2 diabetes mellitus or any disease requiring treatment at the time of study.

The nature, purpose and potential risks of the study were carefully explained to each subject, who gave informed written consent (in appendix 1). The human study protocol was approved by the Ethics Committee of the IPO, Lisboa, Portugal and conformed to the standards set by the last revision of the Declaration of Helsinki.

3.1.2. Subject Preparation

The study was performed with the subjects resting in a comfortable position and standard electrocardiogram (ECG) was monitored.

All experiments began between 9:30 and 10:00am. An intravenous catheter was inserted in the median cubital vein in each forearm (Figure 3.1). One forearm was heated using a circulating water-controlled heating pad to produce "arterialized" venous blood for sampling representative of arterial blood (Nauck *et al.*, 1992). The intravenous catheter inserted in the median cubital vein of this forearm was used only to collect blood samples. A second intravenous catheter, inserted in the median cubital vein, in the contralateral forearm, was used for the administration of insulin and glucose (to evaluate insulin sensitivity) and for atropine administration.

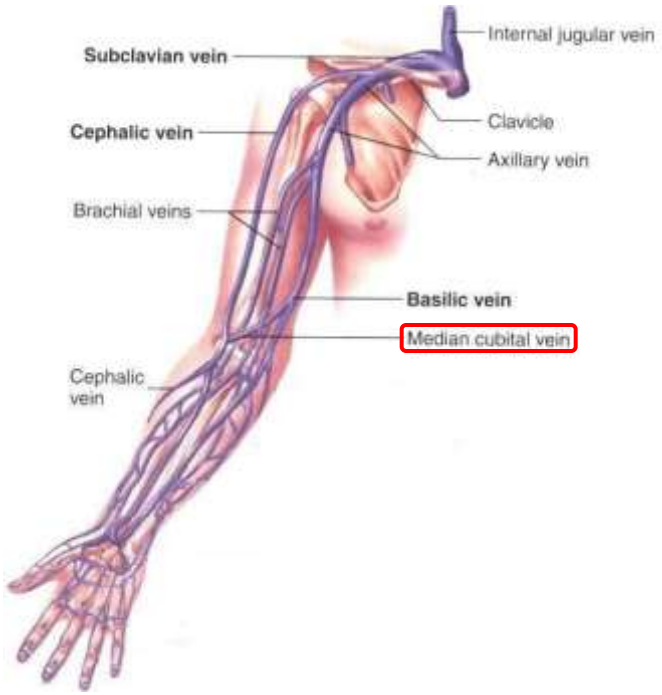


Figure 3.1 - Representation of human veins of the upper limb. Adapted from (Seeley et al., 2003).

3.1.3. Glycemia quantification

The arterial glycemia was analyzed by the oxidase method with a glucose analyzer (1500 YSI SPORT, YSI Inc., USA).

Blood samples were collected from the "arterialized" median cubital vein with a 25μl microsyringe (YSI Model 1501 Syringepet, YSI Inc., USA) and immediately injected into the glucose analyzer.

The Glucose Analyzer

The glucose analyzer 1500 YSI SPORT was developed to quantify glucose levels by the glucose oxidase (GOx) enzymatic method. The probe is fitted with a three-layer membrane containing immobilized GOx in the middle layer. Figure 3.2 shows an exploded view of the membrane and its relationship to the face of the probe.

The face of the probe, covered by the membrane, is situated in a buffer-filled sample chamber into which a sample is injected.

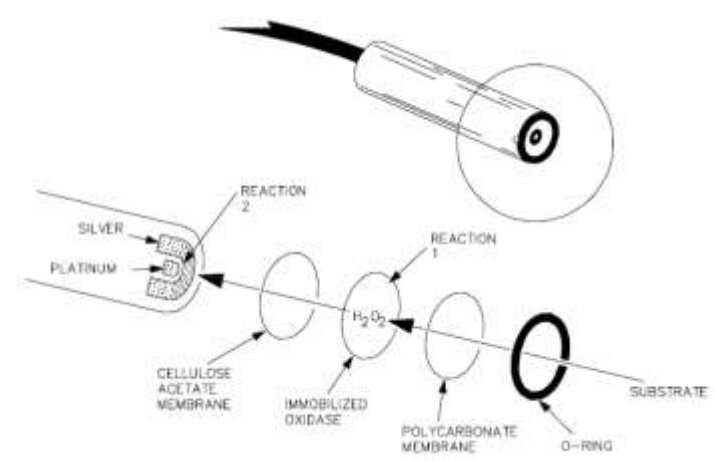


Figure 3.2 - Representation of the glucose analyzer sensor probe and enzyme membrane. The probe contains one membrane with three layers, in which the second layer contains the immobilized glucose oxidase (GOx) enzyme. The method is based on the glucose enzymatic oxidation (Reaction 1) and on the detection of the produced electrons by the formation of hydrogen peroxide. Adapted from Glucose Analyzer YSI 1500 Operations Manual (YSI, 2001).

When the glucose contacts the immobilized enzyme (GOx), it is rapidly oxidized, producing hydrogen peroxide (H_2O_2) (Reaction 1).

$$\beta\text{-D-Glucose} + O_2 \xrightarrow{\textbf{GOx}} \text{Glucono-}\delta\text{-lactone} + H_2O_2 \quad (1)$$

The H_2O_2 is, in turn, oxidized at the platinum anode, producing electrons (Reaction 2).

$$H_2O_2 \xrightarrow{\textbf{Platinum Anode}} 2H^+ + O_2 + 2e^- \quad (2)$$

A dynamic equilibrium is achieved when the rate of H_2O_2 production and the rate at which H_2O_2 leaves the immobilized enzyme layer become constant. The equilibrium is indicated by a steady state response. The electron flow is linearly proportional to the steady state H_2O_2 concentration and, therefore, to the concentration of glucose.

The platinum electrode is held at an anodic potential and is capable of oxidizing many substances other than H_2O_2. To prevent these reducing agents from contributing to sensor current, the membrane contains an inner layer consisting of a very thin film of cellulose acetate. This film readily passes H_2O_2 but excludes chemical compounds with molecular weights above approximately 200Da. The cellulose acetate film also protects the platinum surface from proteins, detergents and other substances that could foul it.

3.1.4. RAPID INSULIN SENSITIVITY TEST (RIST) IN HUMANS

The Rapid Insulin Sensitivity Test (RIST) is a modified euglycemic clamp that quantifies the response to a exogenous bolus of insulin, and the glycemia is kept constant through iv glucose infusion, as previously described by Lautt and colleagues (Lautt *et al.*, 1998a). At the beginning of the experiment (control RIST) and after pharmacological manipulation, the basal glycemia was established and the RIST was performed.

Basal glycemia (Baseline)

Subjects were allowed to stabilize prior to the first blood sampling. Blood samples were collected from the intravenous catheter and glycemia was analyzed by the oxidase method with a glucose analyzer (1500 YSI SPORT, YSI Inc., USA), as previously described. These blood samples were taken at 5min intervals until three successive stable glucose concentrations were obtained. The mean of these three concentrations is referred to as the

basal blood glucose level (baseline), and was used as the euglycemic target to be maintained during the RIST.

The RIST in humans

The RIST is a dynamic euglycemic clamp quantifying the response to an intravenous bolus of insulin (50mU/kg bw), administered over 30sec (t=0min). To maintain euglycemia, a 20% glucose infusion was started at a rate of 3mg glucose/kg/min, 1min after insulin bolus. Blood samples were drawn at t=1min and at 2min intervals for the remainder of the RIST. Based on blood glucose concentration, the infusion rate of glucose was adjusted to clamp the glycemia at baseline values, using an infusion pump (IVAC, Denmark). The RIST was complete when glucose infusion was no longer required to maintain euglycemia.

As previously done in animals (Lautt *et al.*, 1998a), the total amount of glucose infused during the RIST quantifies insulin sensitivity and is referred to as the **RIST Index** (mg glucose/kg bw) and corresponds to the area under the curve of total glucose infusion. The RIST Index is the parameter used to evaluate insulin sensitivity.

3.1.5. QUANTIFICATION OF HISS ACTION

The RIST provides two means of quantitating HISS-dependent insulin action. The first method utilizes the dynamics of the rates of glucose infusion obtained during the RIST. To obtain the mean curves representing the time-course from each test, we plotted the values of glucose infusion rate at 0.1min intervals. The HISS-dependent component curve was obtained by subtracting the fasted curve values from the corresponding fed curve values (Figure 3.3).

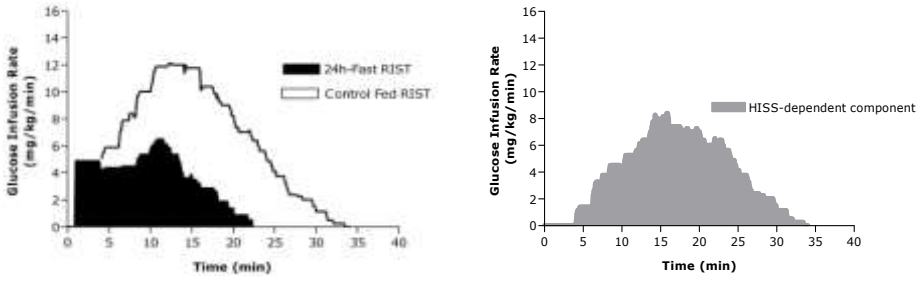


Figure 3.3 – Schematic representation of the typical profile using the dynamic analysis of the pattern of glucose infusion during the Rapid Insulin Sensitivity Test (RIST), in rats. Control fed RIST (white area), 24h-Fast RIST (black area) and HISS-dependent component curve (grey area).

The second method uses the RIST index, that is, the total amount of glucose (mg glucose/kg bw) required to maintain euglycemia after a bolus of insulin (50mU/kg bw).
The RIST index obtained in the fasted state, when HISS release is blocked, represents the HISS-independent component of insulin action. By subtracting the fasted-RIST index from the fed-RIST index, one quantifies the HISS-dependent component of insulin action (Figure 3.4).

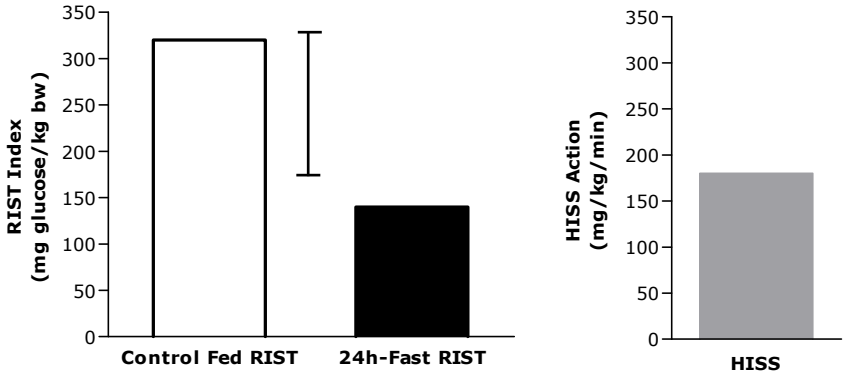


Figure 3.4 - Schematic representation of the typical RIST index obtained in the fed and fasted state, in rats. By subtracting the fasted RIST index from the fed RIST index, the HISS-dependent component of insulin action could be quantified.

3.1.6. Blood Samples

At specific time points (-100, -80, -60, -40, -25, -15, -10, -5, 0, 1, 3, 5, 7, 9, 12, 15, 20, 25, 30, 35, 45, 55 and 70min), when blood was collected for glycemia analysis, plasma and serum were also obtained and stored at -80ºC for determination of insulin, C-peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides concentrations.

The blood was collected by venipuncture into K_3EDTA tubes (BD Vacutainer®, Franklin Lakes, USA) containing EDTA as anticoagulant, to obtain plasma and into a BD SST™ tubes with gel and clot activator (BD Vacutainer®, Franklin Lakes, USA), to obtain serum. The tubes were then centrifuged at 2750*g* for 10 minutes at 0ºC in a Beckman centrifuge (Beckman Coulter Allegra 6R Centrifuge, rotor GH-3.8A).

3.1.7. Human biochemical parameters analysis

The human biochemical parameters analysis was performed using the plasma and serum samples collected at different time points during the experiment. All the samples were evaluated through the fully automated clinical chemistry analyzer (Olympus AU400 Chemistry Analyzer). The principles of the method and the reactions behind each kit for each biochemical parameter analyzed are detailed below.

Lactate

Plasma lactate concentration was measured spectrophotometrically at 505nm using an enzymatic colorimetric kit (bioMérieux, 61192, France) with an Olympus analyzer (Olympus AU400 Chemistry Analyzer, USA).

The principle of the kit is that the lactate present in the plasma sample is determined according to reaction 3:

$$\text{Lactate} + O_2 \xrightarrow{\textbf{Lactate Oxidase}} \text{Pyruvate} + H_2O_2 \quad (3)$$

The H_2O_2 formed is assayed according to a trinder type reaction (Reaction 4):

$$H_2O_2 + \text{4-chlorophenol} + \text{4-aminoantipyrine} \xrightarrow{\textbf{Peroxidase (POD)}} \text{Quinoneimine} + 2H_2O + HCl \quad (4)$$

The intensity of the measured coloration (quinoneimine) is proportional to the quantity of lactate present in the plasma sample.

HDL-Cholesterol

Serum HDL-cholesterol was measured spectrophotometrically at 500nm using an enzymatic colorimetric kit (Olympus, OSR6187, Ireland) with an Olympus analyzer (Olympus AU400 Chemistry Analyzer, USA).

The principle of the kit is that anti-human-β-lipoprotein antibody present in reagent 1 of the kit, binds to lipoproteins other than HDL (LDL, VLDL and chylomicrons) (Reaction 5):

$$\text{LDL, VLDL, Chylomicrons} \xrightarrow{\textbf{Anti-human-β-Lipoprotein antibody}} \text{Antigen–Antibody complexes} \quad (5)$$

The antigen-antibody complexes formed block enzyme reactions, when reagent 2 of the kit is added (Reaction 6). The enzymes responsible for this reaction are cholesterol esterase (CHE) and cholesterol oxidase (CHO), present in the reagent 2 of the kit.

$$\text{HDL-Cholesterol} + H_2O_2 + O_2 \xrightarrow{\textbf{CHE and CHO}} \text{Cholest-4-en-3-one} + \text{Fatty acid} + H_2O_2 \quad (6)$$

HDL-cholesterol is quantified by the presence of an enzyme chromogen system, catalysed by POD (Reaction 7):

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{F-DAOS} \xrightarrow{\textbf{POD}} \text{Blue dye} + 2H_2O \quad (7)$$

LDL-Cholesterol

Serum LDL-cholesterol was measured spectrophotometrically at 500nm using an enzymatic colorimetric kit (Olympus, OSR 6183, Ireland) with an Olympus analyzer (Olympus AU400 Chemistry Analyzer, USA).

The principle of this kit is that a protecting agent, present in the reagent 1 of the kit, protects LDL from enzymatic reactions. All non-LDL lipoproteins (HDL, VLDL, chylomicrons) are broken down by reaction with CHE and CHO (Reaction 8):

$$2\text{LDL-Cholesterol} + 2H_2O_2 + 2O_2 \xrightarrow[\text{Protecting Reagent}]{\textbf{CHE and CHO}} 2\text{Cholest-4-en-3-one} + 2\text{Fatty acid} + 2H_2O_2 \quad (8)$$

The H_2O_2 produced by this reaction is decomposed by catalase, present the in reagent 1 of the kit. When reagent 2 of the kit is added, the protecting reagent is released from LDL and catalase inactivated by sodium azide (Reaction 9):

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{H-DAOS} \xrightarrow{\textbf{POD}} \text{Blue dye} + OH^- + 3H_2O \quad (9)$$

Total Cholesterol

The Olympus Cholesterol reagent utilises an enzymatic colorimetric method to measure total cholesterol in human serum (Olympus, OSR 6116, Ireland) with an Olympus analyzer (Olympus AU400 Chemistry Analyzer, USA).

In this procedure, cholesterol esters in a sample are hydrolysed by CHE (Reaction 10):

$$2\ \text{Cholesterol esters} + 2H_2O \xrightarrow{\textbf{CHE}} 2\ \text{Cholesterol} + 2\ \text{Fatty acid} \quad (10)$$

The free cholesterol produced is oxidised by CHO to cholestene-3-one with the simultaneous production of H_2O_2 (Reaction 11), which oxidatively couples with 4-aminoantipyrine and phenol in the presence of POD to yield a chromophore (Reaction 12):

$$2\ \text{Cholesterol} + 2\ O_2 \xrightarrow{\textbf{CHO}} 2\ \text{Cholestene-3-one} + 2H_2O_2 \quad (11)$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{Phenol} \xrightarrow{\textbf{POD}} \text{Quinoneimine} + 4H_2O \quad (12)$$

The red quinoneimine dye formed can be measured spectrophotometrically at 540/600nm as an increase in absorbance.

Triglycerides

Serum triglyceride was measured spectrophotometrically at 500nm using an enzymatic colorimetric kit (Olympus, OSR 6133, Ireland) with an Olympus analyzer (Olympus AU400 Chemistry Analyzer, USA).

This procedure is based on a series of coupled enzymatic reactions. The triglycerides in the sample are hydrolysed by a combination of microbial lipases to give glycerol and fatty acids (Reaction 13):

$$\text{Triglyceride} + 3H_2O \xrightarrow{\textbf{Lipase}} \text{Glycerol} + 3\ \text{Fatty acid} \quad (13)$$

The glycerol is phosphorylated by adenosine triphosphate (ATP) in the presence of glycerol kinase (GK) to produce glycerol-3-phosphate (Reaction 14):

$$\text{Glycerol} + \text{ATP} \xrightarrow{\textbf{GK}, \textbf{Mg}^{2+}} \text{Glycerol-3-phosphate} + \text{ADP} \quad (14)$$

The glycerol-3-phosphate is oxidised by molecular oxygen in the presence of glycerol phosphate oxidase (GPO) to produce H_2O_2 and dihydroxyacetone phosphate (Reaction 15):

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\textbf{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2 \quad (15)$$

The H_2O_2 is used to oxidatively couple *p*-chlorophenol and 4-aminoantipyrine catalysed by POD to give a red dye with an absorbance maximum at 500nm (Reaction 16):

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{4-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + HCl + 2H_2O \quad (16)$$

The increase in absorbance at 520/600nm is proportional to the triglyceride content of the serum sample.

Insulin

Plasma insulin concentration was measured by a radioimmunoradiometric assay (IRMA) kit (Biosource, INS-Irma KIP1251, Belgium). This assay is based on coated-tube separation and monoclonal antibodies (Mabs) Mab1, the capture antibodies, are attached to the lower and inner surface of the plastic tube. Calibrators or samples added to the tubes will at first show low affinity for Mabs1. Addition of Mab2, the signal antibody labelled with ^{125}I, will complete the system and trigger the immunological reaction. After washing, the remaining radioactivity bound to the tube reflects the antigen concentration. The insulin antibody used is the human-specific antibody with no cross-reactivity to human proinsulin.

The plasma samples (50µl), the kit controls (50µl) and the calibrators (50µl) were added to the coated tubes and incubated for 2h at room temperature. The content of each tube was aspirated. The tubes were washed with 2ml of working wash solution (diluted TRIS-HCl). The content of each tube was again aspirated, washed with 2ml of wash solution (concentrated TRIS-HCl) and decanted. After the final washing, the tubes were stand upright for 2min and the remaining liquid was dropped. Lastly, the tubes were counted in a gamma counter (DPC Gamma Counter, USA) for 1min and the insulin concentration was calculated based on the calibration curve plotted.

C-Peptide

Plasma C-Peptide concentration was measured by the BioSource C-PEP II-RIA-CT kit, which is a radioimmunoassay for the in vitro quantitative measurement of

human C-Peptide in plasma (Biosource, C-Pep II-RIA-CT KIP0409, Belgium). A fixed amount of ^{125}I labelled Tyr-C-Peptide competes with the C-Peptide to be measured present in the plasma sample or in the calibrator for a fixed amount of antibody sites being immobilized to the wall of a polystyrene tube. After 3h incubation at room temperature, an aspiration step terminates the competition reaction. The tubes are then washed with 3ml of

wash solution and aspirated again. A calibration curve is plotted and the C-Peptide concentrations of the samples are determined by dose interpolation from the calibration curve.

The plasma samples (100µl), the kit controls (100µl) and the calibrators (100µl) were added to the coated tubes. 50µl of ^{125}I labeled Tyr-C-Peptide was dispensed into each tube. Tubes were shaked gently to liberate any trapped air bubbles and then incubated for 3h at room temperature. The content of each tube was aspirated. The tubes were washed with 3ml of working wash solution (diluted TRIS-HCl). The content of each tube was again aspirated. After the washing, the tubes were place upright for 2min and the remaining liquid was dropped. Lastly, the tubes were counted in a gamma counter (DPC Gamma Counter, USA) for 1min and the C-peptide concentration was calculated based on the calibration curve plotted.

3.2. ANIMALS STUDIES

The animal experiments were performed at the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Canada.

3.2.1. Sprague-Dawley rats

Male Sprague-Dawley rats weighing 319.4±7.6g (9-weeks old) from Charles River, St. Constant, Quebec, Canada were maintained in the animal house under controlled conditions (22±1ºC) on a 12h light/dark cycle. Rats had *ad libitum* access to standard rat chow diet (Prolab RMH 3000 5P00, Labdiet, USA) and with free access to normal tap water, for one week to adapt to the housing environment.

Animals were treated according to the guidelines of the Canadian Council on Animal Care (CCAC), and the ethics committee on animal care at the University of Manitoba approved all protocols.

The animals were kept anesthetised during the experiment and at the end of the protocols they were euthanized with a lethal injection of sodium pentobarbital in accordance with the guidelines of the CCAC.

3.2.2. Pre-surgical protocol and anesthesia

All rats were fasted for 8h, and then allowed *ad libitum* access to food for 2h immediately before starting the surgical preparation. As HISS is only released in the fed state, the fasting-refeeding protocol assured a high level of HISS release in response to insulin (Lautt *et al.*, 2001). All the experiments started at 8:00am.

The methodology used was described by Lautt (Lautt *et al.*, 1998a). The animals were weighed and anesthetized with an intraperitoneal (ip) injection of sodium pentobarbital (65mg/kg) (Somnotol®, Biomeda-MTC Animal Health Inc., Cambridge, Ontario).

The sodium pentobarbital (2mg/ml) was maintained throughout the experiment by continuous infusion (0.5ml/h/100g bw) into the jugular vein, using a polyethylene catheter (PE50, Intramedic®, Beckton and Dickinson, USA) connected to an infusion pump (Genie, Kent Scientific Corporation, Litchfield, Massachusetts). After induction, anesthesia was tested regularly during the experiment using the tail pinch and the eyelid reflex. When necessary, intravenous (iv) supplements of pentobarbital sodium (65mg/kg) were administered, through the injection of the anesthetic by puncturing the silicone sleeve on the venous side of the arterial-venous loop (Section 3.2.3.3., Figure 3.5).

Sodium pentobarbital was the selected anesthetic since it did not affect blood pressure significantly, nor insulin action (Lautt *et al.*, 1998a). It had a small effect on autonomic nervous system activity (Best *et al.*, 1984; Taborsky *et al.*, 1984) and splanchnic hemodynamics (Kvietys *et al.*, 1982), thus minimizing the introduction of biased results on insulin sensitivity evaluation (Penicaud *et al.*, 1987). Comparing conscious animals and animals anesthetized with sodium pentobarbital, there were no changes regarding to glucose metabolism or in the plasma concentrations of glucose, insulin, glucagon and norepinephrine, as well as on their metabolic actions, since the temperature of the anesthetized animal was maintained approximately at 37ºC (Lang *et al.*, 1987; Latour *et al.*, 2002c; Saha *et al.*, 2005). The effects of sodium pentobarbital in the nutrient absorption were not significant when compared with other anesthetics (Yuasa *et al.*, 1993) and there were no differences on its effects from the fasted to the fed state (Saha *et al.*, 2005).

3.2.3. Surgical protocol

The surgical procedures were adapted from those described by Lautt and colleagues (Lautt *et al.*, 1998a).

In the surgical procedure, all rats received a tracheotomy followed by a femoral artery-femoral vein arterial-venous loop, left internal jugular vein cannulation and portal vein cannulation.

In order to perform the surgical procedure, all the surgery was performed with surgical amplifier glasses (Optivisor DA-7, Donegan Optical Company, USA) and a cold light illuminator (KL750 Schott, USA).

During all the surgical procedures, body temperature was monitored with a rectal probe and kept at 37.0±0.5°C, by means of a heated surgical table (Harvard Apparatus, Kent, England) and overhead lamp.

3.2.3.1. Arterial-venous loop

The arterial–venous loop or arterial-venous shunt, as shown in Figure 3.5, is a vascular shunt where the blood flows between the catheterized artery and vein. The arterial-venous loop allows multiple rapid arterial sampling, iv administration of drugs and solutions, and allows the measurement of mean arterial and venous pressure (Section 3.2.5.). Continuous monitoring of loop pressure identifies arterial or venous loop occlusions due to clotting or mechanical effects.

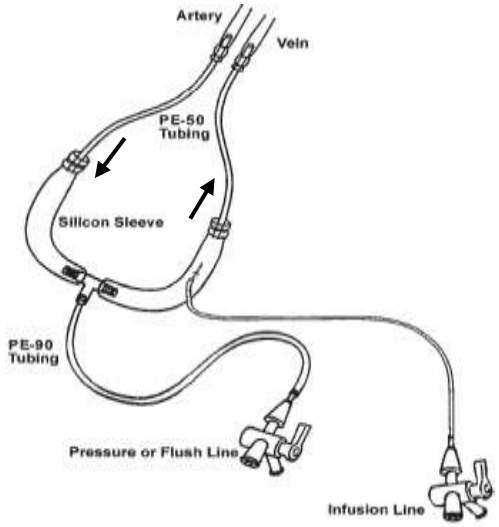


Figure 3.5 - Arterial–venous loop. The blood flows into the loop from the artery into the vein, by a difference in pressure. The loop allows the sampling of arterial blood samples and intravenous administration of drugs by needle puncture into the silicone sleeve, as well as continuous monitoring of loop pressure. Brief occlusion of the venous outflow allows for monitoring arterial pressure. Adapted from (Lautt *et al.*, 1998a).

The arterial-venous circuit consists of one-piece of silicone with 10cm of length to act as a sleeve (MasterFlex Platinum®, Cole Parmer, USA) connected to two pieces of polyethylene tubing PE50, with 12cm in length each (Intramedic®, Beckton and Dickinson, USA), acting as vascular catheters. The two pieces of PE50 tubing are connected to the silicone sleeve with a short reducer of polyethylene tubing, with 4mm in length (Tygon Micro–Bore®, .04ID/.07OD, Cole Parmer, USA).

The silicone sleeve is divided into two equal parts, one corresponding to the arterial side and the other to the venous side. The two parts of the silicone sleeve are attached to a polyethylene T-shaped connector (Raccordo T 4mm, Kartell Labware Division, Italy). The third opening of the connector is attached to a piece of polyethylene tubing PE90 with approximately 20cm long (Intramedic®, Beckton and Dickinson, USA), which is then connected to a pressure transducer for the recording of the loop pressure which, when the silicone tubing toward the venous side of the circuit was closed by clamping, represented the systemic arterial blood pressure (Section 3.2.5.).

Arterial blood continuously flows through the circuit into the venous side. Arterial blood samples can be taken from the loop via puncture of the silicone sleeve.

The arterial-venous loop was prepared before the beginning of the experiment, having been previously primed with a heparin solution (200UI/ml).

3.2.3.2. Tracheotomy

The trachea was cannulated and the anesthetized animals were allowed to breathe spontaneously during all the experiment. It was performed an incision in the anterior wall of the trachea, below the isthmus of the thyroid (Figure 3.6) and the polyethylene tubing (PE240, Intramedic®, Beckton and Dickinson, USA) of 2-2.5cm in length was inserted.

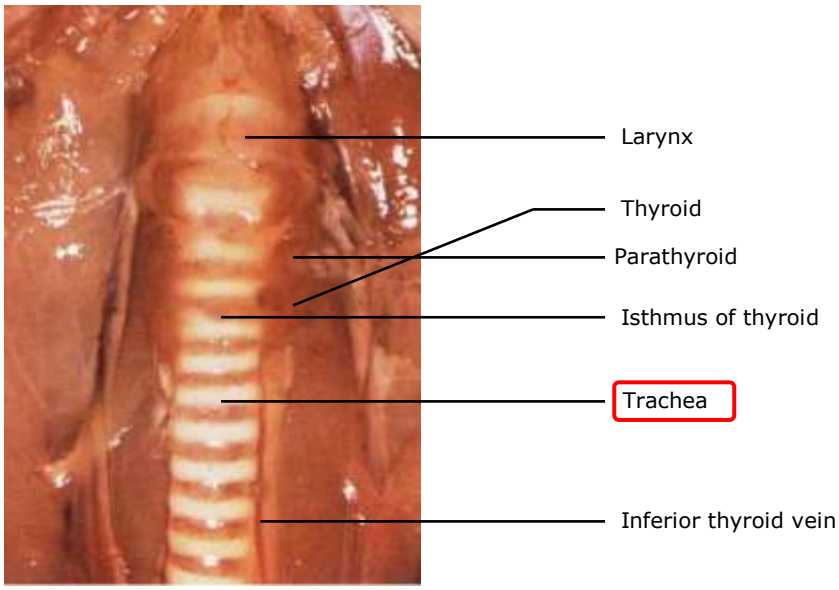


Figure 3.6 – Trachea. The tracheotomy is performed below the isthmus of the thyroid. Adapted from (Olds *et al.*, 1991).

3.2.3.3. Femoral artery and femoral vein cannulation

In order to cannulate the left femoral artery and femoral vein, we performed a small incision at the left rat groin and then the vessels were isolated (Figure 3.7). After the isolation procedure, first the femoral artery and then the femoral vein were cannulated, with the insertion of the polyethylene tubing PE50, connected to arterial-venous loop as described above (Section 3.2.3.1.).

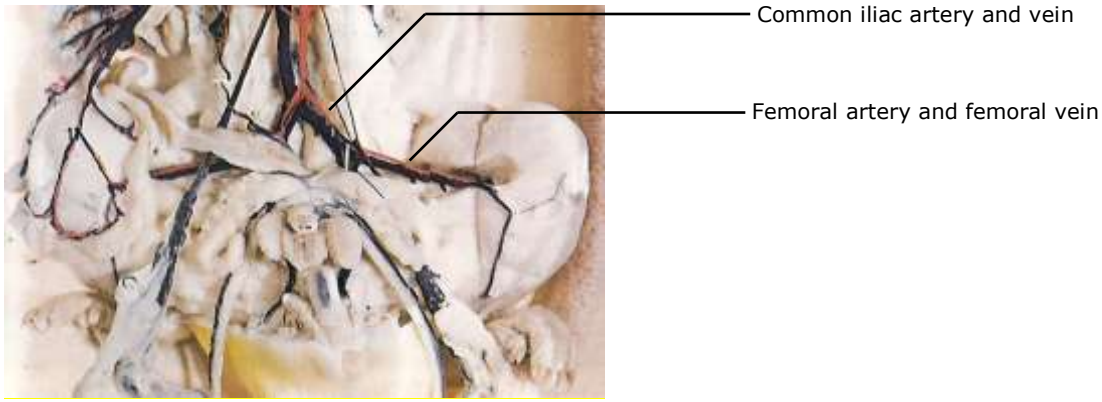


Figure 3.7 – Localization of the femoral artery and femoral vein. Adapted from (Olds *et al.*, 1991).

3.2.3.4. Jugular vein cannulation

In order to cannulate the left internal jugular vein, we performed a small skin incision above the clavical and to the left of midline and then the vein was isolated (Figure 3.8). Following, the vein was cannulated with polyethylene tubing PE50. A short length of silicone sleeve tubing intersects the polyethylene tubing and allows the infusion of drugs directly into the vein, by needle puncture into the silicone sleeve.

The catheter is attached to an infusion pump (Genie, Kent Scientific Corporation, Litchfield, Massachusetts), to maintain anesthesia throughout the experiment by a continuous infusion of sodium pentobarbital and in the small portion of the silicone sleeve, the glucose solution during the insulin sensitivity test is infused, in order to do not contaminate arterial glycemia measured at the arterial-venous loop.

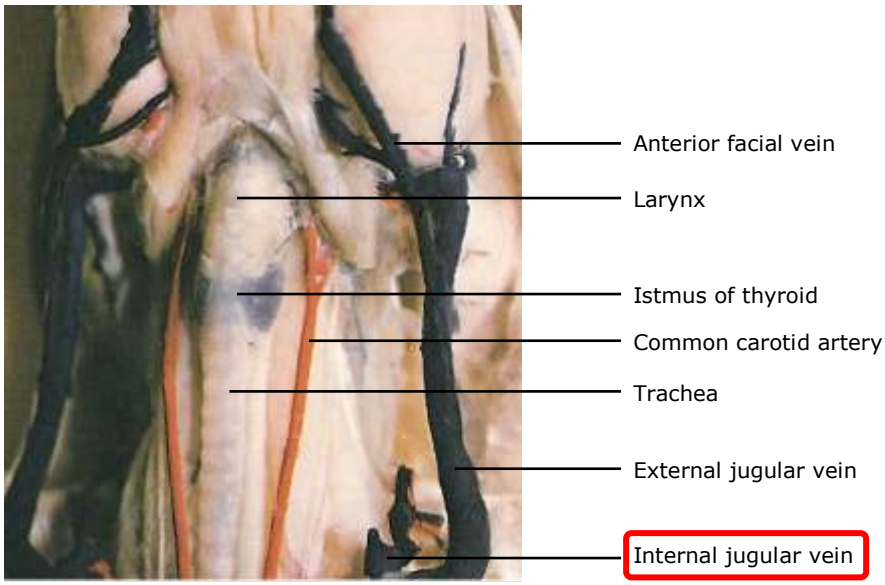


Figure 3.8 – Internal jugular vein and some of the major blood vessels of the rat neck. Adapted from (Olds *et al.*, 1991).

3.2.3.5. Portal vein cannulation

The portal vein (Figure 3.9) was catheterized to administer drugs into the portal circulation and directly to the liver.

After laparotomy, the portal vein was isolated. An intravenous polyurethane catheter (24G Optiva, iv catheter radiopaque ocrilon polyurethane, 19mm, Johnson & Johnson Medical Inc., Arlington, TX) was placed into the portal vein, between the splenic vein and the

cecal vein, to allow ipv infusions of pharmacological agents. The catheter was glued into place with a small drop of tissue adhesive (GluStich, Canada). Attached to the intravenous catheter was a connecting catheter from which ipv infusions were done. The connecting catheter was polyethylene tubing PE50 with a male luer slip end connector attached to the delivery end. This connector was obtained by cutting it from either a stopcock or from a 1ml syringe. The male luer slip end connector was glued to the polyethylene tubing such that the polyethylene tubing extends to the very tip of the male luer slip, thus allowing for low dead volume. A short length of silicone sleeve tubing intersects the polyethylene tubing and allows for direct ipv infusions, by needle puncture into the silicone sleeve. Prior to ipv infusions, a small amount of blood was drawn through the catheter in order to check the effectiveness of the line. The abdominal incision was covered with damp surgical gauze and a covering of Parafilm®.

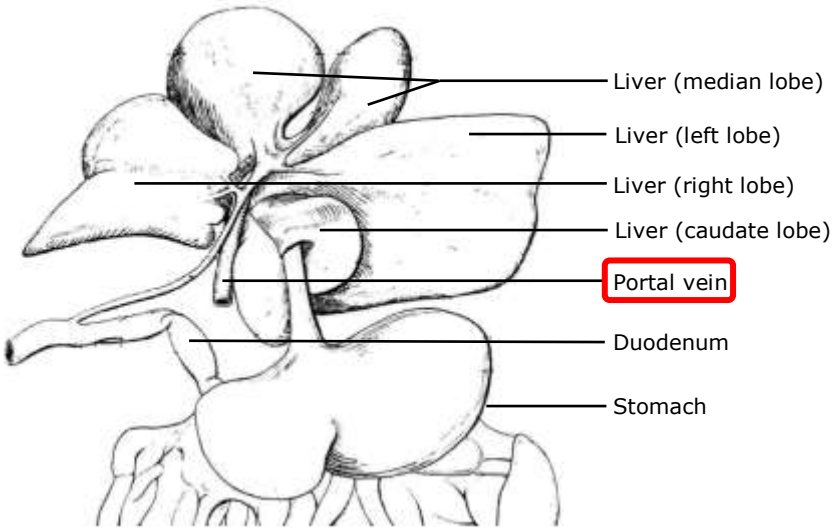


Figure 3.9 – Schematic representation of the liver and the portal vein. The portal vein cannulation allows ipv infusions of pharmacological agents directly into the liver. Adapted from (Greene, 1963).

3.2.4. Post-surgical protocol

Immediately after the arterial-venous loop insertion and jugular vein cannulation, the supplemental sodium pentobarbital infusion was started (into the jugular vein), in order to keep the animal under anesthesia during all the experimental period, as previously described. Once in a while, 0.01-0.02ml of heparin (200UI/ml) was administered to the animals to prevent the clot formation. Following completion of surgery and before doing any type of procedure, the animal was allowed at least a 30min stabilization period.

Body temperature was monitored with a rectal probe and kept at 37.0±0.5°C during all the experiment by means of a heated surgical table (Harvard Apparatus, Kent, England) and overhead lamp.

3.2.5. Blood pressure monitorization

After the insertion of the arterial-venous loop, a polyethylene tube (PE 90) from the T-shaped connector which attaches the silicone sleeve and is designed to monitoring the loop pressure, was joined to a pressure transducer (National Instruments LabView, Austin, USA). A data acquisition system (National Instruments LabView, Austin, USA) combined with application software was used to record and analyze the mean arterial blood pressure.

The pressure measured by the transducer and recorded by the data acquisition system is the mean systolic pressure. The measurement of the mean arterial pressure was performed by reading the pressure indicated during a brief clamping of the silicone sleeve on the venous side of the circuit. The measurement of the mean venous pressure was done by occlusion of the arterial side of circuit. Circuit loop pressure functions as an indicator of circuit resistance and flow. In case of obstruction of the circuit, for example by clot formation, a marked variation of the mean systolic pressure would be observed. If a problem with the loop patency occurs in the circuit, the loop can be cleared and flushed through the side branch connector.

During the insulin sensitivity test, the mean blood pressure was continuously monitored and the blood flow through the arterial-venous loop was checked.

3.2.6. Sampling of arterial blood

Arterial blood samples were collected by puncturing the silicone sleeve on the arterial side of the arterial-venous loop. To avoid hemolysis of the samples, the samples were collected very slowly.

The blood sampling was performed with a 25μl automatic microsyringe (YSI Model 1501 Syringepet, YSI Inc., USA) and the sample was immediately injected into the glucose analyzer. The quantification of arterial glycemia was performed by the oxidase method with a glucose analyzer (1500 YSI SPORT, YSI Inc., USA), as previously described in section 3.1.3.. This method provides a rapid and efficient quantification of arterial glycemia.

3.2.7. Rapid Insulin Sensitivity Test (RIST) in animals

The Rapid Insulin Sensitivity Test (RIST) was the selected method to quantify whole-body insulin sensitivity in animals.

Basal glycemia (Baseline)

After the stabilization period, the baseline glucose levels were determined by samples taken at 5min intervals and continued until 3 successive stable determinations were made. The mean of these three data points was used as the ideal euglycemic baseline to be maintained during the euglycemic clamp, and was used as the euglycemic target.

The RIST in animals

As mentioned earlier, the RIST measures the animal's hypoglycemic response to the exogenous insulin administration and glycemia is kept constant by iv variable infusion of glucose.

After determination of basal glycemia, insulin (Novolin® ge Toronto) 50mU/kg bw infusion is commenced using an infusion pump (Genie, Kent Scientific Corporation, Litchfield, Massachusetts) to administer the dose over 5min (in 0.5ml saline at 0.1ml/min). The beginning of the insulin infusion was considered time zero (t=0min). After 1min of insulin infusion, the first glucose sample is determined, and glucose infusion (100mg/ml, iv) is commenced at the rate of 2.5mg/kg/min with an infusion pump (Genie, Kent Scientific Corporation, Litchfield, Massachusetts). Arterial glucose levels were sampled at 2min intervals throughout the test period with glucose infusion rates adjusted to maintain animal's glycemia near to the baseline value established before starting the RIST. The RIST was considered finished when the blood glucose levels remained near to the baseline without any further glucose infusion. The time line for the RIST is shown in Figure 3.10.

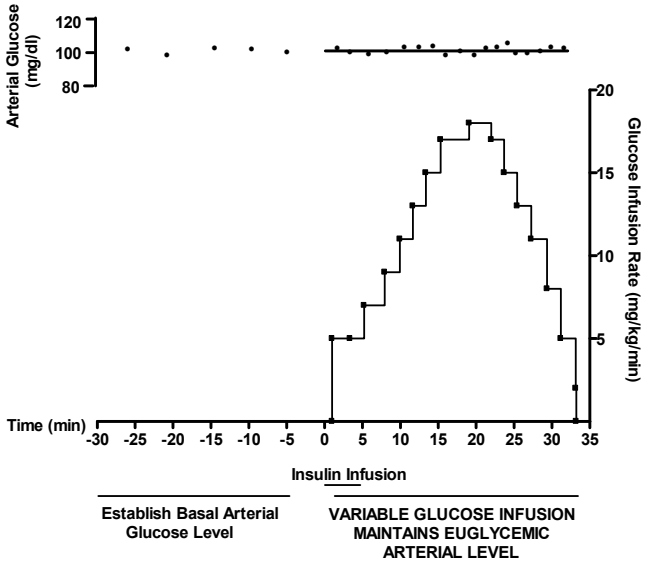


Figure 3.10 – Rat typical profile of the Rapid Insulin Sensitivity Test (RIST). Three stable arterial glucose levels determined at 5min intervals established the ideal euglycemic baseline. Intravenous insulin infusion (50mU/kg bw) is administered over 5min with the glucose infusion and first arterial glucose sample beginning 1min after of insulin infusion. A variable iv glucose infusion is adjusted to maintain euglycemia based on arterial samples taken at 2min intervals throughout the test period. The RIST index is the total amount of glucose infused to maintain euglycemia over the test period, which is terminated when no further glucose infusion is required. From (Lautt *et al.*, 1998a).

The total amount of glucose infused during the RIST quantifies insulin sensitivity and is referred to as the **RIST Index** (mg glucose/kg bw) and corresponds to the area under the curve of total glucose infusion. The RIST Index is the parameter used to evaluate insulin sensitivity.

After each RIST, the animal was allowed to stabilize approximately 30min prior to the following manipulation. Following each intervention or pharmacological manipulation, and to determine their effects on insulin sensitivity, a new RIST was performed, as soon as a new arterial baseline value was reached.

A data acquisition system (National Instruments LabView, Austin, USA) combined with application software was used to record and analyze the mean arterial blood pressure, to calculate the RIST index, and to provide real-time monitoring of adherence to the euglycemic baseline. The software program calculated accuracy and precision of maintenance of the euglycemia target baseline. If either deviated by more than 5%, the entire RIST was determined to be invalid and was discard.

3.2.8. Drug administration

Pharmacological agents were administered intravenously by puncturing the silicone sleeve on the venous side of the arterial-venous loop and intraportally directly into the portal vein. The administration of the drugs into the portal vein was done to directly deliver to the liver, the organ of HISS synthesis/release.

The intraportal infusion rate of drugs was chosen based on preliminary studies done with saline; the continuous infusion at 0.0167ml/min infusion rate and the bolus infusion at 0.04ml/min did not induce either metabolic or hemodynamic alterations. The systemic infusion rate of 0.1ml/min did not change arterial glycemia or mean arterial pressure (MAP).

From the beginning of iv and ipv infusions, experiments have a maximum duration of 3h. The average amount of anesthetic, glucose, insulin and drugs infused in the animals was about 1ml/100g bw/h, which is in accordance with the recommendations to compensate for water loss by surgical intervention and for blood sampling (between 1 and 1.5ml/100g bw/h) (Akerstrom *et al.*, 1989; Diehl *et al.*, 2001; Van Zutphen, 2001).

All the infusions (systemic (iv) and intraportal (ipv)) were performed with Genie pumps from Kent Scientific Corporation, Litchfield, Massachusetts.

3.2.9. Animal biochemical parameters analysis

Blood samples were collected before and after each RIST, for insulin analysis. For the plasma insulin quantification, the blood (30μl) was collected into a 0.5ml eppendorf tube, centrifuged at 10000rpm (5585*g*) (Costar Mini Centrifuge, USA) for 5min at room temperature and the supernatant was placed in another labeled eppendorf and stored at –30°C freezer.

Plasma insulin quantification

The plasma insulin levels were analyzed using the insulin assay kit Mercodia Ultrasensitive Rat Insulin ELISA (Enzyme-Linked Immuno-Sorbent Assay), which is a quantitative and colorimetric method for determination of insulin in rat plasma (Rat Ultrasensitive Insulin ELISA, Mercodia Inc., Sweden).

This method is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the sample

reacts with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitration wells. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine (TMB). The reaction is stopped by adding H_2SO_4 to give a colorimetric endpoint that is read spectrophotometrically at 450nm. The determination of the insulin concentration in the plasma sample was performed using a calibration curve using standard solutions of insulin supplied with the kit. This method has a sensitivity of 0.13μg/l.

On the day of the plasma insulin analysis, 5μl of plasma sample were added to anti-insulin wells (96-well coated plate with mouse monoclonal anti-insulin). 50μl of enzyme conjugate (peroxidase conjugated mouse monoclonal anti-insulin) were added to each well and the plate was incubated at room temperature (18-25ºC) for 2h on a plate shaker at 700-900 cycles per minute in an orbital movement. The plate was washed 6 times with an automatic washer (Multiwash Plus, TriContinent, USA) and 350μl of wash buffer were added to each well. The content of each well was aspirated completely. The previous procedure was repeated 5 times and after the final wash, the plate was inverted and tapped firmly against absorbent paper. 200μl of TMB were added to each well and the plate was again incubated at room temperature (18-25ºC) for 30min on a plate shaker at 700-900 cycles per minute in an orbital movement. The reaction was stopped adding 50μl of stop solution (0.5M H_2SO_4) to each well and the plate was placed on a shaker for approximately 5sec to ensure mixing of substrate and stop solution. Finally, the plate's absorbance was measured at 450nm in a 96 well plate reader with 450nm filter (Biotek, Synergy™ HT, Biotek® Instruments Inc., USA) and the insulin concentration in each well was calculated using the Gen5, Version 1.01.14 software.

3.3. EXPERIMENTAL PROTOCOLS

3.3.1. Human studies

In all human protocols, the body weight, height and mean arterial pressure (MAP) of the subjects were determined. The study was done with the subjects resting in a comfortable position and a standard electrocardiogram (ECG) was monitored.

After a **24h** (chapter 4, 5 and 6) or **14h** (chapter 4 and 5) fasting period, blood samples were collected at -10, -5 and 0min before starting the fasted RIST or starting feeding the standardized test meal (described below), and blood glucose, plasma insulin, plasma C-peptide, plasma lactate, serum HDL-cholesterol, serum LDL-cholesterol, serum total

cholesterol and serum triglycerides were measured, as previously described (section 3.1.7.). These values were referred to as basal levels prior to the fasted RIST (Figure 3.11) or feeding the standardized test meal (Figure 3.12).

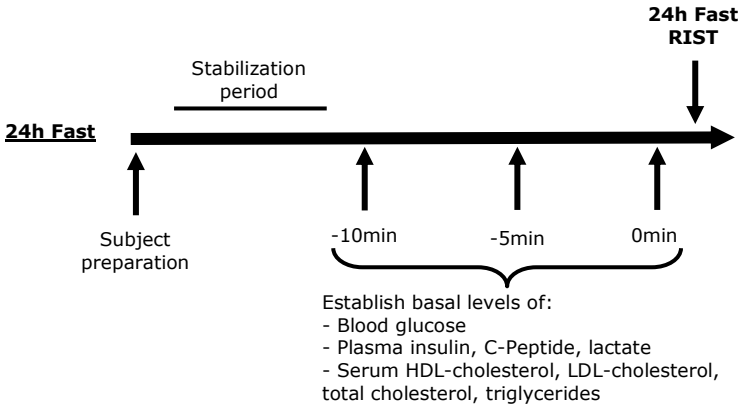


Figure 3.11 – Schematic representation of the experimental protocol before starting the 24h fast RIST.

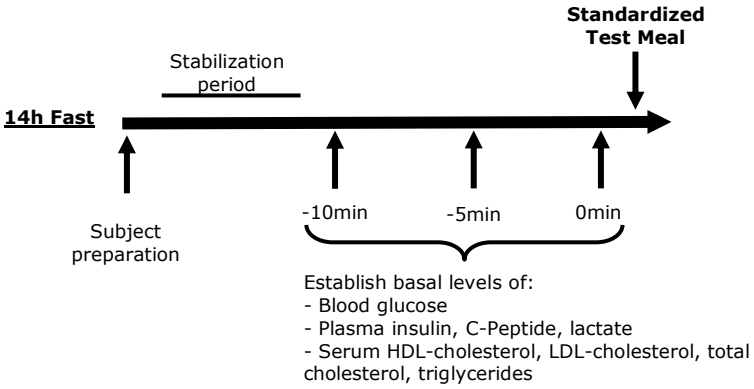


Figure 3.12 – Schematic representation of the experimental protocol before feeding the standardized test meal.

Standardized test meal

The subjects when tested in the postprandial state fed a standardized test meal (16 Proalimentar® cookies, caloric content ≈917kJ) and drank 500ml of mineral water. Table III shows the nutritional composition of the standardized test meal.

Table III – Composition of the standardized test meal according to the supplier (Proalimentar®, Portugal).

Standardized test meal composition	
Proteins	4.0g
Carbohydrates	35.5g
Saturated Lipids	6.8g
Fibers	2.6g
Sodium	0.35g

3.3.1.1. Evaluation of the dynamic response to insulin in the fed state

The experimental protocol described below was performed in lean healthy subjects and the results obtained are presented in Chapter 4.

On the day before the study (14h prior to the beginning of the test), lean healthy male subjects were fed a regular dinner.

On the day of the experiment, after 14h fasted glycemic baseline was achieved, subjects were fed the standardized test meal (described in Section 3.3.1.) and drank 500ml of mineral water.

Blood samples were collected at specific time points during 100min after the standardized test meal, to quantify blood glucose and the biochemical parameters previously described (Section 3.1.7.). After this period of time, and after three successive stable glucose concentrations were obtained, the RIST was performed in the fed state (Figure 3.13).

Each subject was tested on two different days in the same prandial state (fed state).

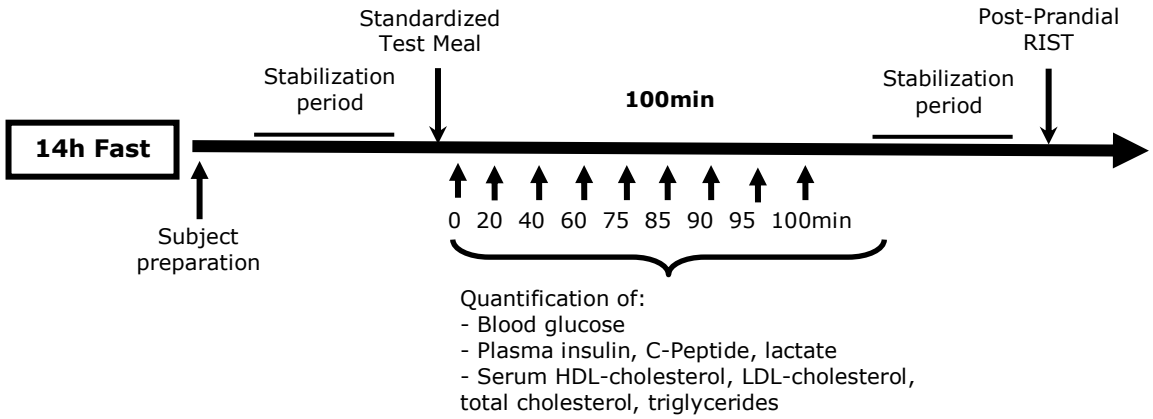


Figure 3.13 – Schematic representation of the experimental protocol for the evaluation of the dynamic response to insulin in the fed state, in lean healthy subjects.

3.3.1.2. Evaluation of the dynamic response to insulin in the fasted and fed states

The experimental protocol described below was performed in lean healthy and overweight subjects and the results obtained are presented in Chapter 4, 5 and 6.

In this protocol, 24h prior to the beginning of the test, lean healthy and overweight male subjects were fed a regular breakfast (ham and cheese sandwich, orange juice and croissant with cheese or ham, ≈2720kJ caloric content).

On the day of the experiment, after 24h fasted glycemic baseline was achieved, the RIST was performed in the fasted state. After the 24h fast RIST, subjects were fed a standardized test meal (described in Section 3.3.1.) and drank 500ml of mineral water.

Blood samples were collected at specific time points during 100min after the standardized test meal, to quantify blood glucose and the biochemical parameters previously described (Section 3.1.7.). After this period of time, and after three successive stable glucose concentrations were obtained, a second RIST was performed in the postprandial state (Figure 3.14).

Each subject was tested on the same day, and in two different nutritional states.

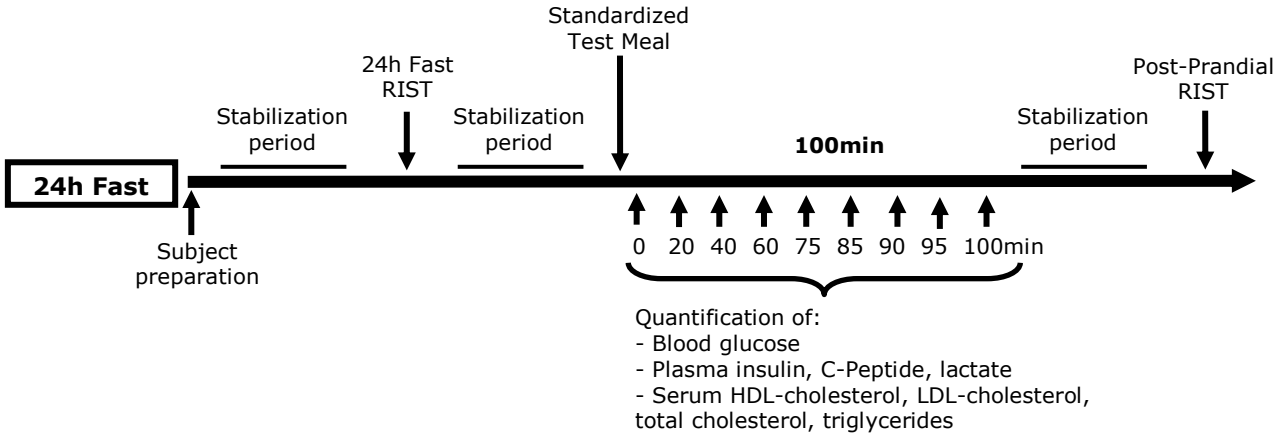


Figure 3.14 – Schematic representation of the experimental protocol for the evaluation of the dynamic response to insulin in the 24h fast and fed state, in lean healthy and overweight subjects.

3.3.1.3. Effect of atropine administration on insulin sensitivity, in lean healthy subjects

The experimental protocol described below was performed in lean healthy subjects and the results obtained are presented in Chapter 5.

On the day before the study (14h prior to the beginning of the test), lean healthy male subjects were fed a regular dinner.

On the day of the experiment, after 14h fasted glycemic baseline was achieved, subjects were fed the standardized test meal (described in Section 3.3.1.) and drank 500ml of mineral water.

Blood samples were collected at specific time points during 100min after the standardized test meal, to quantify blood glucose and the biochemical parameters previously described (Section 3.1.7.). After this period of time, and after three successive stable glucose concentrations were obtained, the RIST was performed in the fed state (Figure 3.15).

Each subject was tested on two different days in a double-blinded protocol. Intravenous infusions over 10min of either atropine (0.5 or 0.75mg) (low therapeutic dose with minor side effects (Brown *et al.*, 1996)) or saline (control group) were administrated 50min after feeding the meal and 50min before starting the fed RIST (Figure 3.15).

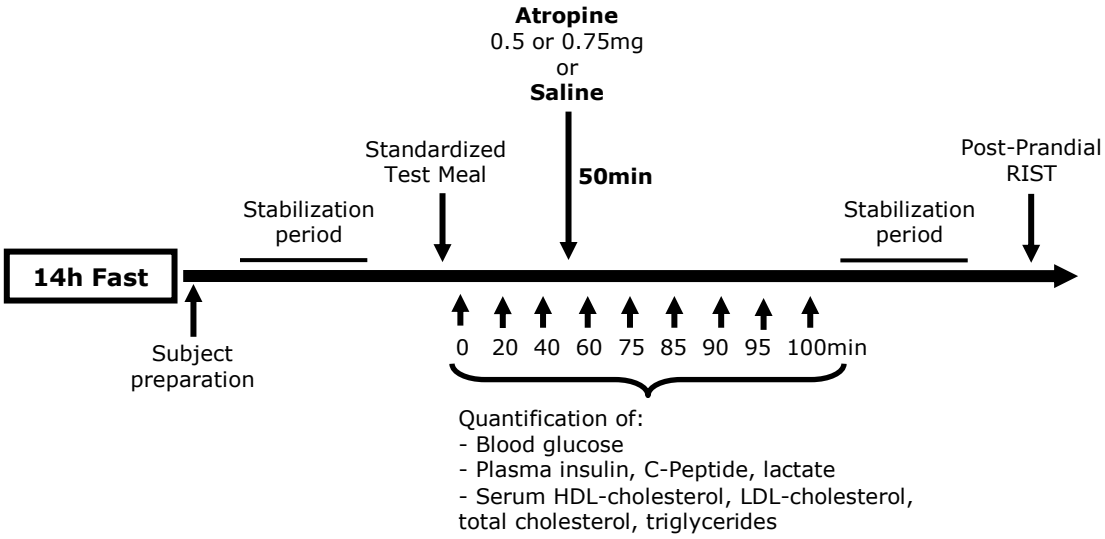


Figure 3.15 – Schematic representation of the experimental protocol for the evaluation of insulin sensitivity after HISS blockade with atropine 0.5 or 0.75mg, or saline, in lean healthy subjects.

3.3.2. Animal Studies

In all animal protocols, male Sprague-Dawley rats weighing 319.4±7.6g (9-weeks old) were used. The animals were fasted for 8h, and then allowed *ad libitum* access to food for 2h immediately before starting the surgical preparation. All the rats were tested under sodium pentobarbital anesthesia.

The surgical procedures were performed as described in section 3.2.3. The RIST was the method used to assess the insulin sensitivity (Section 3.2.7.). The plasma insulin quantifications were performed as previously described in section 3.2.9.

The results obtained with the experimental protocols described below are presented in chapter 7.

3.3.2.1. Hepatic effect of DBcAMP, a cAMP analogue, on insulin sensitivity

After the stabilization period, the baseline glucose levels in the fed state were determined and the first blood sample was taken for insulin analysis. The first control fed RIST was performed. After completing the first RIST, a new glycemic baseline was established and DBcAMP (N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate), a cAMP analog, was infused ipv at different doses ranging from 0.01 to 1.0mg/kg, for 10min at an infusion rate of 0.04ml/min (Cervin *et al.*, 1995). A minimum of a 30min stabilization period was allowed until establishing a new stable glycemic baseline. The second blood sample, after DBcAMP ipv infusion, was taken for insulin quantification. A second RIST was carried out after DBcAMP ipv infusion. Finally, the third blood sample was taken for insulin analysis (Figure 3.16).

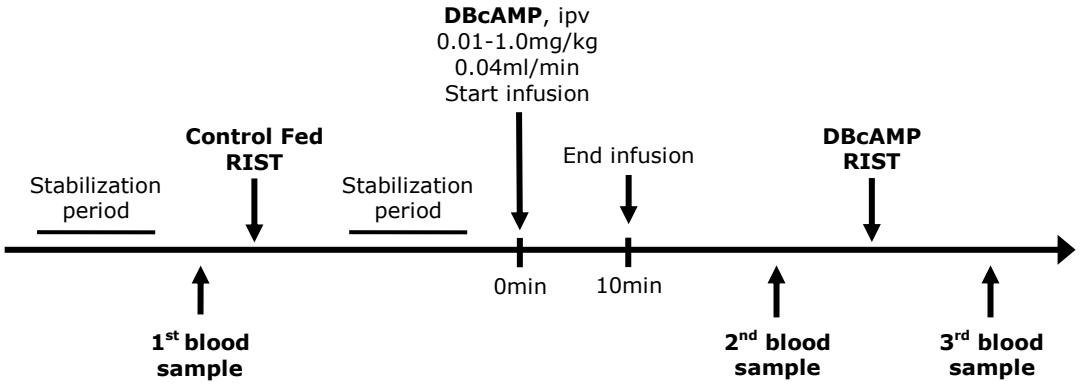


Figure 3.16 – Schematic representation of the experimental protocol for the evaluation of the hepatic effect of different doses of DBcAMP on insulin sensitivity.

3.3.2.2. Hepatic effect of glucagon, on HISS-dependent insulin sensitivity

This protocol was divided into 2 different series. In the first series, after the stabilization period, the baseline glucose levels in the fed state were determined. A control RIST was performed in the fed state. After completing the first RIST, a new glycemic baseline was established and glucagon was infused ipv at different doses ranging from 0.5ng/kg to 20μg/kg, for 10min at an infusion rate of 0.04ml/min (Carrillo *et al.*, 1995). A minimum of a 30min stabilization period was allowed until establishing a new stable glycemic baseline. A second RIST was carried out after glucagon ipv infusion (Figure 3.17).

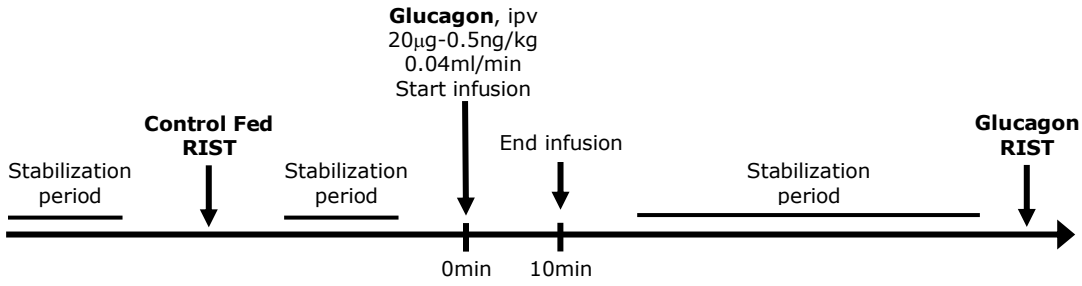


Figure 3.17– Schematic representation of the experimental protocol for the evaluation of the hepatic effect of different doses of glucagon on insulin sensitivity.

In the **second series** of this protocol, after the stabilization period, the baseline glucose levels in the fed state were determined. Then, a control fed RIST was performed. After completing the first RIST, a new glycemic baseline was established and N-monomethyl-L-arginine (L-NMMA), a selective NOS inhibitor, was infused ipv at a dose of 0.73mg/kg for 10min at an infusion rate of 0.04ml/min. The administration of L-NMMA was used to block the HISS pathway (Sadri *et al.*, 1999). A minimum of a 30min stabilization period was allowed until establishing a new stable glycemic baseline. A second RIST was carried out after L-NMMA ipv infusion and a new glycemic baseline was established and then glucagon was infused ipv at 200ng/kg (the dose that produces a maximal inhibition on insulin sensitivity without affecting basal glycemia) for 10min at an infusion rate of 0.04ml/min. A minimum of a 30min stabilization period was allowed until establishing a new stable glycemic baseline and a third RIST was carried out after glucagon ipv infusion (Figure 3.18).

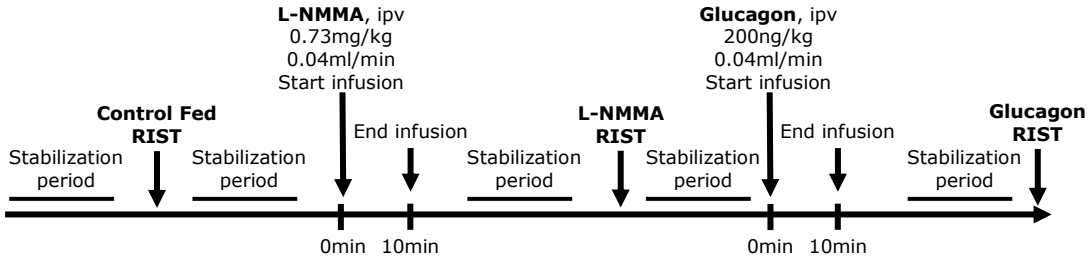


Figure 3.18 – Schematic representation of the experimental protocol for the evaluation of the hepatic effect of glucagon 200ng/kg on HISS-dependent insulin sensitivity.

3.4. STATISTICAL ANALYSIS

The data presented during this dissertation are expressed as means ± SEM of the mean (SEM). As applicable, the significance of the difference between mean values was calculated through Student's *t* tests, paired or unpaired (two-tailed), as the experimental design was, respectively, paired or unpaired and analysis of variance (ANOVA), one-way ANOVA or repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test.

RIST dynamic profiles data were analyzed using repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test in each group. The curves representing the dynamic profile of the RIST were obtained from glucose infusion rates in 0.1min intervals.

Area under the curve (AUC) was calculated for glucose, insulin and C-peptide with a computerized trapezoidal method (GraphPad Software).

Differences were accepted as statistically significant at $p<0.05$. Whenever p value is not indicated, differences are not statistically significant.

The *GraphPad Prism* version 4.0 (GraphPad Software Inc., USA) was the software utilized to elaborate all the graphs and to perform all the statistical analysis.

3.5. REAGENTS AND SOLUTIONS

Human insulin (Humulin® Regular) was obtained from Lilly, Lisbon, Portugal. Heparin, atropine, glucose and saline were purchased from BBraun, Lisbon, Portugal. All drugs were dissolved in saline (BBraun, Portugal).

Sodium pentobarbital (Somnotol®) was obtained from Biomeda-MTC Animal Health Inc., Cambridge, Ontario. Human insulin (Novolin® ge Toronto) was purchased from Novo Nordisk (Mississauga, ON, Canada). Heparin was purchased from Pharmaceutical Partners of Canada, Richmond Hill, Ontario and saline from Baxter Corporation, Toronto, Ontario, Canada.

D-Glucose, L-NMMA, DBcAMP and glucagon were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tissue adhesive was acquired from GluStich Inc., Canada.

All chemicals were of the highest degree of purity on the market. All the solutions for *in vivo* administration were prepared in NaCl 0.9%.

4. THE RAPID INSULIN SENSITIVITY TEST (RIST) IN HUMANS

4. THE RAPID INSULIN SENSITIVITY TEST (RIST) IN HUMANS

4.1. INTRODUCTION AND AIMS

Insulin sensitivity is of central relevance in many areas of clinical medicine and research. A number of techniques to determine insulin sensitivity have been proposed, including the oral glucose tolerance test (OGTT), the hyperinsulinemic-euglycemic glucose clamp method (HIEC) and the insulin tolerance test (ITT) (Soop *et al.*, 2000).

Since its introduction into clinical investigation two decades ago, the HIEC has been established as the "gold standard" for the measurement of insulin sensitivity at the whole body level (Natali *et al.*, 2000). Because HIEC is dependent on steady-state conditions, insulin infusion is continuous for ≈3h and the subjects are kept in the fasted state. The results of the HIEC may be limited by these restraints because physiological insulin release is pulsatile (Juhl *et al.*, 2002; Porksen *et al.*, 1996), and prolonged insulin infusion causes alterations in the autonomic nervous system (Van De Borne *et al.*, 1999).

In the ITT, an intravenous bolus of insulin is administered and insulin sensitivity is measured by the rate of decline of plasma glucose concentration (Hirst *et al.*, 1993). Steeper slopes indicate greater insulin sensitivity. The advantages of the ITT include its simplicity, rapidity, use of a bolus injection of insulin, and the ability to measure insulin sensitivity in the fed or fasting state. Furthermore, because glucose tolerance after a meal is dependent on insulin sensitivity, measuring insulin sensitivity in the prandial state is physiologically relevant. The bolus injection of insulin mimics the physiological pulsatile release of insulin (Hansen *et al.*, 1982). However, it does not allow rapid sequential testing; hypoglycemia and the activation of counter-regulatory hormones are undesired effects of the ITT (Young *et al.*, 1996).

In glucose tolerance tests, an oral glucose load is administered, and glucose and insulin levels are measured. It is important to note that glucose tolerance differs from insulin sensitivity. Glucose tolerance is measured by the rate of glucose disappearance from the blood. Insulin sensitivity involves the measurement of both glucose and insulin kinetics in response to a glucose load. In OGTT, concomitant hyperinsulinemia and normal or impaired glucose tolerance following a glucose load indicate insulin resistance (Bergman *et al.*, 1985). As the other methods described above, it does not allow more than one test per day in a single subject.

Another method to quantify the insulin sensitivity is the Rapid Insulin Sensitivity Test (RIST). It has been only described in the literature in laboratory animals, such as rats, cats and mice (Latour *et al.*, 2002a; Lautt *et al.*, 1998a; Xie *et al.*, 1996c). The RIST has been developed to evaluate the dynamic response to insulin in both the fasted and fed states. The RIST index is the amount of glucose disposed following a bolus administration of insulin, as determined using a euglycemic clamp methodology. This transient euglycemic clamp can be repeated several times in the same animal and is sufficiently sensitive to allow dose-response relationships to be established for the stimulatory effect of insulin (Lautt *et al.*, 2001) and the inhibitory effect of atropine (Takayama *et al.*, 2000). The RIST was developed in order to avoid the hypoglycemia and the activation of counter-regulatory hormones caused by the ITT. The RIST is a rapidly sampled euglycemic clamp in response to a pulse of insulin (Lautt *et al.*, 1998a).

The current paradigm for insulin resistance focuses on peripheral defects in insulin signaling with the majority of studies being carried out in the fasted state. While there can be no question that diabetes imparts an enormous risk factor for the development of several diseases, the continued focus on the fasting state appears misdirected. The importance of the post-meal, rather than the fasting, metabolic status is amply demonstrated in a number of studies (Ceriello *et al.*, 2008b; Lautt, 2004; Leiter *et al.*, 2005; Monnier *et al.*, 2007).

The aim of the study described in this chapter was to develop the RIST as a standard operating procedure in humans, as a new method to assess insulin action *in vivo*, both in the fasted and fed state. This was based on the same procedure that was used in laboratory animals, but with some changes in order to adapt the same methodology to humans. There is not a gold standard test for evaluation of glucose homeostasis in the postprandial state, where major glucose excursions are deleterious. The need to develop a new test became emergent and was the first aim of the present thesis.

4.2. PROTOCOLS

4.2.1. Evaluation of the RIST reproducibility on 2 different days, in lean healthy subjects

The RIST was the method used to evaluate insulin sensitivity (Chapter 3, Section 3.1.4.).

For the evaluation of the RIST reproducibility, each lean healthy subject was tested on two different days in the fed state.

After a 14h fasting period, the subject was fed a standardized test mixed-meal and 100min after the meal, the RIST was performed in the fed state. The detailed experimental protocol is described in chapter 3, section 3.3.1 and 3.3.1.1.

4.2.2. Assessment of the RIST implementation, in lean healthy subjects

In order to implement the RIST in humans, each lean healthy subject was tested on the same day in the fasted state and after ingesting the previously described test meal.

In brief, after a 24h fasting period, the RIST was performed in the fasted state. After, the subject fed a standardized test meal and 100min after the meal, the RIST was performed in the fed state. The detailed experimental protocol is described in chapter 3, section 3.3.1 and 3.3.1.2.

4.3. RESULTS

The aims of the study presented in this chapter were to test the Rapid Insulin Sensitivity Test (RIST) reproducibility and implement the RIST in human subjects, revealing the possibility to perform the test, in different prandial states, and more than one test in the same day.

To evaluate the RIST reproducibility, the same subject was tested in the fed state on two different days. However, to implement the RIST in humans, in the same day, the volunteer was tested in the fasted state and after feeding a standardized test meal (fed state).

Lean healthy male subjects (aged 27.2±2.3 years, BMI 22.5±0.9 kg/m^2, n=6) admitted into this protocol had normal systolic (119.3±5.6mmHg, on day 1 and 114.0±4.0mmHg, on day 2) and diastolic blood pressure (65.7±2.9mmHg, on day 1 and 59.0±3.9mmHg, on day 2).

Basal fasting glucose levels measured on day 1 and day 2 were 81.0±3.4 and 74.5±2.7mg/dl, respectively, and basal glucose levels measured on day 1 and day 2 100min after the meal were 106.0±6.3 and 101.1±2.5mg/dl, respectively.

Basal glycemia, insulin and C-peptide levels increased with feeding, which is consistent with the ingestion and absorption of food. The other biochemical parameters did not change from the fasted to the fed state, even between the 2 different days.

Table IV shows the fasting and postprandial basal levels of the biochemical parameters analyzed, measured in 2 different days, in the lean healthy subjects.

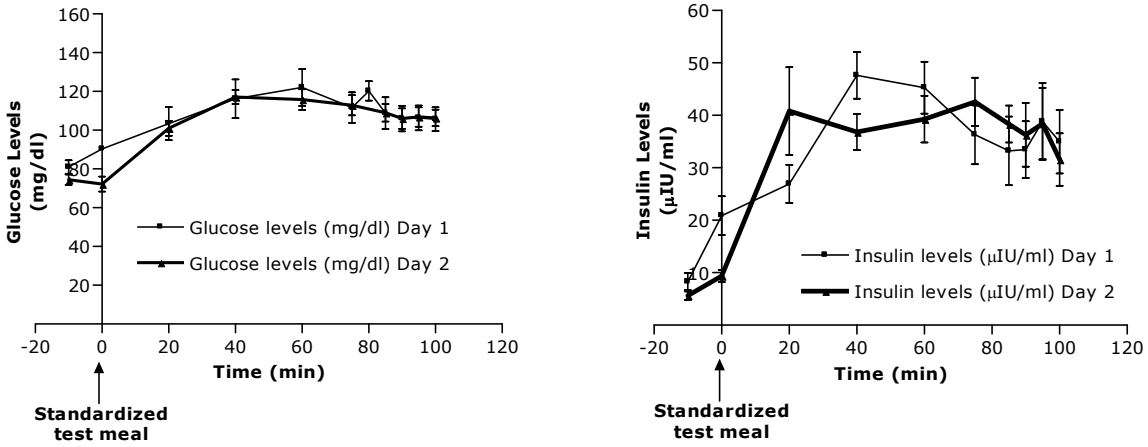
Table IV – Fasting and postprandial basal values of glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides of the lean healthy subjects, on day 1 and day 2 (n=6). Values are means±SEM. Unpaired *t*-test between day 1 and day 2.

Prandial State	Glycemia (mg/dl)	Insulin (μIU/ml)	C-Peptide (ng/ml)	Lactate (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Fasted Day 1 (n=6)	81.0±3.4	8.3±1.6	1.8±0.2	10.2±0.2	52.4±2.4	65.6±5.8	155.9±4.1	40.5±6.0
Fed Day 1 (n=6)	106.0±6.4	36.1±6.6	7.6±0.9	9.9±0.8	48.5±2.6	68.9±6.9	143.4±4.8	42.3±4.9
Fasted Day 2 (n=6)	74.5±2.7	5.6±0.8	1.7±0.3	8.2±0.5	48.7±3.9	84.5±7.0	148.7±10.1	31.7±2.7
Fed Day 2 (n=6)	101.1±2.5	35.4±5.8	8.5±1.1	9.4±0.7	45.6±4.3	78.0±7.7	138.6±12.5	31.9±5.6

4.3.1. Evaluation of the RIST reproducibility on 2 different days, in lean healthy subjects

Biochemical profiles after feeding the standardized test meal, on day 1 and day 2

After subject's stabilization period and in regard to test the reproducibility of the RIST in the fed state, we first evaluated and compared the profiles of the biochemical parameters (glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides) obtained after ingestion of the standardized test meal, on day 1 and day 2 (Figure 4.1).



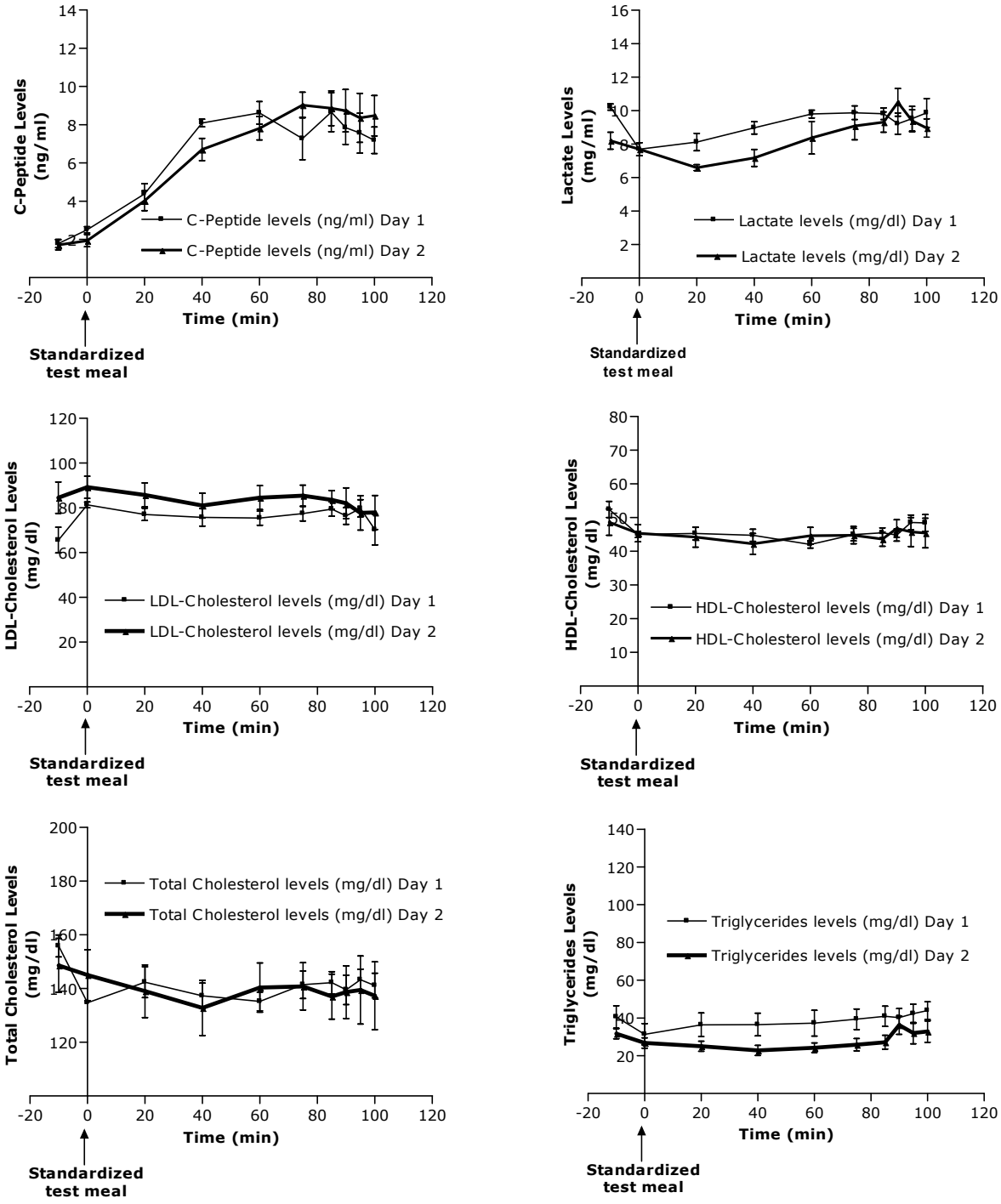


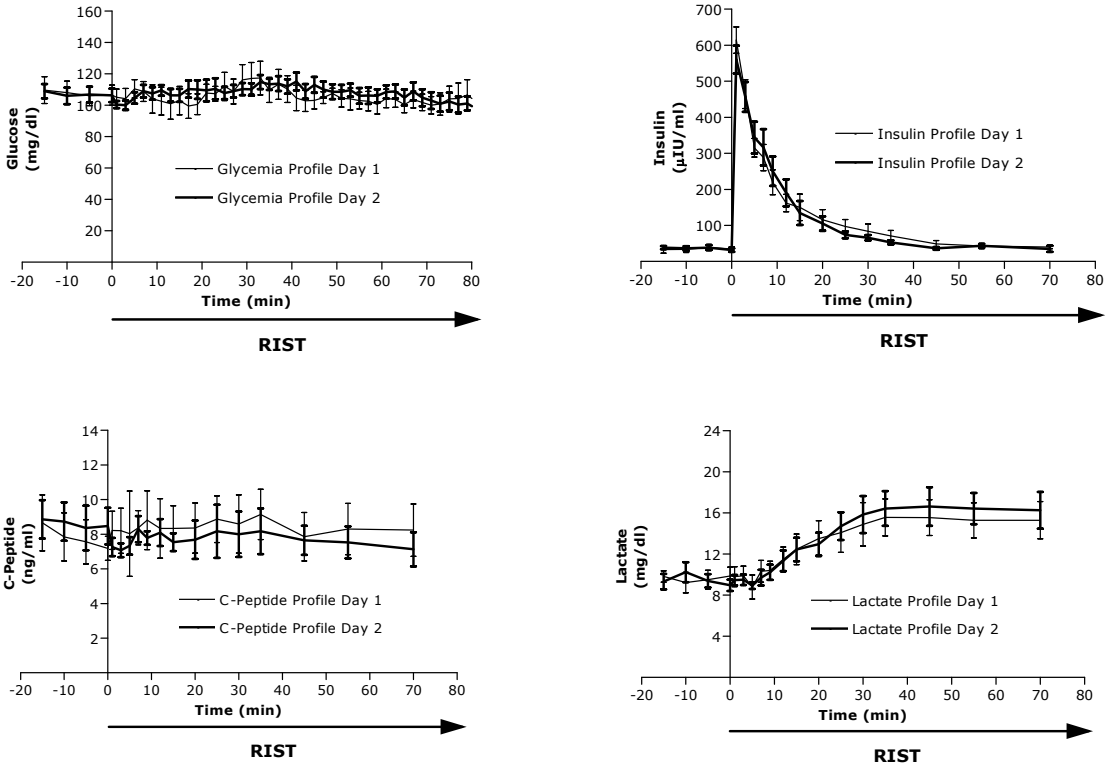
Figure 4.1 – Biochemical parameters (glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides) profiles measured at specific time points during 100min after ingestion of the standardized test meal (0min) and before performing the RIST in the fed state (100min), for the evaluation of the RIST reproducibility, in lean healthy subjects (n=6). Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

As shown in Figure 4.1, the biochemical parameters profiles after ingestion of the standardized test meal were not statistically different when evaluated in the same subjects, on 2 different days.

Comparison of the biochemical profiles during the postprandial RIST, on day 1 and day 2

The evaluation of the biochemical profiles during the RIST, on day 1 and day 2, is essential to test the reproducibility of the RIST in the fed state.

The RIST is an euglycemic clamp, therefore euglycemia after the exogenous bolus of insulin was maintained throughout the test, through a variable rate of exogenous glucose infusion. The insulin levels, after the exogenous insulin bolus, reached a peak that returned to the baseline value afterwards. The C-peptide levels were maintained during the fed RIST. The lactate levels showed a slight increase during the postprandial RIST. The HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides profile was also preserved during the fed RIST (Figure 4.2).



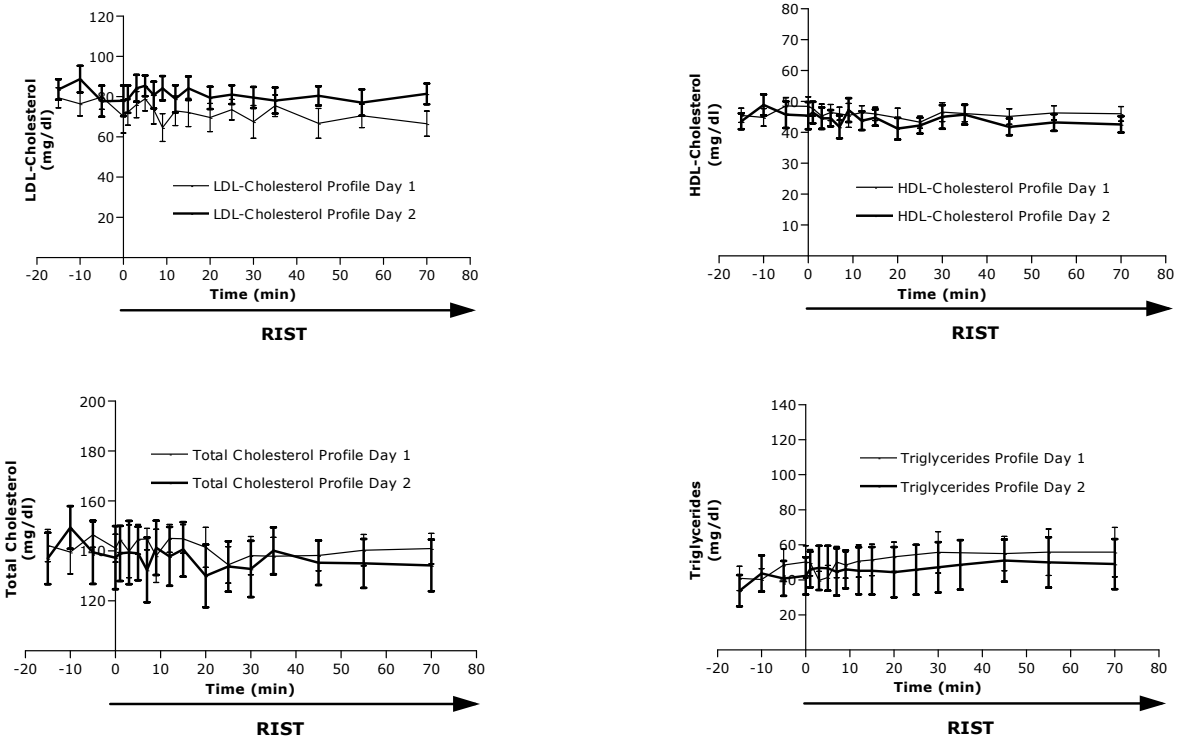


Figure 4.2 – Biochemical parameters (glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL cholesterol, total cholesterol and triglycerides) profiles measured at specific time points during the RIST in the fed state, on day 1 and day 2, in lean healthy subjects (n=6). Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

Variability of the dynamic profiles and postprandial insulin action of the RIST, on day 1 and day 2

Since the usual means of assessing insulin sensitivity required testing in the fasted state, it becomes very valuable to develop a new method to estimate the insulin sensitivity in the fed state.

The glucose intake profile during the RIST can be estimated from the rate of glucose infusion required to maintain the euglycemia during the RIST. The quantification of the total amount of glucose (mg glucose/kg bw) required to maintain euglycemia after a bolus of insulin (50mU/kg bw), that corresponds to the area under the curve, and is called RIST index.

Below, on Figure 4.3, are represented the mean dynamic curves of postprandial RIST for lean healthy subjects in day 1 and day 2. Additionally, the mean characteristics of the dynamic curves (action peak magnitude, peak time and action curve duration) are shown on Table V.

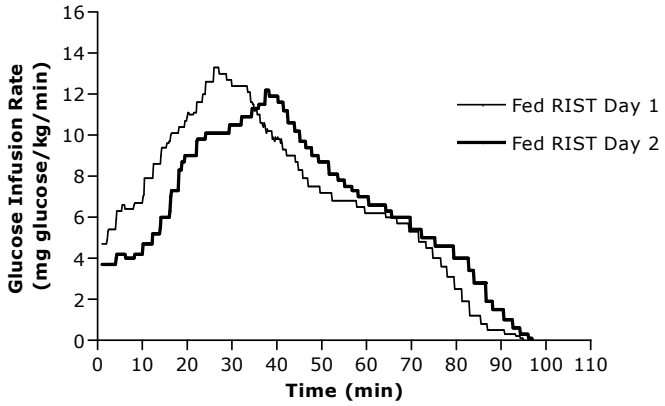


Figure 4.3 – Comparison of the RIST profiles on the fed state in day 1 and day 2. Mean profile using the dynamic analysis of the pattern of glucose infusion during the Rapid Insulin Sensitivity Test (RIST). The mean RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test. Fed RIST Day 1 (simple line) and the RIST obtained in the fed state on day 2 (bold line), n=6.

As shown in Figure 4.3, the dynamic profiles for the postprandial RIST in day 1 and day 2 were practically superimposed, which shows that the RIST in the postprandial state can be reproduced. This allows us to proceed with pharmacological manipulation, as it will be shown in chapter 5.

Table V - Comparison of the RIST dynamic curve main properties on the fed state in day 1 and day 2, n=6. Values are means±SEM. Paired t-test.

Fed RIST Day 1	Peak (mg glucose/kg/min)	13.3±0.6
	Peak time (min)	25.9±1.6
	Duration (min)	94.9 ± 6.8
Fed RIST Day 2	Peak (mg glucose/kg/min)	12.2±1.1
	Peak time (min)	37.5±7.5
	Duration (min)	97.0±4.7

The results aforementioned can be further analyzed by calculating the area under each dynamic curve. That parameter is called RIST index, and the RIST indexes obtained from the fed day 1 and fed day 2 dynamic curves, are presented bellow, on Figure 4.4. The RIST index obtained in the fed state, on both day 1 and day 2, showed similar values (700.3±74.6 vs 672.6±62.1mg glucose/kg bw).

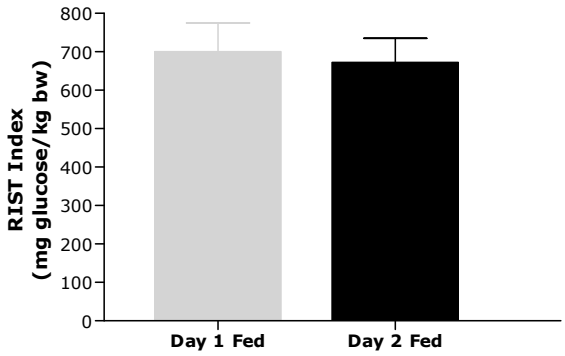


Figure 4.4 - Each volunteer was submitted to the RIST, but at 2 different days, in the fed state. The results obtained showed that there is a variability which is negligible between days, indicating that the RIST in the fed state is reproducible, in lean healthy humans, n=6. Results are means±SEM. Paired t-test.

4.3.2. Assessment of the RIST implementation, in lean healthy subjects

Since the reproducibility of the RIST in the postprandial state was appraised, the subsequent step was to implement the RIST as a means of demonstrating the dynamic action of insulin and meal-induced insulin sensitization (MIS). The subjects were analyzed in the 24h fasted and postprandial states, in the same day.

Lean healthy male subjects (aged 27.7±1.7 years, BMI 24.6±0.4 kg/m^2, n=3) admitted into this protocol had normal systolic (119.7±4.7mmHg) and diastolic blood pressure (65.3±4.7mmHg).

Basal fasting glucose levels were 75.3±1.8mg/dl and glucose levels measured 100min after the meal were 91.0±1.0mg/dl ($p<0.05$).

Table VI shows the fasting and postprandial levels of the biochemical parameters analyzed, in lean healthy subjects.

Table VI - Basal values of glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides of the lean healthy subjects, in the fasted and fed state (n=3). Values are means±SEM. *=p<0.05 and **=p<0.01 between fasted and fed state. Paired *t*-test.

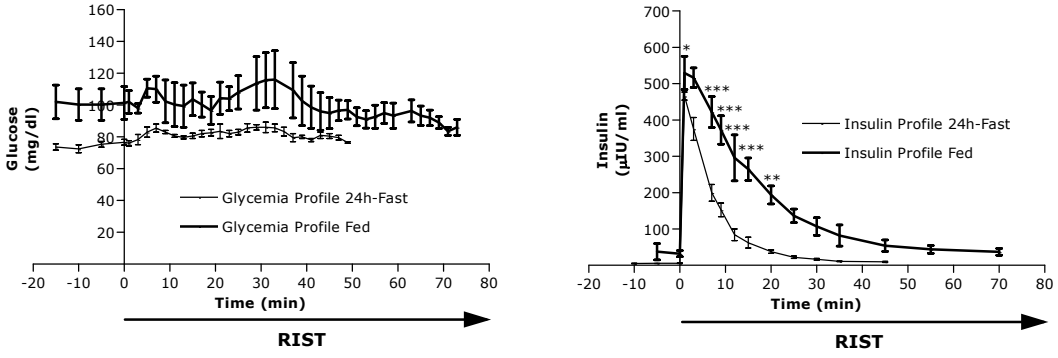
Prandial State	Glycemia (mg/dl)	Insulin (μIU/ml)	C-Peptide (ng/ml)	Lactate (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Fasted	75.3±1.8*	5.3±0.4**	1.7±0.3**	10.3±0.2**	58.6±2.6	57.9±1.7**	158.8±4.7	67.2±24.7
Fed	100.7±10.0*	32.9±11.1**	7.6±0.6**	11.4±0.1**	55.5±1.9	52.3±1.9**	149.3±4.5	59.2±10.9

Comparison of the biochemical profiles during the fasted and postprandial RIST

The evaluation of the biochemical profiles during the RIST, at this time, in the fasted and fed state was achieved. The biochemical parameters analyzed during the fasted and the fed RIST were: glycemia, insulin, C-peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides.

Inasmuch as the RIST was performed in two different prandial states, intrinsic dissimilarities were expected. The food ingestion led to higher levels of glucose and to an increase of insulin and C-peptide secretion. The lactate levels at the beginning of each RIST were similar, and increased with the same profile in both RIST's; the lactate levels were higher in the fed RIST than in the fasted RIST. The lipid profile, given by the HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides levels, did not evidence differences between the fast and fed state (Figure 4.5).

All the biochemical parameters, except insulin and lactate levels, analyzed during the RIST in the fasted and fed state, remained stable in comparison to their basal levels.



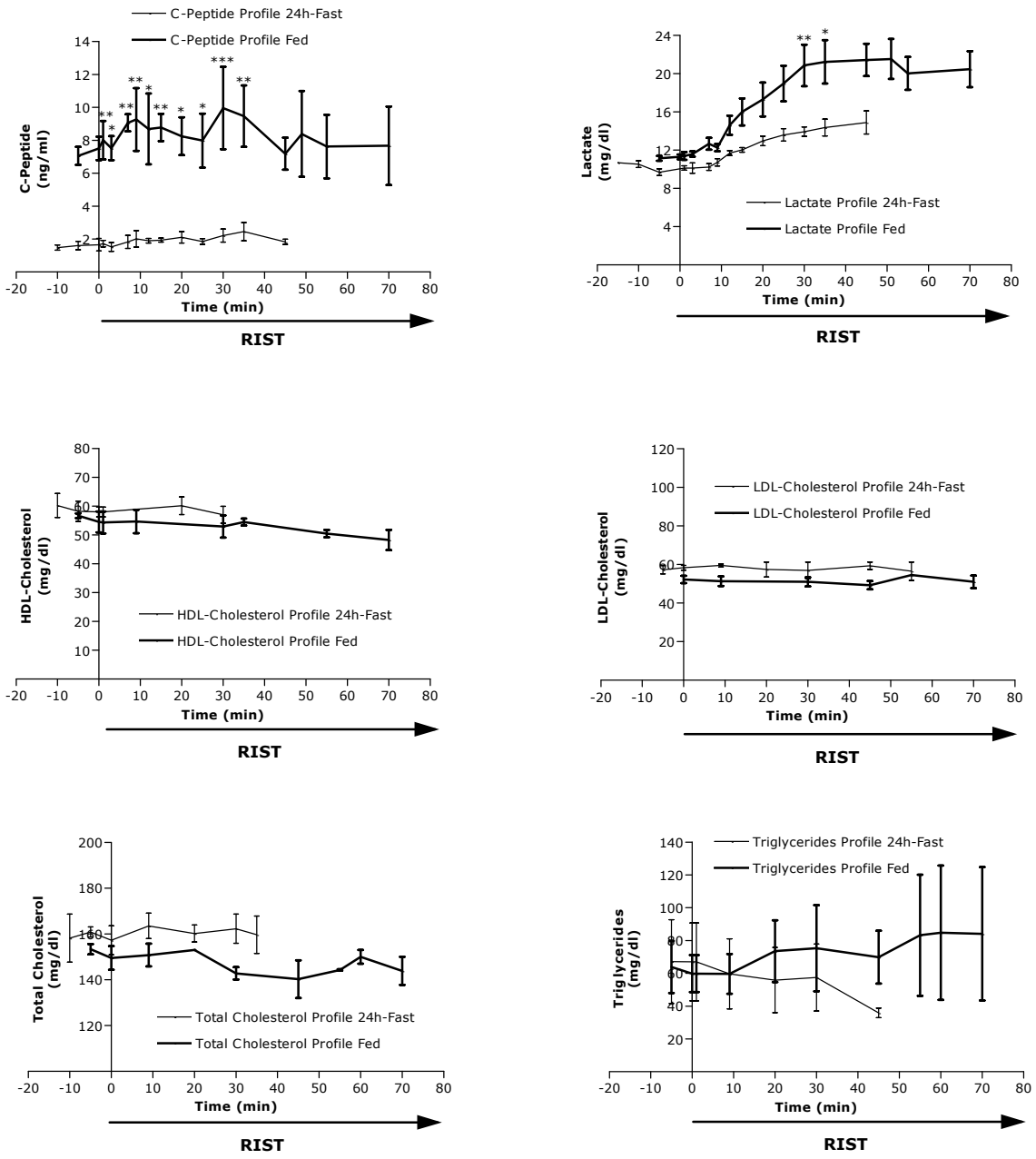


Figure 4.5 – Biochemical parameters (glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides) profiles measured at specific time points during the RIST in the fasted and fed state, in lean healthy subjects (n=3). Results are means±SEM. *=p<0.05, **=p<0.01 and ***=p<0.001 between fasted and fed state. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

Assessement of the RIST dynamic profiles in different prandial states, in lean healthy subjects

A dynamic curve was obtained at the end of each RIST. Below (Figure 4.6) are represented the mean dynamic curves calculated for each experimental protocol condition: the fasted and fed state.

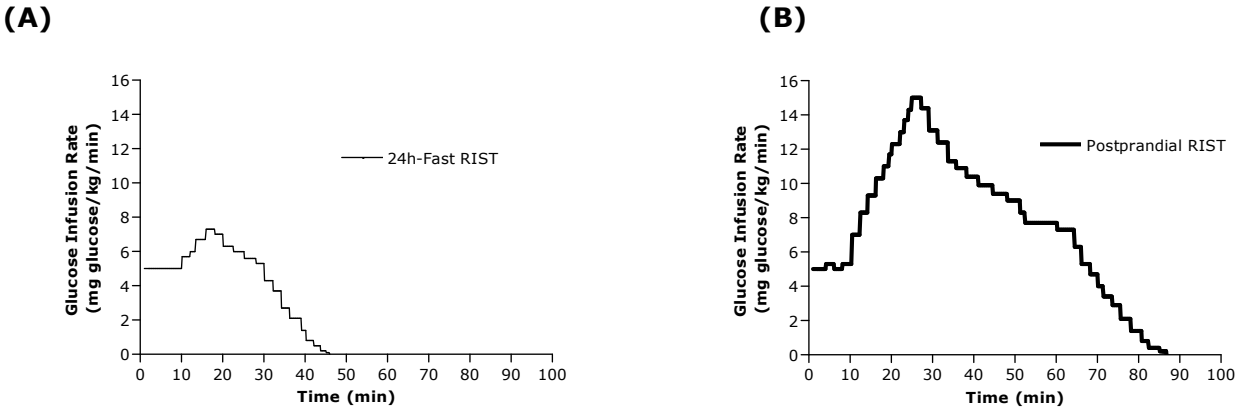


Figure 4.6 – Comparison of the RIST profiles on the 24h-fast (A) and fed state (B). Mean profile using the dynamic analysis of the pattern of glucose infusion during the 24h fast and fed Rapid Insulin Sensitivity Test (RIST). The mean RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test. RIST obtained after 24h-fast (simple line) and postprandial RIST (bold line), n=3.

As shown in Figure 4.6, the RIST dynamic curves were different in the 24h-fasted and fed group. The action peak magnitude, peak time and action curve duration were higher in the postprandial group in comparison with the 24h-fast group.

On Table VII are detailed the main characteristics of the abovementioned profiles.

Table VII - Comparison of the RIST dynamic curve main properties on the 24h-fast and fed state, n=3. Values are means±SEM. $^{*,£,\&}$ =p<0.05 between fasted and fed state. Paired t-test.

24h-Fast RIST	Peak (mg glucose/kg/min)	7.3 ± 1.2*
	Peak time (min)	15.9 ± 1.3$^{£}$
	Duration (min)	44.2 ± 5.0$^{\&}$
Postprandial RIST	Peak (mg glucose/kg/min)	15.0 ± 0.6*
	Peak time (min)	25.0 ± 0.6$^{£}$
	Duration (min)	86.4 ± 8.7$^{\&}$

Effect of fasting and feeding on insulin sensitivity, in lean healthy subjects

The representation of the area under the fasted and fed RIST dynamic curves showed in Figure 4.6 corresponds to the total amount of glucose infused in each RIST (RIST index).

After the 24h fasting period, the RIST index was 224.4±38.9mg glucose/kg bw (n=3). After feeding the standardized test meal the RIST index increased to 647.7±104.9mg glucose/kg bw, $p<0.05$ (Figure 4.7) which represents an increase of 193.6±25.8% in insulin sensitivity from the fasted to the fed state.

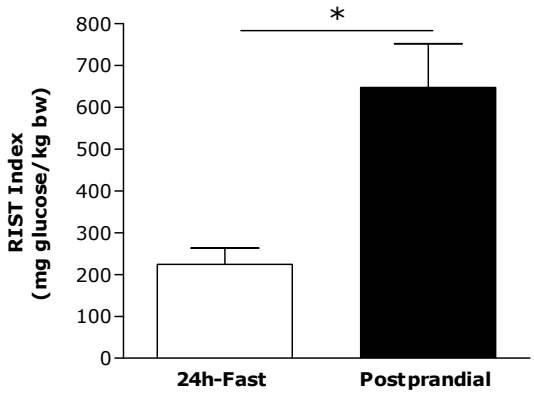


Figure 4.7 - Standardized test meal increases insulin sensitivity in healthy volunteers (n=3). Results are means±SEM. *=p<0.05. Paired t-test.

4.4. DISCUSSION

The results showed in this chapter describe the rapid insulin sensitivity test (RIST), as a new method for quantification of insulin sensitivity both in fasted and fed human subjects, providing results in close agreement with data from rats (Latour *et al.*, 2002c; Lautt *et al.*, 2001; Sadri *et al.*, 2006), cats (Xie *et al.*, 1996c) and mice (Latour *et al.*, 2002a).

Methodological considerations

The RIST in laboratory animals was performed using arterial blood samples. However, in humans arterial sampling is not routinely feasible therefore we used a method that is well recognized for the standard hyperinsulinemic euglycemic clamp determinations. The procedure is based on the principle that heating the forearm results in a thermal-induced vasodilatation that essentially bypasses the nutritive capillary bed of the forearm and

provides venous blood from which significant amounts of glucose have not been extracted (Nauck *et al.*, 1992)[23].

Biochemical profiles before and during the fasted and fed RIST

The RIST just started when stable glucose concentrations were obtained. This glucose value is the euglycemic target to be maintained during the RIST. Since the RIST is an euglycemic clamp, the euglycemia after a bolus of insulin should be kept during the test, by a variable rate of glucose infusion. This is the major parameter to take in account to perform the RIST. If the euglycemia is not achieved during the RIST, the test can not be considered reliable. The accurancy and precison of the RIST should not be higher than 5%, otherwise the entire test is determined to be invalid and is discard.

After the exogenous insulin bolus of 50mU/kg bw, the insulinemia peaked in a similar way both in the fasted and fed state, coming back to initial basal level. However, the fasted and fed insulin profiles were not superimposed, since the way that the insulin levels declined was different. The C-peptide levels, that indicate how endogenous insulin is secreted, were maintained during the fasted and fed RIST and were higher in the fed than in the fasted state. More studies need to be performed in order to ascertain if some factor other than the direct effect of insulin must account for the sensitized glucose disposal response to insulin after the standardized test meal (Figure 4.5 and Figure 4.7).

Lactate levels were higher during the fed than in the fasted RIST and showed a slight increase during both fasted and postprandial RIST. Although lactate is usually seen as a metabolic by-product that serves as a substrate for gluconeogenesis, lactate has an important role in modulating insulin sensitivity (Choi *et al.*, 2002). Basal lactate levels are elevated in patients with glucose intolerance (Consoli *et al.*, 1992), high lactate levels reduce glucose uptake by skeletal muscle (Choi *et al.*, 2002; Lombardi *et al.*, 1999) and mRNA of GLUT4 glucose transporters (Lombardi *et al.*, 1999). Lactate infusion in the artery induces an increase in pancreatic secretion of insulin (Federspil *et al.*, 1980), which is consistent with the hyperinsulinemia observed in situations of insulin resistance.

Since the lactate levels were similar and stable before starting the fasted and fed RIST, it seems that the exogenous insulin bolus infusion (transitory hyperinsulinemia) was the stimulus for the lactate output from skeletal muscle. The results are in agreement with the

[23] Our preliminary data showed that before heating the forearm, pO_2 was 34.0±0.8mmHg and increased to 85.2±1.9mmHg after heating the forearm (Patarrão *et al.*, unpublished observations).

observations from Consoli *et al.*, where the researchers showed that the skeletal muscle is a major site of lactate uptake and release during hyperinsulinemia (Consoli *et al.*, 1992).

In these lean healthy subjects, the lipid profile, given by HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides profile, was preserved during both fasted and fed RIST.

The RIST versus other methods to evaluate insulin sensitivity

Comparing the RIST with other insulin sensitivity techniques, the RIST has a shorter time duration. In the fasted state, the glucose infusion required to maintain baseline euglycemia after insulin administration (50mU/kg bw) reached a peak after 15.9±1.3min and the response was completed after 44.2±5.0min (Table VII); in the fed state, the glucose infusion required after insulin administration reached a peak after 25.9±1.6, 37.5±7.5 or 25.0±0.6min and the response was completed after 94.9±6.8, 97.0±4.7 or 86.4±8.7min, as shown in Table V and Table VII, respectively.

All of the other currently used methods evaluate insulin sensitivity only in the fasted state at the time when physiological insulin sensitivity would be expected to be at its lowest level. This study confirms that the RIST is an effective tool to quantify human whole-body insulin sensitivity in both fasted and fed state. The RIST could be performed in the fed state and can be reproduced on two different days, having the same results. On the other hand, more than one RIST could be performed in the same subject and on the same day, allowing us to design paired experiments, such as the comparison of the degree of insulin sensitivity in the fasted and fed state (Figure 4.7).

Since the RIST involves maintaining arterial euglycemia throughout the entire test period, it does not cause an uncomfortable situation, because the subjects were not subjected to hypoglycemia and there is no counter-regulatory response, providing an advantage over the insulin tolerance test (ITT) (Gelding *et al.*, 1994; Hirst *et al.*, 1993).

Although the hyperinsulinemic euglycemic clamp (HIEC) is currently considered the "gold standard" technique used to evaluate insulin sensitivity, it is non-physiological since high insulin levels are not usually sustained for long periods after a meal (Clark *et al.*, 2003).

Insulin delivery pattern and the RIST

Insulin is secreted in discrete secretory bursts at ≈4min intervals with regulation accomplished through modulation of burst size (Song *et al.*, 2000). As a result, hepatocytes (directly exposed to portal venous blood in hepatic sinusoids) are exposed to an insulin concentration wavefront with oscillations of ≈29–72μIU/ml in the fasting state and increasing to 144–720μIU/ml after meal ingestion (Porksen *et al.*, 1996; Song *et al.*, 2000). Regulation of insulin delivery to extrahepatic tissues depends on both the rate of insulin secretion and the extent of hepatic insulin clearance (Meier *et al.*, 2005). In addition to minute-by minute regulation of insulin secretion, pancreatic β-cells also adapt over a longer period to overall demand.

Increased actions of insulin on muscle, adipose and liver tissues have been reported when the hormone is delivered in a pulsatile versus a constant manner; thus indicates a possible role for the insulin release pattern in the modulation of insulin action at circumstances with special needs, in terms of glucose homeostasis (Juhl *et al.*, 2002). The prolonged insulin infusion causes suppression of hepatic glucose production and endogenous insulin release (DeFronzo *et al.*, 1979). With the HIEC, a steady-state glucose level cannot be maintained at a constant glucose infusion rate using constant insulin infusion over a 3h period (Bergman *et al.*, 1985; Doberne *et al.*, 1981). Furthermore, it is stated that hyperinsulinemia reduces the cardiac vagal tone, suggesting that the prolonged infusion of insulin that occurs during the HIEC may also inhibit the hepatic branch of the vagus (Van De Borne *et al.*, 1999), resulting in insulin resistance mechanisms.

The current study suggests that the RIST provides a new powerful tool to characterize insulin action not only in the fasted, but also in the fed state, in the same day at the same human subject, with reproducible results. With the RIST, the same subject can be submitted to the test at two different days, in the same prandial state, having no differences between days. Therefore, with the RIST one can design paired experiments, allowing us to proceed with pharmacological manipulations.

In the future, the RIST can be used in clinical practice and may provide a mean to detect the prediabetic state. At this stage, early insulin resistance can be detected well before the impairment of the direct effect of insulin, at a time when lifestyle interventions can be readily tested.

5. THE HISS IN LEAN HEALTHY HUMANS

5. THE HISS IN LEAN HEALTHY HUMANS

5.1. INTRODUCTION AND AIMS

The published observations made in animals, suggest that the dynamic response to insulin is approximately doubled following a meal. This phenomenon of meal-induced insulin sensitization (MIS) and the signalling systems that regulate MIS have been demonstrated in rats (Lautt *et al.*, 2001; Sadri *et al.*, 2006). Administration of a mixed meal to rats, either by voluntary consumption of solid rat chow (Latour *et al.*, 2002c), or by intragastric injection (Sadri *et al.*, 2006) results in an approximate doubling of the dynamic glucose disposal in response to insulin. The MIS phenomenon is explained by the HISS (Hepatic Insulin Sensitizing Substance) hypothesis. HISS is suggested to be released from the liver in response to pulses of insulin. In rats and dogs, denervation of the liver or atropine administration results in insulin resistance that is not dependent upon altered glucose uptake of the splanchnic system, including the liver, but is a defect occurring, mostly, in skeletal muscle (Moore *et al.*, 2002; Xie *et al.*, 1996b). Thus, HISS acts in skeletal muscle, but not liver or intestine, to stimulate glucose uptake (Xie *et al.*, 1996b).

It has been suggested that the absence of HISS release following a meal, indicating a state of HISS-dependent insulin resistance (HDIR), represents a prediabetic state that accounts for postprandial hyperglycemia and compensatory hyperinsulinemia (Lautt, 2007). In rats, HISS release occurs only in the fed state and accounts for approximately 55% of the glucose disposal response to insulin. In conscious and anaesthetized rats, HISS-dependent component of insulin action decreases progressively to insignificance by 24h of fasting (Latour *et al.*, 2002c; Lautt *et al.*, 2001).

Consistent with the parasympathetic permissive feeding signal, hepatic denervation impaired the development of HISS action in response to a meal (Sadri *et al.*, 2006). The MIS process is abolished by the administration of atropine so that the RIST index in fed animals after atropine is similar to the RIST index determined in the fasted state (Sadri *et al.*, 2006).

The central objective of study described in this chapter was to test the hypothesis that, in lean healthy humans, the ingestion of a mixed meal results in significant insulin sensitization (MIS). Furthermore, this MIS is inhibited using a cholinergic antagonist, atropine. This study shows, for the first time, the physiological relevance of the HISS-dependent mechanism for overall insulin action in humans.

5.2. PROTOCOLS

5.2.1. Evaluation of the dynamic response to insulin in the fasted and fed states, in lean healthy subjects

In these experiments, the main goal was to compare the dynamic response to insulin in the fasted and fed states.

Therefore, each lean healthy subject was tested on the same day in the fasted and fed state.

Shortly, a first RIST was performed after a 24h fasting period. The subject was then fed a standardized test meal and 100min afterwards, a second RIST was performed (in the fed state). The detailed experimental protocol is described in chapter 3, section 3.3.1 and 3.3.1.2.

5.2.2. Effect of atropine administration on insulin sensitivity, in lean healthy subjects

The major objective in these experiments was to evaluate the effect of atropine infusion on HISS action.

Thereby, each lean healthy subject was tested on two different days in a double-blinded protocol.

Summarily, after a 14h fasting period, the subject fed a standardized test meal and intravenous infusions over 10min of either atropine (0.5 or 0.75mg) or saline (control group) were administrated 50min after feeding the meal and 50min before starting the fed RIST. The detailed experimental protocol is described in chapter 3, section 3.3.1 and 3.3.1.3.

5.3. RESULTS

Lean healthy male subjects (aged 27.0±1.9 years, BMI 22.7±1.1 kg/m^2, n=7) admitted into this protocol had normal systolic (117.1±3.8mmHg) and diastolic blood pressure (63.6±2.9mmHg).

Basal fasting glucose levels were 72.9±1.8mg/dl and glucose levels measured 100min after the meal increased to 104.8±5.1mg/dl ($p<0.001$).

Table VIII shows basal fasting and postprandial levels of the biochemical parameters, of the lean healthy subjects.

Table VIII – Fasting and postprandial basal values of glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides of the lean healthy subjects (n=7). Values are means±SEM. ***=p<0.001, **=p<0.01 and *=p<0.05 between fasted and fed state. Paired *t*-test.

Prandial State	Glycemia (mg/dl)	Insulin (μIU/ml)	C-Peptide (ng/ml)	Lactate (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Fasted	72.9±1.8***	5.4±0.6**	1.5±0.2***	9.3±0.5	52.7±2.9*	76.1±8.0**	158.6±6.7*	36.5±3.8
Fed	104.8±5.1***	39.5±6.4**	9.1±1.0***	10.1±0.7	48.9±3.7*	68.3±8.3**	145.8±10.3*	35.0±5.5

5.3.1. Evaluation of the dynamic response to insulin in the fasted and fed states, in lean healthy subjects

5.3.1.1. Comparison of the fasted and fed RIST dynamic profile, in lean healthy subjects

Figure 5.1 shows the mean dynamic curves of 24h-fast and fed insulin action for lean healthy subjects. The mean dynamic curve for the HISS-dependent component is also presented, calculated by subtracting each 24h-fast dynamic curve from the corresponding fed dynamic curve (Figure 5.2). Moreover, the main characteristics of the dynamic curves (action peak magnitude, peak time, and action curve duration for 24h-fast and postprandial curves; and action peak magnitude, peak time, action curve onset and duration for HISS-dependent component curves) are shown on Table IX.

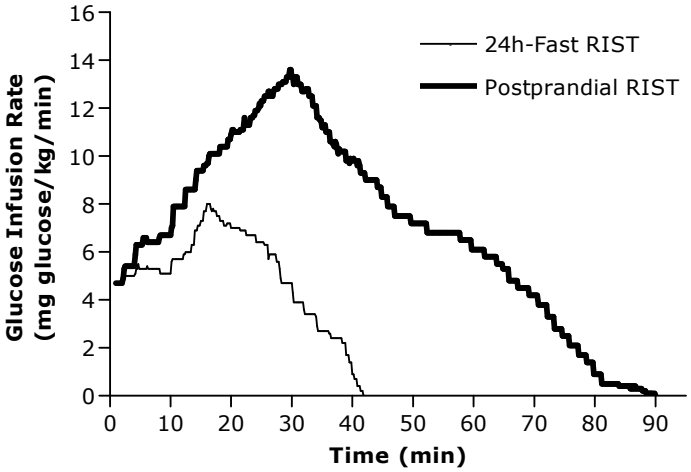


Figure 5.1 - Mean dynamic curves for the Rapid Insulin Sensitivity Test (RIST) performed on 24h-fast (simple line) and postprandial (bold line) states, in lean healthy subjects, n=7. The mean dynamic RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test.

In the postprandial group, all the dynamic parameters (action peak magnitude, peak time, and action curve duration) were higher in comparison to the 24h-fast group (Figure 5.1 and Table IX).

Based on animal studies, the difference between the 2 curves from Figure 5.1, was plotted to show the dynamic action of the HISS-dependent component of insulin action as shown in Figure 5.2 and Table IX.

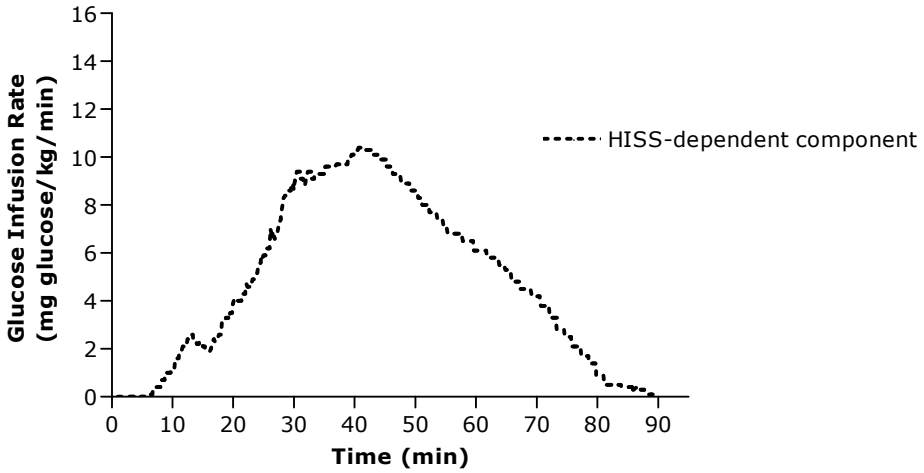


Figure 5.2 - Mean dynamic profile curve for the HISS-dependent component of insulin action, calculated from the difference between the curves in Figure 5.1, in lean healthy subjects. HISS action began at 6.3±2.3min after the onset of insulin administration, n=7.

The mean characteristics of the dynamic profiles of the RISTs obtained both in the fasted and in the fed states are summarized in Table IX.

Table IX - Dynamic profile characteristics of the 24h-fast RIST, postprandial RIST and the HISS-dependent component of insulin action, n=7. Values are means±SEM. $^{\#,\S,@,€}$=p<0.001 between fasted state, fed state and HISS-dependent component. $^{\$,\&,£}$=p<0.05 between fasted state, fed state and HISS-dependent component. Repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test.

24h-Fast RIST	Peak (mg glucose/kg/min)	8.0 ± 0.8$^{\#}$
	Peak time (min)	16.1 ± 2.2$^{\&,\S}$
	Duration (min)	41.9 ± 2.9$^{@,€}$
Postprandial RIST	Peak (mg glucose/kg/min)	13.3 ± 0.7$^{\#,\$}$
	Peak time (min)	28.4 ± 3.0$^{\&,£}$
	Duration (min)	90.5 ± 5.0$^{@}$
HISS-dependent component	Onset (min)	6.3 ± 2.3
	Peak (mg glucose/kg/min)	10.4 ± 0.8$^{\$}$
	Peak time (min)	40.8 ± 4.7$^{\S,£}$
	Duration (min)	90.4 ± 5.0$^{€}$

5.3.1.2. Effect of fasting and feeding on insulin sensitivity, in lean healthy subjects

The area under each dynamic curve, from the results abovementioned (Figure 5.1), provides the RIST index for each prandial state. There was a significant increase in the total insulin sensitivity after the mixed meal.

After the 24h fasting period, the RIST index was 215.5±20.8mg glucose/kg bw (n=7). By contrast, the standardized test meal ingestion led to an increase of the RIST index to 681.2±60.9mg glucose/kg bw, p<0.001 (Figure 5.3), which represents an increase in 232.1±46.3% in insulin sensitivity.

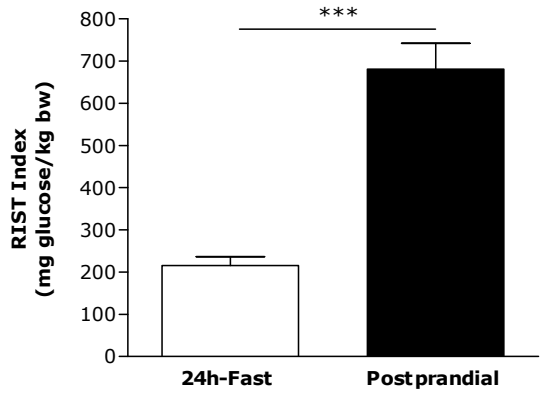


Figure 5.3 - Standardized test meal increases insulin sensitivity in lean healthy volunteers (n=7). Results are means±SEM. ***=p<0.001. Paired t-test.

If one considers that the 24h-fast induces a complete abolishment of HISS mechanism, then the contribution of the HISS-dependent component of total insulin action in the postprandial state was 67.5±3.0%, and the contribution of the HISS-independent component to total insulin action was 32.5±3.0%.

5.3.1.3. Plasma insulin and C-Peptide levels during the fasted and fed RIST, in lean healthy subjects

The evaluation of plasma insulin and C-peptide levels during the RIST was done in both the fasted and the fed state (n=7). As long as the RIST was performed in the fasted and fed state, inherent differences were anticipated. The meal ingestion led to an increase of insulin and C-peptide secretion.

In the fasted state, the basal plasma insulin and C-peptide levels were 5.4±0.6μIU/ml and 1.5±0.2ng/ml, respectively. Immediately after the bolus injection of 50mU/kg bw of insulin, the plasma insulin levels reached a value of 487.3±44.0μIU/ml and plasma C-peptide levels remained stable and not statistically different from basal levels throughout the 24h-fast RIST (1.5±0.2ng/ml); by the end of the fasted RIST (≈42min), plasma insulin levels had returned to basal plasma levels (8.5±0.9μIU/ml).

Subjects were fed a standardized test meal and 100min after (the time required to achieve stable glucose), the basal plasma insulin and C-peptide levels in the fed state were 39.5±6.4μIU/ml ($p<0.01$) and 9.1±1.0ng/ml ($p<0.001$), respectively. Immediately after the bolus injection of 50mU/kg bw of insulin (1min after starting the RIST), insulinemia reached a value of 501.6±30.2μIU/ml, that was not statistically different from the peak level obtained

in the fasted state (487.3±44.0μIU/ml) and plasma C-peptide levels remained stable and not statistically different from basal levels (8.5±0.9ng/ml, $p<0.001$).

Insulin levels in both fed and fasted state had returned to baseline ≈42min after insulin administration (fasted: 8.5±0.9μIU/ml vs fed: 43.8±5.9μIU/ml, $p<0.05$). At this time (≈42min), plasma C-peptide levels were also stable (fasted: 1.5±0.2ng/ml vs fed: 8.8±1.1ng/ml, $p<0.05$).

Although the RIST in the fed state continued until 90min, the insulin levels at ≈42min had already returned to baseline levels and at the end of the postprandial RIST, plasma insulin levels were maintained at basal plasma levels (40.5±5.5μIU/ml) and C-peptide levels were not altered from fed basal levels (8.9±1.3ng/ml).

The plasma insulin and C-peptide profiles for both fasted and fed state are illustrated in Figure 5.4 and Figure 5.5, respectively.

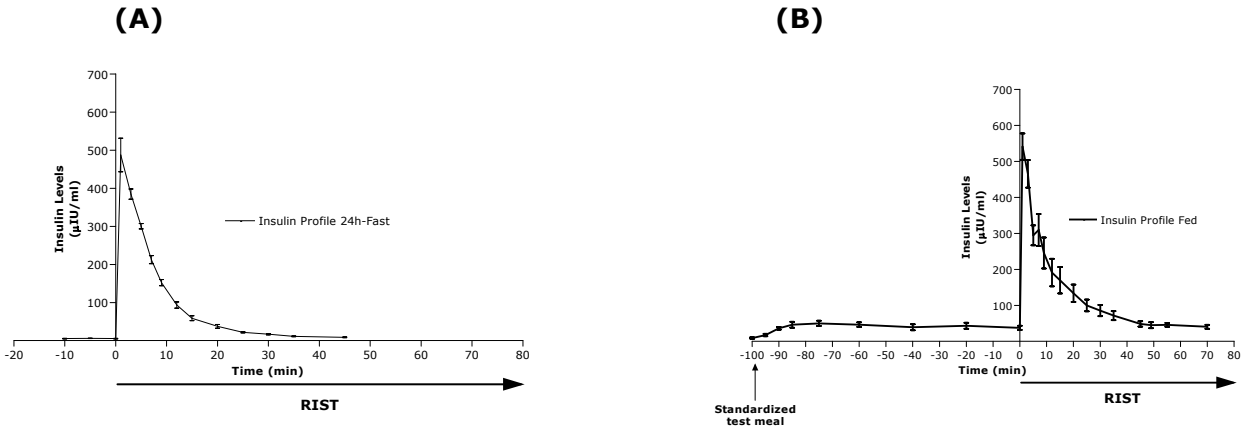


Figure 5.4 - Plasma insulin level profiles obtained before (from -100min to 0min) and during the RIST. The regular line represents the insulin level profile obtained for the 24h-fast RIST (A) and the bold line represents the insulin level profile obtained for the fed RIST (B). Results are means±SEM, n=7.

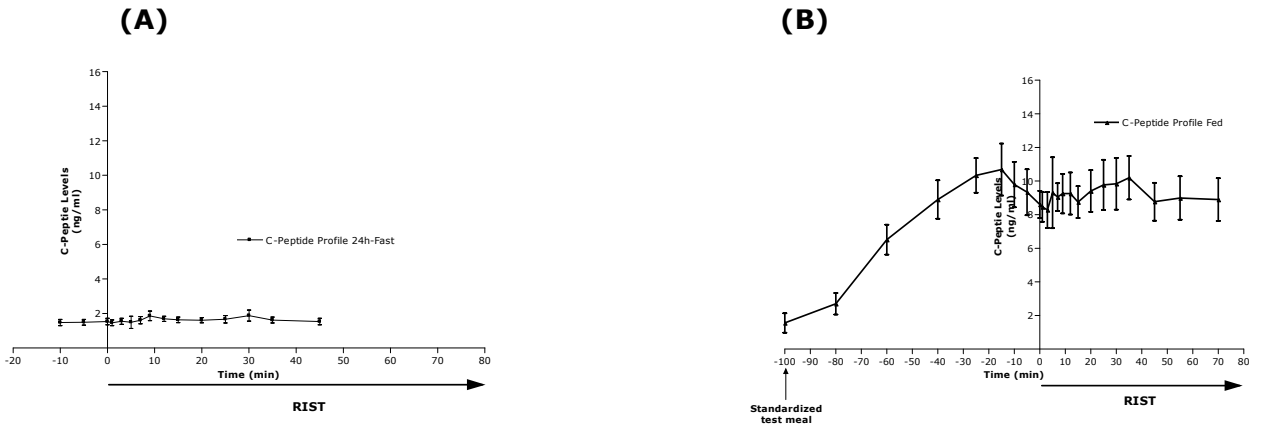


Figure 5.5 - Plasma C-peptide level profiles obtained before (from -100min to 0min) and during the RIST. The regular line represents the C-peptide level profile obtained for the 24h-fast RIST (A) and the bold line represents the C-peptide level profile obtained for the fed RIST (B). Results are means±SEM, n=7.

Insulin levels were measured when insulin action was completed at the end of the 24h-fast RIST (≈42min) and at this same time point at the fed RIST. These values were not statistically different from basal levels (24h-Fast RIST – basal: 5.4±0.6μIU/ml vs end of 24h-fast RIST: 8.5±0.9μIU/ml; Fed RIST – basal: 39.5±6.4μIU/ml vs end of 24h-fast RIST: 43.8±5.9 μIU/ml).

5.3.2. Effect of atropine administration on insulin sensitivity, in lean healthy subjects

The lean healthy male subjects (aged 29.7±1.5 years, BMI 23.9±0.6 kg/m^2) admitted into this protocol had normal systolic (115.5±2.3mmHg, n=10) and diastolic blood pressure (61.7±2.0mmHg, n=10).

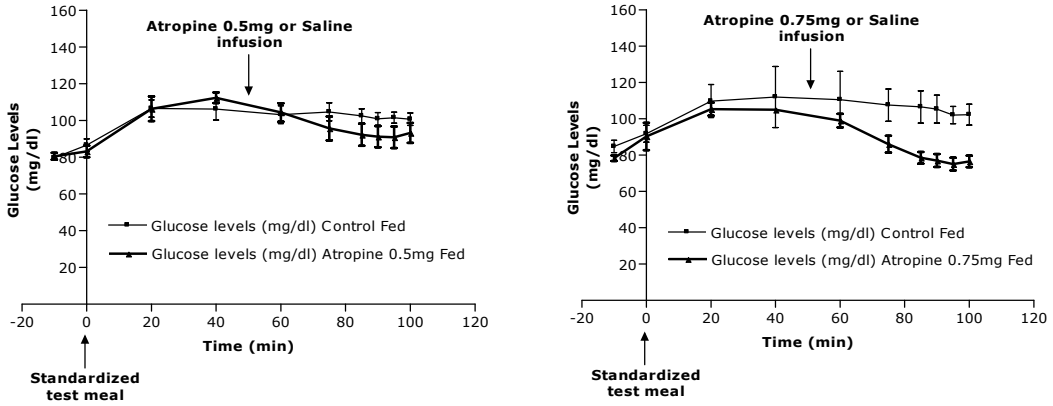
Table X shows basal control and atropine 0.5 (n=6) and 0.75mg (n=4) fed levels of the biochemical parameters, of the lean healthy subjects.

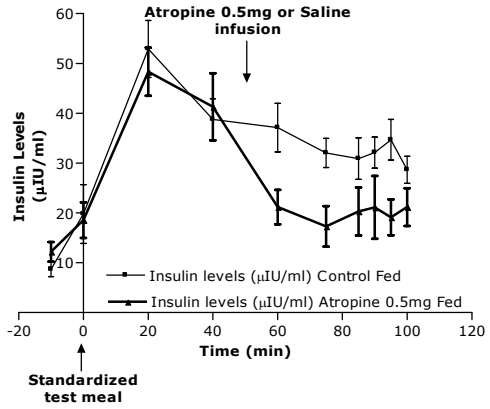
Table X - Basal values of glycemia, insulin, C-peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides values of the lean healthy subjects, in the control fed, atropine 0.5 (n=6) and 0.75mg (n=4) fed state. Values are means±SEM. *=p<0.05 between control postprandial and atropine 0.5mg and control postprandial and atropine 0.75mg. Paired *t*-test.

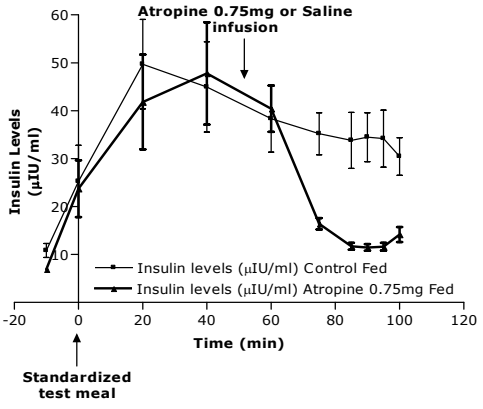
	Glycemia (mg/dl)	Insulin (μIU/ml)	C-Peptide (ng/ml)	Lactate (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control Postprandial (n=6)	101.1±3.1	28.7±2.7	8.6±1.1*	8.7±0.6	46.6±3.1	83.5±3.4	136.9±3.4	55.7±7.9*
Atropine 0.5mg Postprandial (n=6)	94.0±5.7	21.2±3.8	4.7±0.6*	7.5±0.6	48.1±3.4	75.9±5.5	131.4±4.4	37.6±3.8*
Control Postprandial (n=4)	101.9±4.9*	30.5±3.9*	7.3±1.5	9.1±0.9	50.6±2.0	82.9±2.6	143.5±3.9	52.6±13.1
Atropine 0.75mg Postprandial (n=4)	78.3±2.7*	14.2±1.6*	4.7±0.6	11.5±1.8	52.5±3.6	92.5±5.0	170.0±8.1	48.2±9.6

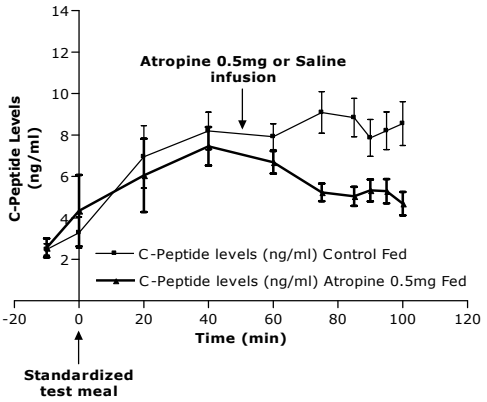
5.3.2.1. Biochemical profiles after feeding the standardized test meal, on control fed and atropine 0.5 and 0.75mg fed groups

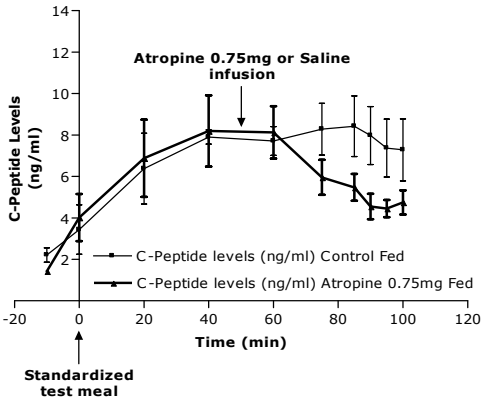
The evaluation of the biochemical profiles, obtained after ingestion of the standardized test meal (t=0min), was performed in the control fed and atropine 0.5 and 0.75mg fed groups (Figure 5.6).

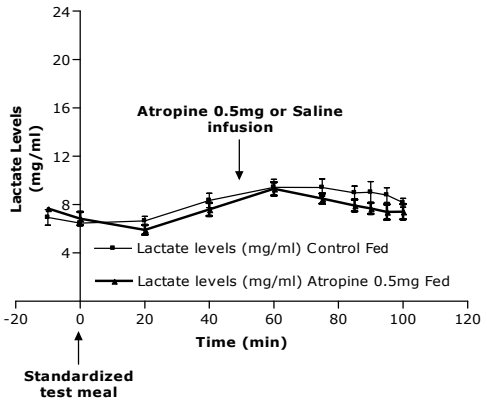


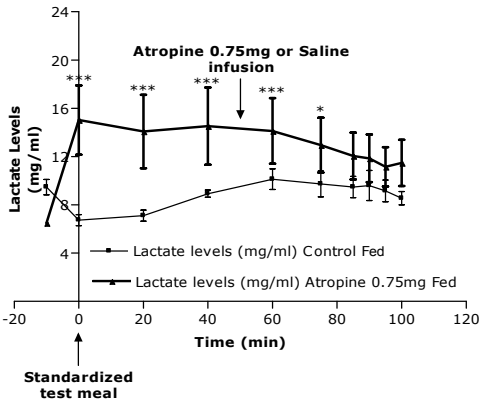

Atropine 0.5mg or Saline infusion
Insulin Levels (μIU/ml)
Insulin levels (μIU/ml) Control Fed
Insulin levels (μIU/ml) Atropine 0.5mg Fed
Time (min)
Standardized test meal

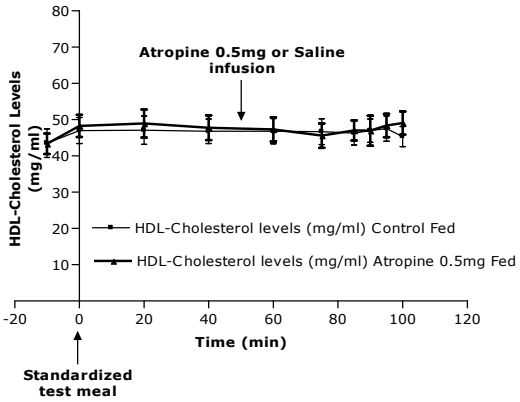

Atropine 0.75mg or Saline infusion
Insulin Levels (μIU/ml)
Insulin levels (μIU/ml) Control Fed
Insulin levels (μIU/ml) Atropine 0.75mg Fed
Time (min)
Standardized test meal

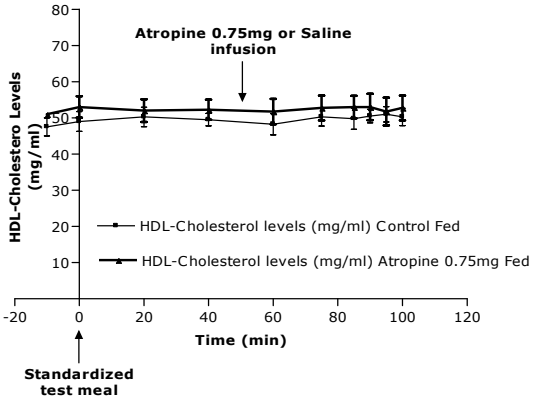

Atropine 0.5mg or Saline infusion
C-Peptide Levels (ng/ml)
C-Peptide levels (ng/ml) Control Fed
C-Peptide levels (ng/ml) Atropine 0.5mg Fed
Time (min)
Standardized test meal


Atropine 0.75mg or Saline infusion
C-Peptide Levels (ng/ml)
C-Peptide levels (ng/ml) Control Fed
C-Peptide levels (ng/ml) Atropine 0.75mg Fed
Time (min)
Standardized test meal


Atropine 0.5mg or Saline infusion
Lactate Levels (mg/ml)
Lactate levels (mg/ml) Control Fed
Lactate levels (mg/ml) Atropine 0.5mg Fed
Time (min)
Standardized test meal


Atropine 0.75mg or Saline infusion
Lactate Levels (mg/ml)
Lactate levels (mg/ml) Control Fed
Lactate levels (mg/ml) Atropine 0.75mg Fed
Time (min)
Standardized test meal


Atropine 0.5mg or Saline infusion
HDL-Cholesterol Levels (mg/ml)
HDL-Cholesterol levels (mg/ml) Control Fed
HDL-Cholesterol levels (mg/ml) Atropine 0.5mg Fed
Time (min)
Standardized test meal


Atropine 0.75mg or Saline infusion
HDL-Cholestero Levels (mg/ml)
HDL-Cholesterol levels (mg/ml) Control Fed
HDL-Cholesterol levels (mg/ml) Atropine 0.75mg Fed
Time (min)
Standardized test meal

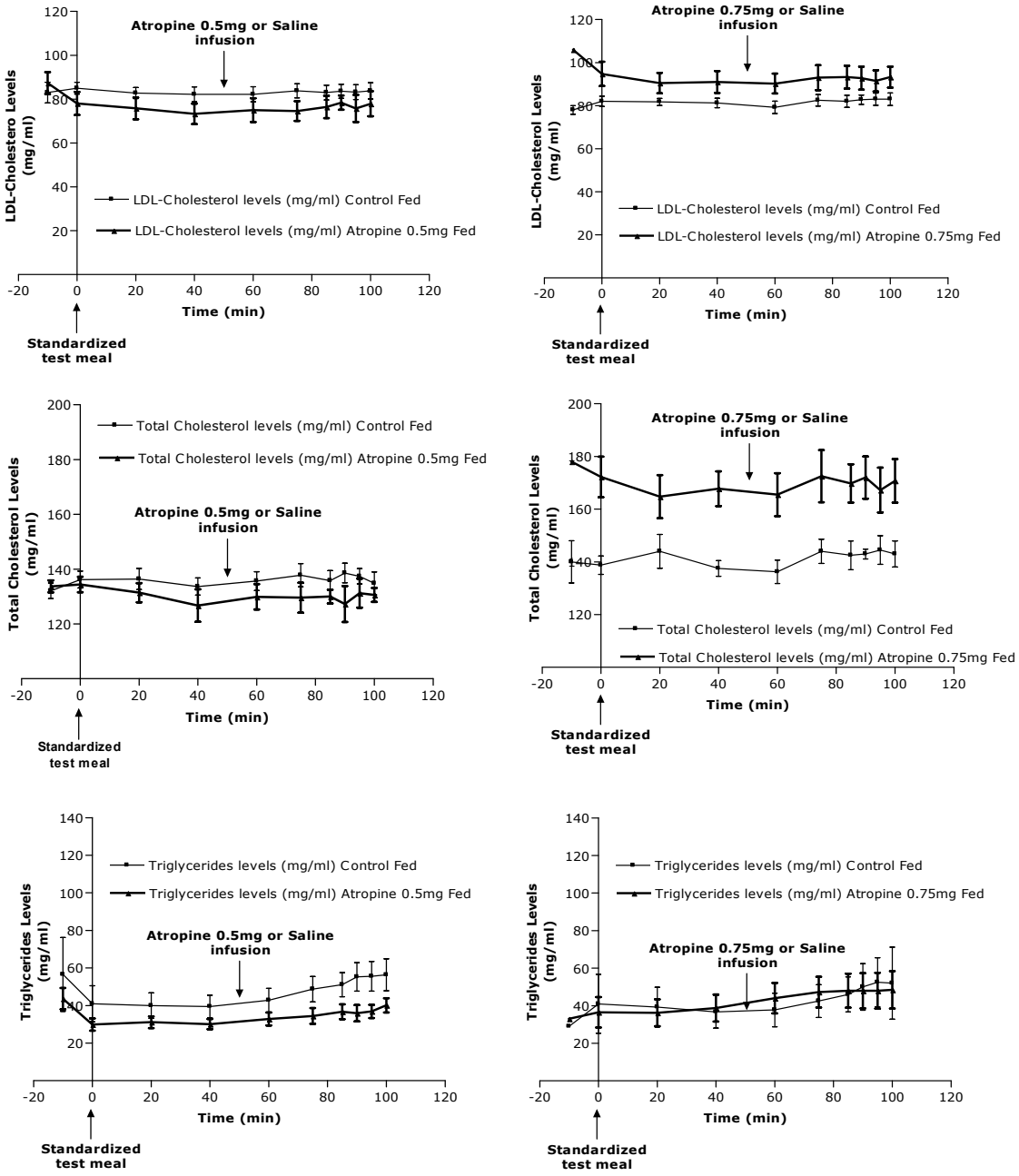


Figure 5.6 – Biochemical parameters (glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides) profiles measured at specific time points during 100min after ingestion of the standardized test meal (0min) and before performing the RIST in the fed state (100min), on control fed and atropine 0.5mg (n=6) and 0.75mg (n=4) fed groups, in lean healthy subjects. Results are means±SEM. *=p<0.05 and ***=p<0.001 between control fed and atropine 0.5 or 0.75mg fed state. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

As shown in Figure 5.6, the infusion of saline (control fed) *versus* infusion of atropine 0.5mg and atropine 0.75mg after ingestion of the standardized test meal, only the atropine dose of 0.75mg altered the lactate profile. Nonetheless, 100min after the food ingestion all the levels returned to their basal levels.

5.3.2.2. Comparison of the control fed and atropine 0.5mg fed RIST dynamic profiles, in lean healthy subjects

The mean dynamic profiles of total postprandial insulin action as well as the dynamic profiles obtained after atropine 0.5mg administration are shown below, in Figure 5.7. The mean dynamic curve for the HISS-dependent component is also presented, calculated by subtracting each atropine 0.5mg dynamic curve from the corresponding control fed dynamic curve (Figure 5.8).

Additionally, the main characteristics of the dynamic curves (action peak magnitude, peak time, and action curve duration for postprandial and atropine 0.5mg postprandial curves; and action peak magnitude, peak time, action curve onset and duration for HISS-dependent component curves) are shown in Table XI.

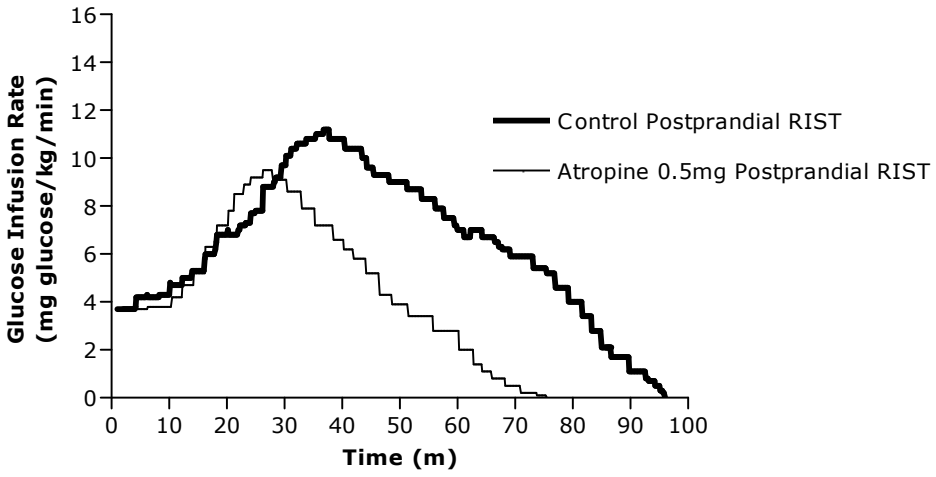


Figure 5.7 – Effect of saline (control) and atropine 0.5mg on postprandial RIST profiles. Mean dynamic curves for the Rapid Insulin Sensitivity Test (RIST) performed on control fed (bold line) and post-atropine 0.5mg fed (simple line) conditions, in lean healthy subjects, n=6. The mean RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test.

In the post-atropine 0.5mg group, all the dynamic parameters (action peak magnitude, peak time, and action curve duration) were lower in comparison to the postprandial group. This was due to the inhibition of the HISS-dependent component (Figure 5.7 and Table XI).

Assuming that atropine 0.5mg abolished HISS action, the difference between the 2 curves from Figure 5.7, was plotted to show the dynamic action of the HISS-dependent component of insulin action (Figure 5.8 and Table XI).

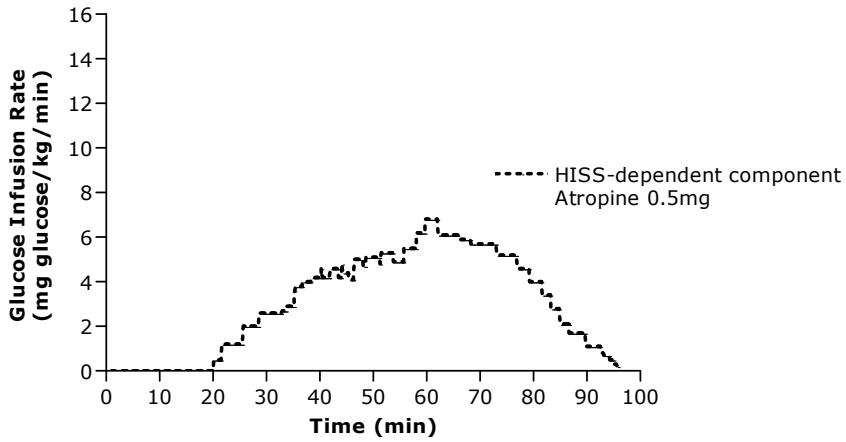


Figure 5.8 - Mean dynamic profile curve for the HISS-dependent component of insulin action calculated from the difference between the curves in Figure 5.7, in lean healthy subjects after atropine 0.5mg administration. HISS action begins at 20.0±4.8min after the onset of insulin administration, n=6.

The characteristics of the dynamic profiles of the RISTs obtained, in lean healthy subjects, both in the fed state and after atropine 0.5mg infusion are summarized in Table XI.

Table XI - Dynamic profile characteristics for the control postprandial RIST, atropine 0.5mg postprandial and the HISS-dependent component of insulin action, in lean healthy subjects (n=6). Values are means±SEM. $^{\$,\#}$=p<0.05 between control fed state, atropine 0.5mg postprandial state and HISS-dependent component.$^{€,£,\&}$=p<0.01 between control fed state, atropine 0.5mg postprandial state and HISS-dependent component. Repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test.

Control Postprandial RIST	Peak (mg glucose/kg/min)	11.2 ± 0.8$^{€}$
	Peak time (min)	37.5 ± 7.1
	Duration (min)	95.6 ± 5.4$^{£}$
Atropine 0.5mg Postprandial RIST	Peak (mg glucose/kg/min)	9.5± 0.9$^{\$}$
	Peak time (min)	26.7 ± 2.0$^{\#}$
	Duration (min)	73.5 ± 6.9$^{£,\&}$
HISS-dependent component	Onset (min)	20.0 ± 4.8
	Peak (mg glucose/kg/min)	6.8 ± 0.9$^{€,\$}$
	Peak time (min)	61.0 ± 8.5$^{\#}$
	Duration (min)	95.9 ± 5.3$^{\&}$

5.3.2.3. Effect of atropine 0.5mg infusion on insulin sensitivity, in lean healthy subjects

The glucose uptake induced by the insulin action after blocking the HISS pathway, with atropine, corresponds to the HISS-independent insulin sensitivity.

The total postprandial insulin sensitivity was significantly decreased after atropine 0.5mg infusion. Therefore, the atropine 0.5mg dose was able to induce inhibition of HISS action. These results, which are consistent with animal data, showed that atropine blocks MIS by blocking HISS release.

In the control fed group (after saline infusion), the RIST index was 627.4±83.9mg glucose/kg bw (n=6). In the atropine 0.5mg group, the RIST index decreased to 395.1±64.3mg glucose/kg bw (p<0.01) (Figure 5.9).

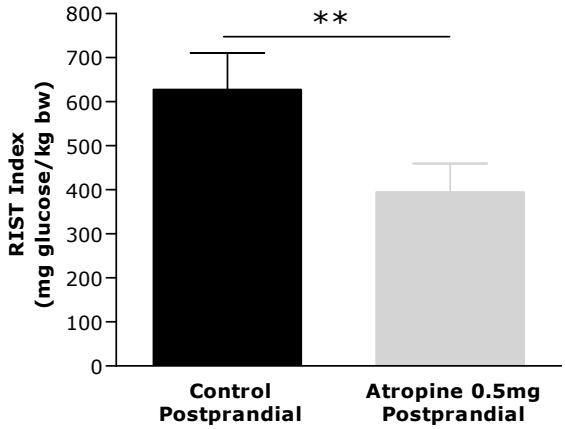


Figure 5.9 - Atropine 0.5mg decreased postprandial insulin sensitivity in lean healthy subjects (n=6). Results are means±SEM. *=p<0.05. Paired t-test.

These results represent a partial inhibition of HISS release induced by atropine 0.5mg of approximately 56.5±11.6%, based on the assumption that full HISS blockade would result in a similar RIST index to the 24h fasting level from fasted and fed lean healthy subjects protocol.

5.3.2.4. Comparison of the control fed and atropine 0.75mg fed RIST dynamic profiles, in lean healthy subjects

Figure 5.10 shows the mean dynamic profiles of total postprandial insulin action as well as the dynamic profiles obtained after atropine 0.75mg administration. Further, the mean dynamic curve for the HISS-dependent component is also presented, calculated by subtracting each atropine 0.75mg dynamic curve from the corresponding control fed dynamic curve (Figure 5.11).

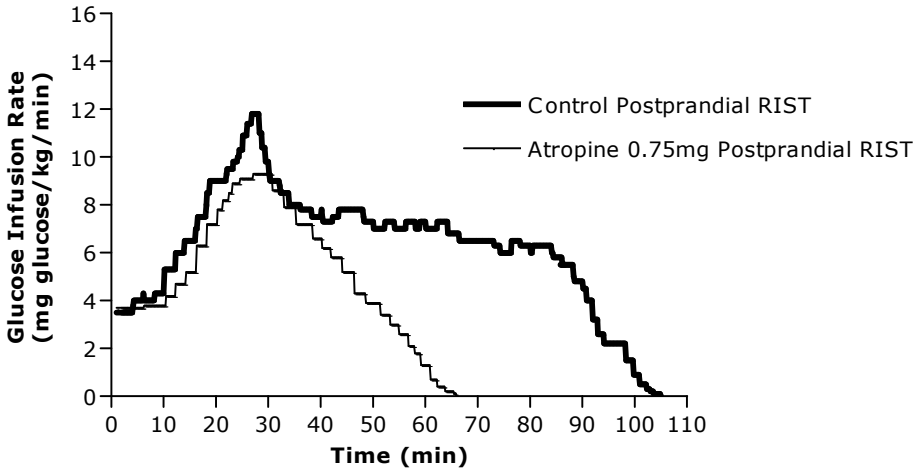


Figure 5.10 – Effect of saline (control) and atropine 0.75mg on postprandial RIST profiles. Mean dynamic curves for the Rapid Insulin Sensitivity Test (RIST) performed on control fed (bold line) and post-atropine 0.75mg fed (simple line) conditions, in lean healthy subjects, n=4. The mean RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test.

In addition, the main characteristics of the dynamic curves (action peak magnitude, peak time, and action curve duration for postprandial and atropine 0.75mg postprandial curves; and action peak magnitude, peak time, action curve onset and duration for HISS-dependent component curves) are shown on Table XII.

As seen previously in section 5.3.2.2. for the post-atropine 0.5mg group, in the post-atropine 0.75mg group all the dynamic parameters (action peak magnitude, peak time, and action curve duration) were lower in comparison to the postprandial group. However, the main difference between the 2 dynamic profiles was the action curve duration that was lower in the atropine 0.75mg fed group (Figure 5.10 and Table XII).

Assuming that atropine 0.75mg abolished HISS action, the difference between the 2 curves from Figure 5.10, was plotted to show the dynamic action of the HISS-dependent component of insulin action (Figure 5.11 and Table XII).

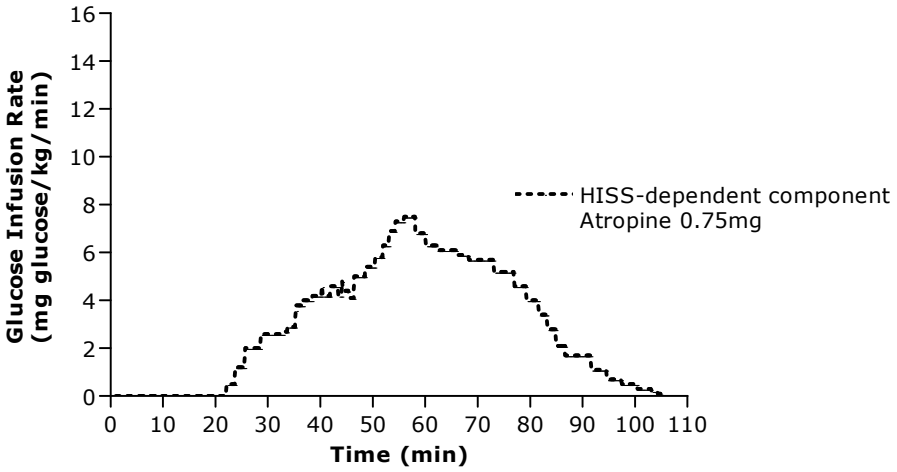


Figure 5.11 – Mean dynamic profile curve for the HISS-dependent component of insulin action calculated from the difference between the curves in Figure 5.10, in lean healthy subjects after atropine 0.75mg administration. HISS action begins at 22.6±7.0min after the onset of insulin administration, n=4.

The characteristics of the dynamic profiles of the RISTs obtained, in lean healthy subjects, both in the fed state and after atropine 0.75mg infusion are summarized in Table XII.

Table XII - Dynamic profile characteristics for the control postprandial RIST, atropine 0.75mg postprandial and the HISS-dependent component of insulin action, in lean healthy subjects (n=4). Values are means±SEM. $^{£,§}$=p<0.001 between control fed state, atropine 0.75mg postprandial state and HISS-dependent component. $^{\$}$=p<0.05 between control fed state, atropine 0.75mg postprandial state and HISS-dependent component. Repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test.

Control Postprandial RIST	Peak (mg glucose/kg/min)	11.8 ± 1.3
	Peak time (min)	27.2 ± 3.2$^{\$}$
	Duration (min)	103.5± 5.8$^{£}$
Atropine 0.75mg Postprandial RIST	Peak (mg glucose/kg/min)	9.3± 1.3
	Peak time (min)	30.3 ± 3.8
	Duration (min)	65.9 ± 9.4$^{£,§}$
HISS-dependent component	Onset (min)	22.6 ± 7.0
	Peak (mg glucose/kg/min)	7.5 ± 1.3
	Peak time (min)	57.7 ± 11.8$^{\$}$
	Duration (min)	103.3 ± 5.7§

5.3.2.5. Effect of atropine 0.75mg infusion, on insulin sensitivity, in lean healthy subjects

In this section, the effect of atropine 0.75mg infusion (in the fed state) on insulin sensitivity is presented. Since the atropine 0.5mg dosage was already tested in terms of insulin sensitivity, the following aim of this study was to analyze if a higher dose of atropine (0.75mg) is able to decrease even more the total postprandial insulin sensitivity in comparison to the atropine 0.5mg infusion results, due to the atropine-induced inhibition of HISS release/action.

In the control fed group (after saline infusion), the RIST index was 669.4±90.0mg glucose/kg bw (n=4). In the atropine 0.75mg group, the RIST index decreased to 378.8±107.1mg glucose/kg bw ($p<0.05$) (Figure 5.12).

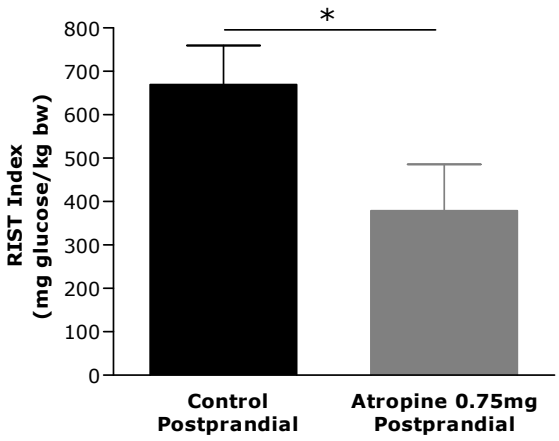


Figure 5.12 - Atropine 0.75mg decreased postprandial insulin sensitivity in lean healthy subjects (n=4). Results are means±SEM. *=$p<0.05$. Paired t-test.

These results represent a partial inhibition of HISS release induced by atropine 0.75mg of approximately 68.5±21.9%, based on the assumption that full HISS blockade would result in a similar RIST index to the 24h fasting level from fasted and fed healthy lean protocol. The atropine 0.5mg dose only induced an inhibition of HISS release of 56.5±11.6% and with a dose increase, the percentage of inhibition of insulin sensitivity also increased (Figure 5.13).

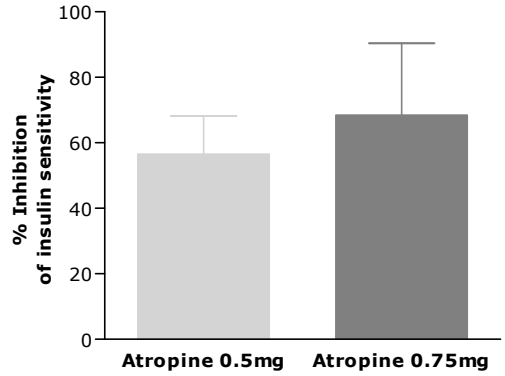


Figure 5.13 - Insulin sensitivity decreases after atropine 0.5mg infusion of 56.5±11.6% (n=6) and after atropine 0.75mg of 68.5±21.9% (n=4), in lean healthy volunteers. Results are means±SEM. Unpaired t-test.

5.3.2.6. Effect of atropine 0.5mg on plasma insulin and C-Peptide levels after feeding the standardized test meal and during the fed RIST, in lean healthy subjects

Since it is well described that atropine infusion affects endogenous insulin secretion (Teff *et al.*, 1999b), the assessment of plasma insulin and C-peptide levels was done in both the control fed state and after atropine 0.5mg infusion (n=6).

The insulin and C-peptide levels determined 100min after the standardized test meal and 50min after either control saline or atropine 0.5mg infusion were: insulin levels - 28.7±2.7 and 21.2±3.8μIU/ml, respectively and C-peptide levels - 8.6±1.1 and 4.7±0.6ng/ml ($p<0.01$), respectively.

The administration of atropine 0.5mg significantly decreased postprandial plasma insulin and C-peptide levels, during the meal absorption. The mean area under the curve (AUC) for insulin measured from 60 to 100min postingestion decreased from 1316±114.4 for control fed to 768.1±150.6μIU/ml/40min for post-atropine 0.5mg fed, $p<0.05$ (Figure 5.14, left insert).

AUCs for C-peptide were also significantly reduced from 60 to 100min postingestion (341.0±33.5 to 202.6±25.7ng/ml/40min for control fed and post-atropine 0.5mg fed, respectively, $p<0.01$) (Figure 5.15 , left insert).

However, the molar ratio of C-peptide to insulin after the standardized test meal was not significantly different between the control fed and atropine 0.5mg fed groups (0.266±0.026 and 0.301±0.052, respectively), suggesting that hepatic extraction of insulin was not different between the two groups.

Moreover, 1min after the exogenous insulin bolus, the insulin levels increased to similar concentration and the C-peptide levels did not differ from basal plasma levels in both groups (666.6±81.2μIU/ml and 8.4±1.2ng/ml in the control fed group and 583.6±64.8μIU/ml and 5.6±0.9ng/ml in the post-atropine 0.5mg group).

At the end of the control fed RIST and the post-atropine 0.5mg RIST, plasma insulin levels returned to basal plasma levels (control fed RIST: 38.3±5.3μIU/ml; post-atropine 0.5mg RIST: 27.2±4.2μIU/ml) and the C-peptide levels did not change from basal plasma levels (control fed RIST: 7.6±0.8ng/ml; post-atropine 0.5mg RIST: 5.8±0.9ng/ml).

The effect of atropine 0.5mg during the fed RIST did not alter the insulin and C-peptide AUCs in the control fed and atropine 0.5mg fed RIST. The mean AUC for insulin measured during the control fed and atropine 0.5mg fed RIST was similar (5431±572.4 and 5260±451.7μIU/ml, respectively) (Figure 5.14, right insert). AUCs for C-peptide were not altered during the control fed and atropine 0.5mg fed RIST (80.3±24.5 and 82.5±27.9ng/ml, respectively) (Figure 5.15, right insert).

The molar ratio of C-peptide to insulin during the RIST was not significantly different between the control fed and atropine 0.5mg fed groups (0.014±0.004 to 0.016±0.005), suggesting that hepatic extraction of insulin was not different during the control fed and atropine 0.5mg RIST.

During the RIST, the insulin concentration profiles were super-imposable and not significantly different at any time point (Figure 5.14). The plasma C-peptide profiles for both the control fed state and after atropine 0.5mg are represented in Figure 5.15.

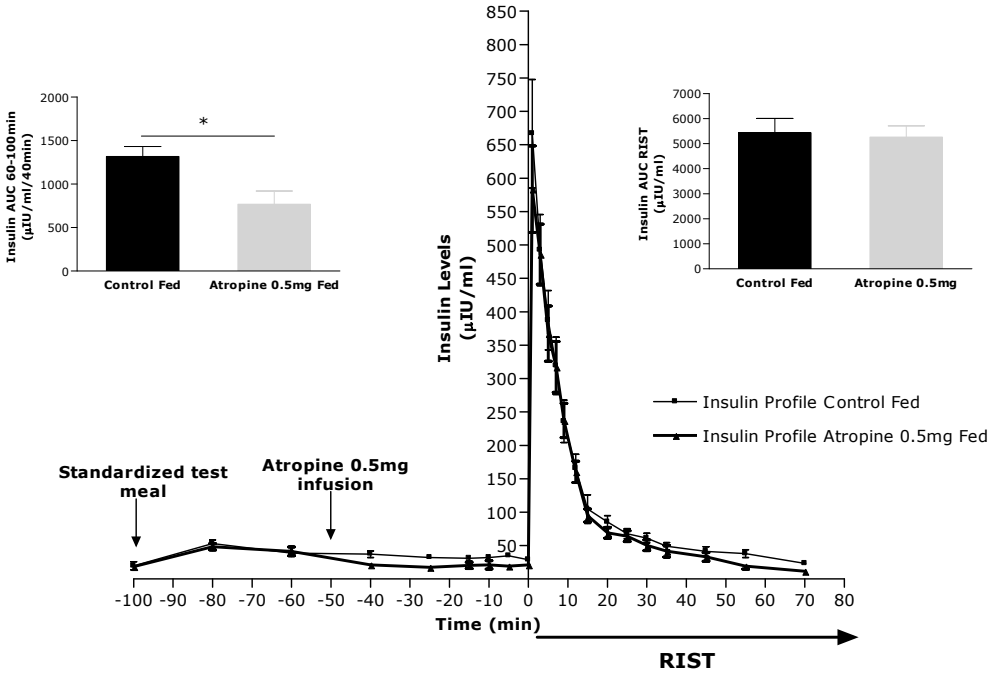


Figure 5.14 - Plasma insulin level profiles obtained after standardized test meal, atropine 0.5mg infusion and during the RIST. The regular line represents the insulin level profile obtained for the control fed RIST and the bold line represents the insulin level profile obtained for the post-atropine 0.5mg RIST. Results are means±SEM, n=6. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for the 60 to 100min after saline (Control Fed) or atropine 0.5mg (Atropine 0.5mg Fed) infusion and before starting the fed RIST. Intravenous infusions over 10min of either atropine 0.5mg or saline were administrated 50min after feeding the meal and 50min before starting the fed RIST. Right insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for Control Fed and Atropine 0.5mg Fed RIST. Results are means±SEM, n=6. *=p<0.05. Paired t-test.

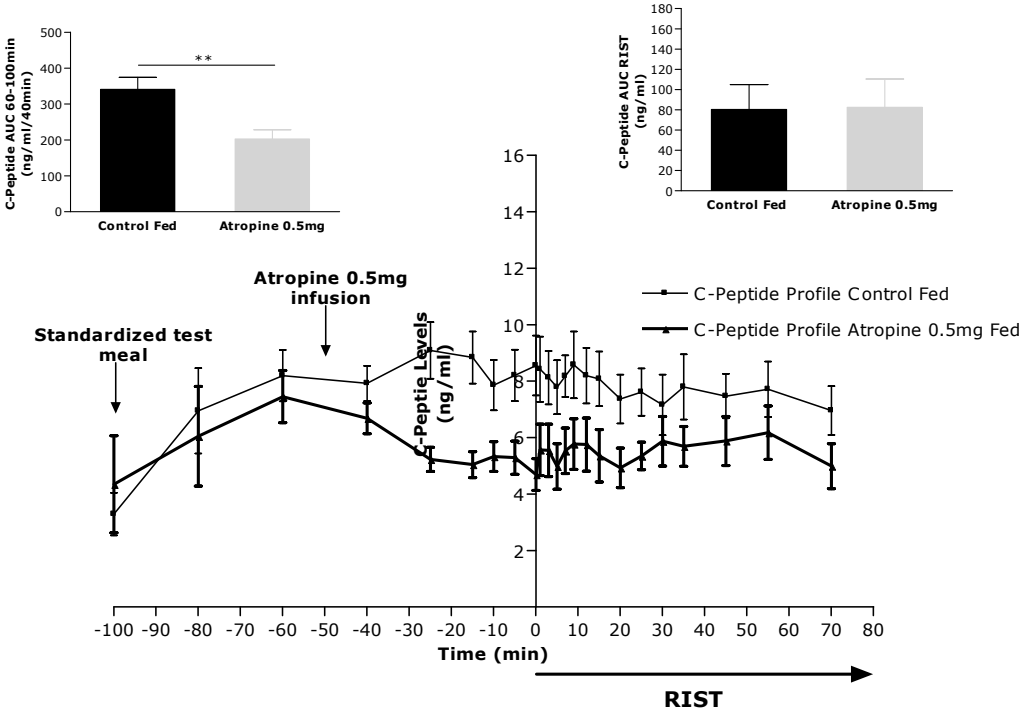


Figure 5.15 - Plasma C-peptide level profiles obtained after standardized test meal, atropine 0.5mg infusion and during the RIST. The regular line represents the C-peptide level profile obtained for the control fed RIST and the bold line represents the C-peptide level profile obtained for the post-atropine 0.5mg RIST. Results are means±SEM, n=6. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for the 60 to 100min after saline (Control Fed) or atropine 0.5mg (Atropine 0.5mg Fed) infusion and before starting the fed RIST. Intravenous infusions over 10min of either atropine 0.5mg or saline were administrated 50min after feeding the meal and 50min before starting the fed RIST. Right insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for Control Fed and Atropine 0.5mg Fed RIST. Results are means±SEM, n=6. **=p<0.01. Paired t-test.

5.3.2.7. Effect of atropine 0.75mg on plasma insulin and C-Peptide levels after feeding the standardized test meal and during the fed RIST, in lean healthy subjects

As previously described for the atropine 0.5mg dose and with the purpose to estimate the atropine's effect on insulin secretion, the assessment of plasma insulin and C-peptide levels were done in both the control fed state and after atropine 0.75mg infusion (n=4).

The insulin and C-peptide levels determined 100min after the standardized test meal and 50min after either atropine 0.75mg or control saline administration, that is, before starting the postprandial RIST were: insulin levels - 30.5±3.9 and 14.2±1.6μIU/ml, respectively and C-peptide levels - 7.3±1.5 and 4.7±0.6ng/ml, respectively.

As previously stated for the atropine 0.5mg dose, the administration of atropine 0.75mg also significantly decreased postprandial plasma insulin and C-peptide levels, during the meal absorption. The mean AUC for insulin measured from 60 to 100min postingestion decreased from 1401±185.9 for control fed to 746.9±57.2μIU/ml/40min for post-atropine 0.75mg fed, $p<0.05$ (Figure 5.16, left insert).

The C-peptide AUC for the atropine 0.75mg fed group was not found to be statistically different from the C-peptide AUC for the control fed group from 60 to 100min postingestion, although there was a trend toward an attenuation (319.5±49.0 to 233.3±28.9ng/ml/40min for control fed and post-atropine 0.75mg fed, respectively) (Figure 5.17, left insert).

However, the molar ratio of C-peptide to insulin after the standardized test meal was not significantly different between the control fed and atropine 0.75mg fed groups (0.238±0.040 to 0.282±0.016), suggesting that hepatic extraction of insulin was again not different between the two groups.

Additionally, 1min after the exogenous insulin bolus, insulin levels spiked to similar concentration and C-peptide levels did not alter from the basal plasma levels in both groups (738.2±85.2μIU/ml and 7.9±1.7ng/ml in the control fed group, and 673.7±69.2μIU/ml and 4.6±0.4ng/ml in the post-atropine 0.75mg group).

At the end of the control fed and the post-atropine RIST, plasma insulin levels returned to basal plasma levels (control fed RIST: 37.6±5.5μIU/ml; post-atropine 0.75mg RIST: 22.0±1.7μIU/ml) and C-peptide levels did not change from basal plasma levels (control fed RIST: 6.8±1.2ng/ml; post-atropine 0.75mg RIST: 3.9±0.5ng/ml).

The effect of atropine 0.75mg during the fed RIST did not alter the insulin and C-peptide AUCs in the control fed and atropine 0.75mg fed RIST. The mean AUC for insulin measured during the control fed and atropine 0.75mg fed RIST was similar (5404±681.9 and

4736±596.9μIU/ml, respectively) (Figure 5.16, right insert). AUCs for C-peptide were not altered during the control fed and atropine 0.75mg fed RIST (46.0±14.0 and 44.4±7.3ng/ml, respectively) (Figure 5.17, right insert).

The molar ratio of C-peptide to insulin during the RIST was not significantly different between the control fed and atropine 0.75mg fed groups (0.008±0.002 to 0.010±0.002), suggesting that hepatic extraction of insulin was not different during the control fed and atropine 0.75mg RIST.

During the RIST, the insulin concentration profiles were super-imposable and not significantly different at any time point (Figure 5.16). The plasma C-peptide profile for both the control fed state and after atropine 0.75mg is represented in Figure 5.17.

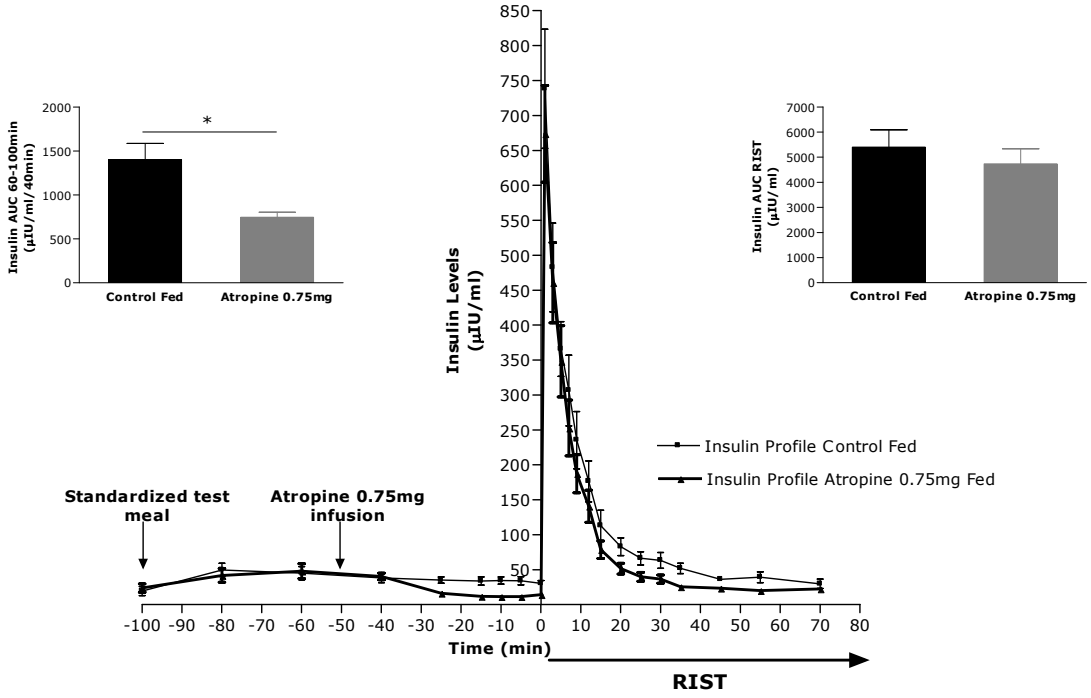


Figure 5.16 - Plasma insulin level profiles obtained after standardized test meal, atropine 0.75mg infusion and during the RIST. The regular line represents the insulin level profile obtained for the control fed RIST and the bold line represents the insulin level profile obtained for the post-atropine 0.75mg RIST. Results are means±SEM, n=4. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for the 60 to 100min after saline (Control Fed) or atropine 0.75mg (Atropine 0.75mg Fed) infusion and before starting the fed RIST. Intravenous infusions over 10min of either atropine 0.75mg or saline were administrated 50min after feeding the meal and 50min before starting the fed RIST. Right insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for Control Fed and Atropine 0.75mg Fed RIST. Results are means±SEM, n=4. *=p<0.05. Paired t-test.

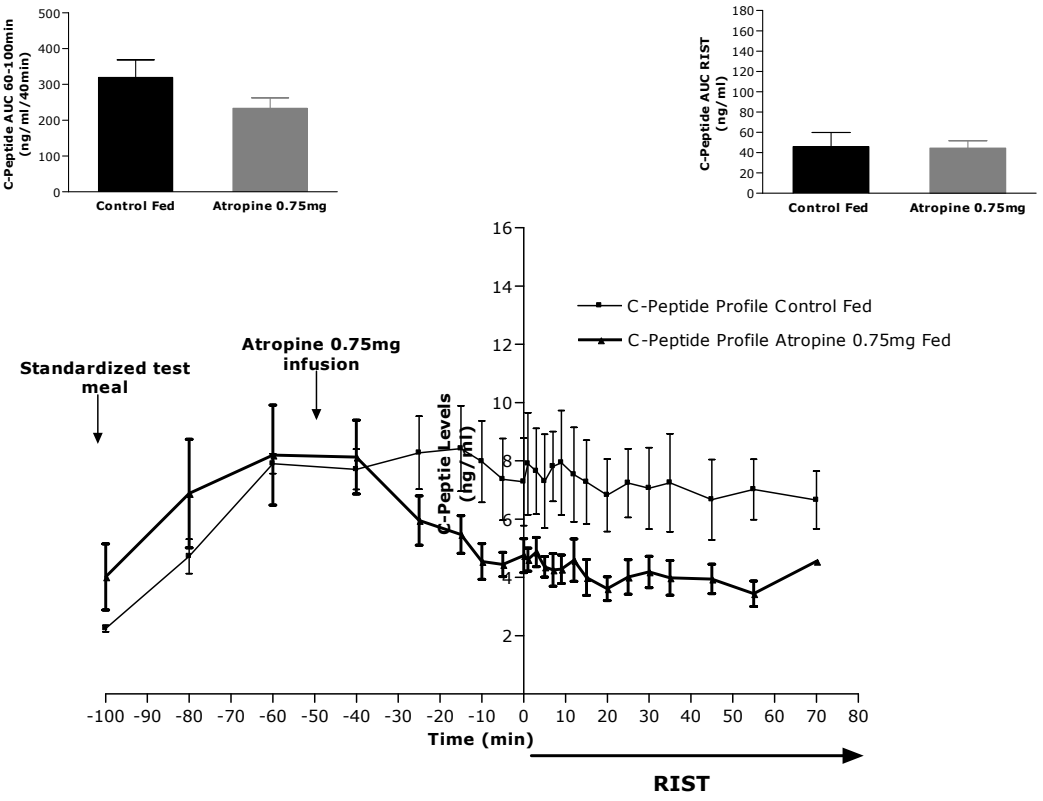


Figure 5.17 - Plasma C-peptide level profiles obtained after standardized test meal, atropine 0.75mg infusion and during the RIST. The regular line represents the C-peptide level profile obtained for the control fed RIST and the bold line represents the C-peptide level profile obtained for the post-atropine 0.75mg RIST. Results are means±SEM, n=4. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for the 60 to 100min after saline (Control Fed) or atropine 0.75mg (Atropine 0.75mg Fed) infusion and before starting the fed RIST. Intravenous infusions over 10min of either atropine 0.75mg or saline were administrated 50min after feeding the meal and 50min before starting the fed RIST. Right insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for Control Fed and Atropine 0.75mg Fed RIST. Results are means±SEM, n=4. Paired t-test.

5.4. DISCUSSION

The current chapter demonstrated for the first time the existence of the meal-induced insulin sensitization (MIS) and also the pharmacological blockade of HISS release using atropine, in humans, using the RIST as the index of dynamic insulin sensitivity.

Similar to animal studies, the RIST index in these human studies was dramatically increased following a meal. These data suggest that postprandial dynamic response to insulin is accounted by the MIS process, resulting in 232.1±46.3% sensitization to the insulin bolus compared to responses determined after a 24h fast.

The present data further suggests that, as in animals, the MIS process in humans has been confirmed to be dependent upon cholinergic mechanisms.

Our data are consistent with the hypothesis that the decreased physiological insulin sensitivity observed in the fasted state is potentiated following a meal and iv atropine administration suppresses the MIS. The partial blockade of MIS by atropine is consistent with the hypothesis that a hepatic parasympathetic "feeding signal" is necessary for insulin to cause the release of HISS from the liver. The differences observed between the dynamic profiles of glucose infusion in the fasted and fed state suggest that HISS action has an additive insulin-like effect.

The feeding signal in humans

The use of the RIST in humans provided data consistent with results derived from cats, rats, and mice (Latour *et al.*, 2002a; Lautt *et al.*, 1998a; Xie *et al.*, 1996c). Blockade of HISS-dependent insulin action results in a decrease of glucose disposal of approximately 55% in the fed state in rats (Lautt *et al.*, 2001), cats (Xie *et al.*, 1995a) and dogs (Moore *et al.*, 2002). The HISS-dependent component of insulin action decreases progressively with the duration of fasting to become insignificant after a 24h fast in rats (Lautt *et al.*, 2001).

For the purpose of estimating the HISS-dependent and HISS-independent components of insulin action in the human studies and based on animal data, we have assumed that a state of full physiological HISS-dependent insulin resistance exists after a 24h-fast (Latour *et al.*, 2002c; Lautt *et al.*, 2001) and the only glucose disposal effect producedin the fastes state is due to the direct action of insulin.

The RIST index in response to insulin was increased after the meal and represents the HISS contribution for the total response which was approximately 67.5% in humans. The MIS shown here is compatible with the same phenomenon shown in animal models and is consistent with the HISS hypothesis.

Feeding stimulates the release of acetylcholine (Ach) from hepatic parasympathetic nerves, which activates muscarinic receptors (Xie *et al.*, 1996a), leading to the production of hepatic nitric oxide (NO) and subsequent release of HISS (Guarino *et al.*, 2004; Sadri *et al.*, 1999). Guarino and colleagues showed that hepatic glutathione (GSH) elevation in response to a meal was also essential for HISS release (Guarino *et al.*, 2003). Regarding the nature of the feeding signal, it was shown, in rats, that in the fed state, MIS can be completely inhibited by blocking hepatic muscarinic receptors, hepatic nitric oxide synthase or hepatic GSH synthesis, resulting in a RIST index similar to that seen in the fasted state (Guarino *et*

al., 2003; Lautt *et al.*, 2001; Sadri *et al.*, 1999; Sadri *et al.*, 2000a). Thus it is clear that both elevated hepatic GSH and NO, which are increased in the fed state, are required for insulin to result in HISS release (Guarino *et al.*, 2006).

We show here that in fed human subjects, MIS was partially inhibited by blocking the muscarinic receptors using atropine at the doses of 0.5 and 0.75mg (56.5±11.6% and 68.5±21.9%, respectively), as seen in Figure 5.13. The HISS-independent component of insulin action obtained after iv infusion of atropine was not completely abolished, since the RIST index after atropine 0.5 and 0.75mg infusion was not the same as the one obtained after a 24h-fast RIST; these doses of atropine used were the highest therapeutic doses ethically permissible and that had minor side effects (Brown *et al.*, 1996)[24].

Atropine produces dose-related suppression of HISS action in rats (Takayama *et al.*, 2000) and cats (Xie *et al.*, 1995a) and thus is able to completely block MIS (Sadri *et al.*, 2006). Atropine produces no notable effect in animals where HISS release is already inhibited by fasting or inhibition of NO synthase or denervation.

Based on rat and cat dose-response curves, we observed that, in humans, the inhibition obtained after atropine 0.5 and 0.75mg infusion was partial and, to achieve a total blockade of HISS, so that the post-meal RIST index returns to levels seen in the fasted state, the atropine dose should be increased. We estimate that an atropine dose of 4mg in humans should produce a full block of HISS release. However, this experimental protocol in humans is not acceptable and the doses of atropine of 0.5 and 0.75mg have already a significant effect on insulin sensitivity.

The atropine 0.5 and 0.75mg effect on the biochemical parameters after feeding the standardized test meal

At the onset of and during meal ingestion, the parasympathetic nervous system is activated, eliciting ACh release at multiple tissue sites, including the pancreas (Ahrén *et al.*, 1986; Teff, 2000; Teff, 2008) and liver (Lautt, 1983; Lautt, 1980; Puschel, 2004). Vagal efferent activity at the level of the pancreas stimulates neurally mediated insulin release, which results in significant increases of this hormone in the portal vein (Campfield *et al.*, 1983).

In this study, during food assimilation, the two doses of atropine (0.5 and 0.75mg) infused 50min after food ingestion, generated similar biochemical profiles (glycemia, insulin,

[24] Takayama *et al.* showed a sensitive response to atropine with the ED_{50} for the intraportal route being much less than for the iv route, thus confirming that the atropine-induced inhibition of HISS action is secondary to blockade of hepatic cholinergic receptors (Takayama *et al.*, 2000).

C-peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides) and, there were no differences in the profiles after saline (in the control fed group) or atropine 0.5 and 075mg infusion.

Following meal ingestion, there was a rise in the glycemia, insulin and C-peptide plasma levels, which were expected due to food absorption; and 100min after food ingestion the plasma levels returned to their postprandial basal levels. The lipid profile given by the HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides levels, was maintained stable over the 100min after the meal. However, the lactate levels for the atropine 0.75mg fed group (Figure 5.6) showed a different profile in comparison to the control fed group. This difference could be attributable to the interindividual variability (due to the lower number of individuals that were included in this protocol), since it was described that following atropine administration the lactate levels were not altered (Boyle *et al.*, 1988).

Concerning the basal values for all the biochemical parameters (Table X), postprandial levels of glucose were lowered by atropine 0.75mg ($p<0.05$), possibly the result of atropine delaying gastric empting (Katschinski, 2000; Teff *et al.*, 1999a) or inhibiting intestinal glucose absorption (Stumpel *et al.*, 1997). On the other hand, the atropine 0.5mg dose did not change the basal glucose levels, which are in accordance with the results from Boyle *et al.* (Boyle *et al.*, 1988).

Neither plasma insulin nor C-peptide basal levels were altered by atropine infusion.

The atropine 0.75mg dose decreased basal plasma insulin levels. It is well described that the effect of muscarinic blockade on plasma insulin was the result of multiple factors and not solely of a direct inhibition on insulin release. One contributing factor may have been the inhibition of the insulin secretagogue, glucagon-like peptide-1 (GLP-1), which was shown to be under direct cholinergic control (Balks *et al.*, 1997).

Moreover, the basal plasma C-peptide levels decreased after atropine 0.5 infusion, which is in agreement with results from other authors (Schneeberger *et al.*, 1991; Teff *et al.*, 1996; Teff *et al.*, 2004).

The postprandial basal levels of triglycerides were decreased by atropine 0.5mg. These differences could be attributable to the interindividual variability and additional studies will be necessary to address this difference.

In summary, since the basal levels (used as baseline values to start the RIST) of the biochemical parameters analyzed before control fed and atropine fed RIST were similar (Table X), the different responses observed in the insulin action could not be assigned to them.

The infusion of atropine 0.5 or 0.75mg, 50min after feeding the standardized test meal decreased insulin and C-peptide AUC (Figure 5.14, left insert; Figure 5.15, left insert;Figure 5.16, left insert; Figure 5.17, left insert). However, the molar ratio of C-peptide to insulin after the meal was not different between the control fed and atropine 0.5 or 0.75mg groups, suggesting that the hepatic extraction of insulin was not affected during the meal assimilation by the atropine administration.

HISS pharmacodynamics and insulin action

During the RIST, the plasma insulin and C-peptide profiles and the AUC for insulin and C-peptide following the bolus administration of insulin in the fed state were not altered by atropine 0.5 or 0.75mg infusion (Figure 5.14, right insert; Figure 5.15, right insert; Figure 5.16, right insert; and Figure 5.17, right insert). The molar ratio of C-peptide to insulin during the control fed and atropine 0.5 or 0.75mg fed RIST was not altered between the two groups, indicating that hepatic insulin extraction was not affecting the insulin response. Even with the superimposable insulin profiles during the control fed and atropine 0.5 or 0.75mg fed RIST, there was a significant decrease in the response to insulin in the fed state after atropine 0.5 and 0.75mg (Figure 5.9 and Figure 5.12). This decrease in insulin sensitivity, indicates that this insulin response to the exogenous bolus of insulin could not be only due to the plasma insulin levels.

The plasma insulin and C-peptide levels profiles obtained during the RIST for the two doses of atropine tested were similar. However, looking at the RIST index values and percentage of inhibition obtained for the two doses, they were different and dose-related, that is, increasing the atropine dose leads to a decrease of insulin sensitivity and an increase of the percentage of inhibition. The lack of effect of atropine on baseline and spike insulin levels confirms data from cats (Xie *et al.*, 1995a) and rats (Latour *et al.*, 2002b). In cats, atropine produced a dose-related HDIR but did not alter baseline insulin, glucagon, or catecholamines nor the peak insulin levels attained following insulin administration (Xie *et al.*, 1995a)

These data suggest, for the first time in humans, that some factor other than insulin accounts for the difference in dynamic insulin action.

Peak insulin levels in fed and fasted state (1min after administration of the insulin bolus) did not differ significantly either with or without previous atropine administration (Figure 5.4 (A) and (B), Figure 5.14 and Figure 5.16). Although the insulin levels were higher during the RIST in the fed state, the data are not consistent with the MIS being accounted for only by

reduced insulin clearance. Termination of insulin action from the administered bolus is determined by the time at which no further administration of glucose is required to maintain euglycemia. In the fasted state, this time point was reached after ≈40min (Figure 5.1 and Table IX). Insulin concentrations determined after 50min had returned to baseline levels (Figure 5.4 (A)). To determine if the prolonged effect of the insulin bolus in the fed state could be attributed to prolonged elevation in insulin, insulin levels were also determined at the ≈40min time point of the postprandial RIST, at which time the glucose disposal response was well maintained at approximately 50% of the maximal response and continued until the end of the postprandial RIST. However, the insulin concentration measured in the fed state at ≈40min had already returned to the pre-administration basal concentration (Figure 5.4 (B)). Moreover, endogenous insulin secretion during the 24h-fast and fed RIST was not altered, since the C-peptide levels did not change along the RIST (Figure 5.5 (A) and Figure 5.5 (B)). Thus, the glucose disposal in the fasted state can be attributed only to insulin whereas the prolonged action in the fed state cannot. The differences observed related to the peak magnitude, peak time, and total duration between the fasted and fed RIST suggest that HISS has an additive rather than a synergistic insulin-like action (Table IX).

The increased glucose disposal determined from the second RIST (performed on the same day, after the meal) is not secondary to delayed effects from the first fasted RIST. The RIST index in the fed state in 24h fast vs fed protocol was the second RIST and was equivalent to the control fed RIST in the atropine 0.5 and 0.75mg protocol, carried out in the same subjects, where only one RIST was carried out. The RIST indexes in these fed subjects were 681.2±60.9mg glucose/kg bw in 24h fast vs fed protocol, 627.4±83.9mg glucose/kg bw in fed controls from the atropine 0.5mg protocol and 669.4±90.0mg glucose/kg bw in fed controls from the atropine 0.75mg protocol (not statistically different). Insulinemia was reduced to baseline at ≈40min in both the fed and fasted states, yet the glucose disposal effect was considerably prolonged after the meal.

In animal studies, it has been demonstrated that four sequential RISTs are reproducible with no "priming effects" detectable (Lautt *et al.*, 1998a). Further, insulin action tested in rats using a similar MIS protocol as used here showed a fasting RIST index that was dramatically sensitized following a meal but which was then returned to the level seen in the fasting state following blockade of HISS release by atropine (Sadri *et al.*, 2006).

Concerning that the bolus administration of insulin results in a non-physiological spike of insulin and that may have numerous indirect effects was also addressed in the animal studies, where dose-response curves for insulin showed that HISS-dependent component of insulin action in the fed state was similar (≈55%) over the entire insulin dose range of 5-

100mU/kg bw (Lautt *et al.*, 2001). If the insulin dose of 50mU/kg is administered over 30 seconds, 5 minutes or 10 minutes, the RIST index is similar, thus showing lack of impact of a bolus spike in insulin concentration (Reid *et al.*, 2004). Thus, the human data reported in this chapter are consistent with the animal data, indicating that some factor other than the direct effect of insulin must account for the sensitized glucose disposal response to insulin after a meal.

In these studies, we tested the MIS and HISS hypothesis demonstrating the ability to compare dynamic insulin action in the fed and fasted state. We report the first evidence of MIS and support the utility of the RIST in humans (Patarrão *et al.*, 2007). This study is consistent with the studies in several other species demonstrating that the MIS process is regulated by hepatic parasympathetic nerves.

6. THE HISS IN OVERWEIGHT HUMANS

6. THE HISS IN OVERWEIGHT HUMANS

6.1. INTRODUCTION AND AIMS

As shown in the previous chapters, in the normal healthy state, feeding results in a rapid meal-induced insulin sensitization (MIS). The MIS process, in lean healthy subjects, resulted in approximately 232% sensitization in response to a pulse of insulin, compared to responses determined after a 24h fast and it is dependent upon cholinergic mechanisms (Patarrão *et al.*, 2008). The mechanism of MIS has been demonstrated to result from the release and action of the hepatic insulin sensitizing substance (HISS). For MIS to occur, a permissive feeding signal must be delivered to the liver through parasympathetic nerves, mediated by cholinergic muscarinic receptor activation and generation of nitric oxide (Sadri *et al.*, 2006) in the presence of an increase of hepatic glutathione (GSH), which result in HISS release (Guarino *et al.*, 2006). The most straightforward method to quantify MIS is to compare insulin sensitivity in the fasted and in the fed state. Alternatively, HISS can be readily quantified by determining insulin sensitivity in the fed state and then tested again after the parasympathetic signal has been eliminated by surgical denervation of the liver, or atropine blockade of hepatic muscarinic receptors, or inhibition of hepatic nitric oxide production (Lautt, 1999; Lautt, 2003b).

The prandial state insulin sensitivity (and consequently MIS) as become more relevant in recent years (Ceriello *et al.*, 2008b; Ceriello *et al.*, 2004). In fact, the first pathophysiological alterations in glucose homeostasis are observed in the fed rather than in the fasted state, which highlights the need to study postprandial insulin sensitivity.

The transition from the early metabolic abnormalities that forerun diabetes, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), to overt diabetes may take many years; however, current estimates indicate that most individuals (perhaps up to 70%) with these prediabetic states eventually develop diabetes (Nathan *et al.*, 2007). The natural history of both IGT and IFG is variable, with ≈25% progressing to diabetes, 50% remaining in their abnormal glycemic state, and 25% reverting to normal glucose tolerance over an observational period of 3–5 years (Gabir *et al.*, 2000; Stern *et al.*, 2005). Individuals who are older, overweight, obese and have other diabetes risk factors are more likely to progress towards diabetes (Nathan *et al.*, 2007).

Obesity has long been recognized to be associated with insulin resistance (Afonso *et al.*, 2007a; Afonso *et al.*, 2010; Kahn *et al.*, 2006; Utzschneider *et al.*, 2006); however, the mechanisms underlying such relationship remain unclear and can vary according to the way obesity is induced.

Moreover, studies in rats suggested that the insulin resistance seen in the high-fat diet (HFD)-induced obese rat model is quite different from the genetically modified obese Zucker (OZR) and diabetic Zucker (ZDF) rat: the HFD rats are mainly HISS-dependent insulin resistant while the OZR and ZDF show impairment of both HISS action as well as the insulin action *per se* (independent of HISS) to the same extent (Afonso *et al.*, 2007a; Afonso *et al.*, 2010). Furthermore, adiposity correlates strongly and negatively with the degree of HISS action (Afonso *et al.*, 2010; Lautt *et al.*, 2008; Ming *et al.*, 2010).

Therefore, the main objective of the study presented in this chapter was to test the hypothesis that insulin resistance in moderately overweight subjects, in comparison with the control lean subjects, is due to MIS impairment and that impairment will only be seen in the postprandial state.

6.2. PROTOCOLS

6.2.1. Evaluation of the dynamic response to insulin in the fasted and fed state, in lean healthy and overweight subjects

In these experiments, the main goal was to compare the dynamic response to insulin in the fasted and fed states, in both lean healthy and overweight subjects.

Therefore, each lean healthy and overweight subject was tested on the same day in the fasted and fed state.

Shortly, after a 24h fasting period, the RIST was performed in the fasted state. After, the subject was fed a standardized test meal and 100min after, a second RIST was performed (postprandial RIST). The detailed experimental protocol is described in chapter 3, section 3.3.1 and 3.3.1.2.

6.3. RESULTS

Four overweight male subjects (aged 24.3±0.9 years, n=4) admitted into this protocol had normal systolic blood (133.0±6.5mmHg) and diastolic blood pressure (69.0±5.5mmHg) and seven lean healthy male subjects (aged 27.0±1.9 years, n=7) admitted into this protocol had normal systolic blood (117.1±3.8mmHg) and diastolic blood pressure (63.6±2.9mmHg). The body mass index between lean healthy and overweight subjects were different (22.7±1.1kg/m^2 and 27.7±0.4kg/m^2, respectively, $p<0.01$).

Basal fasting glucose levels and glucose levels measured after the meal were identical for the overweight and lean healthy subjects (Table XIII).

Table XIII shows basal fasting and postprandial levels of biochemical parameters, of both lean and overweight subjects. Beyond plasma insulin levels, all the biochemical parameters remained stable during the RIST in comparison to their basal levels.

Table XIII – Fasting and postprandial basal values of glycemia, insulin, C-Peptide, lactate, HDL-cholesterol, LDL-cholesterol, total cholesterol and triglycerides of both overweight (n=4) and lean subjects (n=7). Values are means±SEM. $^{*,\#}$=p<0.05 between lean and overweight subjects. Unpaired *t*-test between fasted lean and fasted overweight, and fed lean and fed overweight.

Prandial State	Subjects	Glycemia (mg/dl)	Insulin (μIU/ml)	C-Peptide (ng/ml)	Lactate (mg/dl)	HDL-Cholesterol (mg/dl)	LDL-Cholesterol (mg/dl)	Total Cholesterol (mg/dl)	Triglycerides (mg/dl)
Fasted	**Lean**	72.9±1.8	5.4±0.6	1.5±0.2	9.3±0.5	52.7±2.9	76.1±8.0	158.6±6.7$^{\#}$	36.5±3.8
	Overweight	69.3±3.5	7.7±1.7	2.3±0.5	8.5±0.6	45.7±3.4	100.6±4.6	197.0±14.3$^{\#}$	45.9±1.5
Fed	**Lean**	104.8±5.1	39.5±6.4	9.1±1.0	10.1±0.7	48.9±3.7	68.3±8.3	145.8±10.3*	35.0±5.5
	Overweight	113.9±8.3	39.9±8.1	9.5±0.6	9.2±0.9	48.2±4.1	93.2±1.9	195.0±9.1*	46.7±6.2

6.3.1. Characterization of insulin action, in overweight and lean healthy subjects

6.3.1.1. Evaluation of glucose, insulin and C-Peptide profiles after feeding the standardized test meal, in overweight and lean subjects

The assessment of glucose, insulin and C-peptide profiles, obtained after ingestion of the standardized test meal (t=0min), was performed in lean and overweight groups and it is shown in Figure 6.1 and Table XIV.

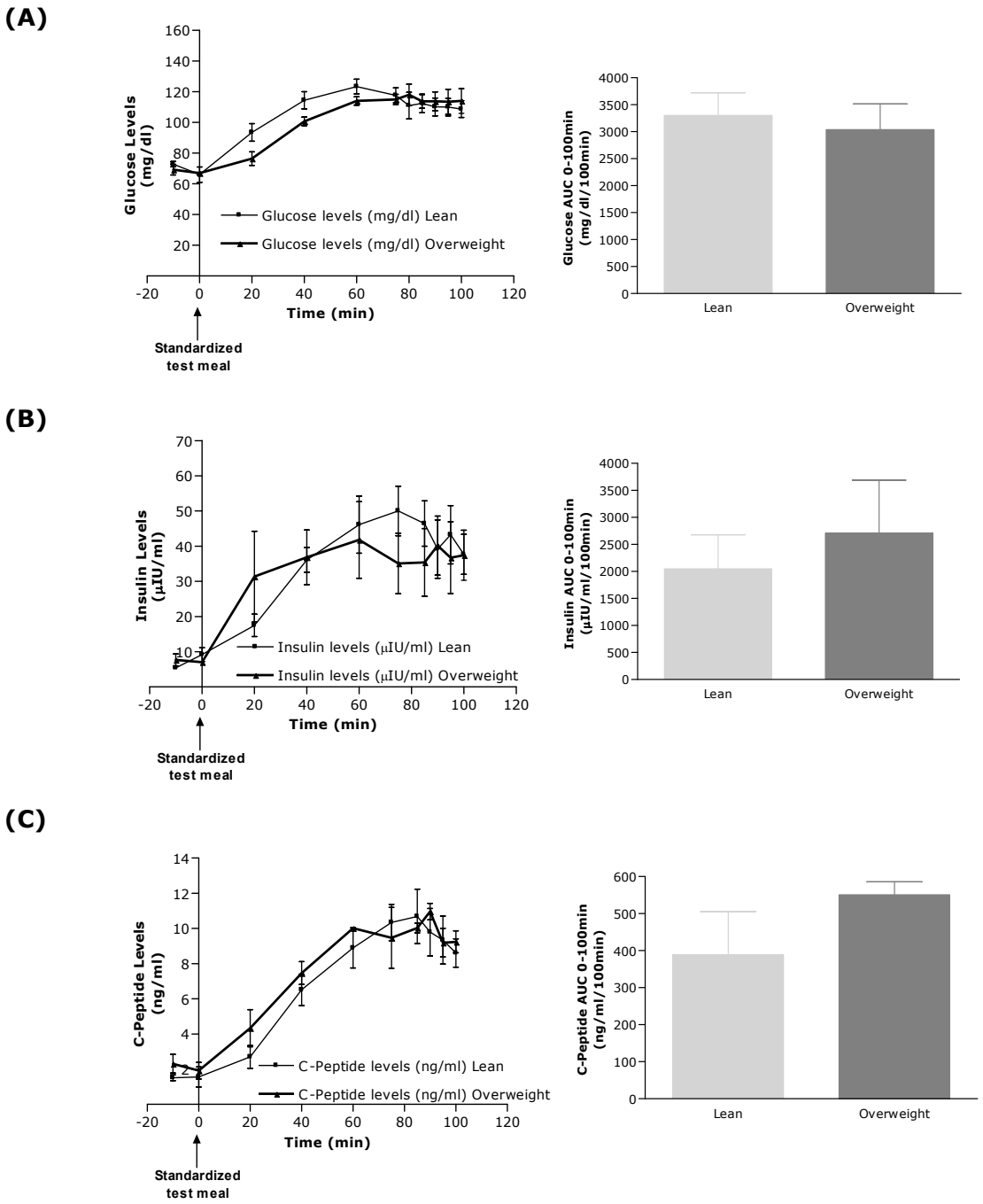


Figure 6.1 - Biochemical parameters (glycemia (A), insulin (B) and C-Peptide (C)) profiles measured at specific time points during 100min after ingestion of the standardized test meal (0min) and before performing the RIST in the fed state (100min), in lean (n=7) and overweight (n=4) subjects. The right inserts correspond to the area under the curve (AUC) of glucose, insulin and C-peptide, respectively, calculated by the trapezoid rule for the 100min after feeding the meal. Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

As shown in Figure 6.1 and Table XIV, the incremental changes in glucose, insulin and C-peptide plasma concentrations at different time points after ingestion of the standardized test meal had a similar increase in both lean and overweight subjects, and the corresponding AUCs were not statistically different when evaluated in the two groups (AUC Glucose - Lean vs Overweight: 3303±419.6 vs 3039±477.9mg/dl/100min; AUC Insulin - Lean vs Overweight: 2048±629.6 vs 2708±979.5μIU/ml/100min; AUC C-Peptide - Lean vs Overweight: 388.5±116.8 vs 550.8±34.9ng/ml/100min). In addition, the molar ratio of C-peptide to insulin in response to the standardized test meal was not significantly different between the lean and overweight groups (0.230±0.053 and 0.229±0.070, respectively), suggesting that neither insulin secretion nor hepatic extraction was different between the two groups during the meal absorption.

Table XIV - 24h fast basal values and specific time points following standardized test meal ingestion of glucose, insulin and C-peptide values, of both lean (n=7) and overweight subjects (n=4). Values are means±standard error. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

			Time after standardized test meal (min)								
		Baseline 24h Fast	0	20	40	60	75	85	90	95	100
Glucose Levels (mg/dl)	Lean	72.9±1.8	66.0±5.0	93.5±5.7	114.4±5.6	123.4±4.8	117.7±4.8	112.4±6.1	110.1±5.9	110.0±5.6	104.8±5.1
	Overweight	69.3±3.5	67.0±1.0	76.5±4.4	100.8±2.8	114.0±2.7	115.0±3.4	113.8±4.5	113.8±6.1	113.5±8.2	113.9±8.3
Insulin Levels (μIU/ml)	Lean	5.4±0.6	9.2±2.0	17.5±3.2	36.1±3.5	46.1±8.1	50.0±7.0	46.4±6.5	39.2±8.3	43.2±8.3	39.5±6.4
	Overweight	7.7±1.7	7.0±0.4	31.3±12.9	36.8±7.8	41.8±10.9	35.1±8.6	35.4±9.6	40.2±8.4	36.7±10.2	39.9±8.1
C-Peptide Levels (ng/ml)	Lean	1.5±0.2	1.6±0.6	2.7±0.6	6.5±0.9	8.9±1.1	10.3±1.0	10.7±1.5	9.8±1.3	9.3±1.4	9.1±1.0
	Overweight	2.3±0.5	1.9±0.5	4.3±1.1	7.5±0.6	10.0±0.1	9.5±1.7	10.0±0.3	11.0±0.5	9.2±0.8	9.5±0.6

6.3.1.2. Comparison of the fasted and fed RIST dynamic profiles, in overweight and lean subjects

On Figure 6.2, are represented the mean dynamic profiles of 24h-fast and fed insulin action for overweight subjects. In overweight subjects, the RIST dynamic profiles, both in the fasted and fed state, were slightly different in comparison with the ones obtained in lean healthy subjects (Chapter 5, Figure 5.3). Assuming that in the 24h-fasted state, HISS action

is absent, the mean dynamic curve for the HISS-dependent component[25] can be calculated by subtracting each 24h-fast dynamic curve from the corresponding fed dynamic curve (Figure 6.3). Moreover, the comparison of the main characteristics of the dynamic curves for lean and overweight subjects (action peak magnitude, peak time, and action curve duration for 24h-fast and postprandial curves; and action peak magnitude, peak time, action curve onset and duration for HISS-dependent component curves) are shown on Table XV.

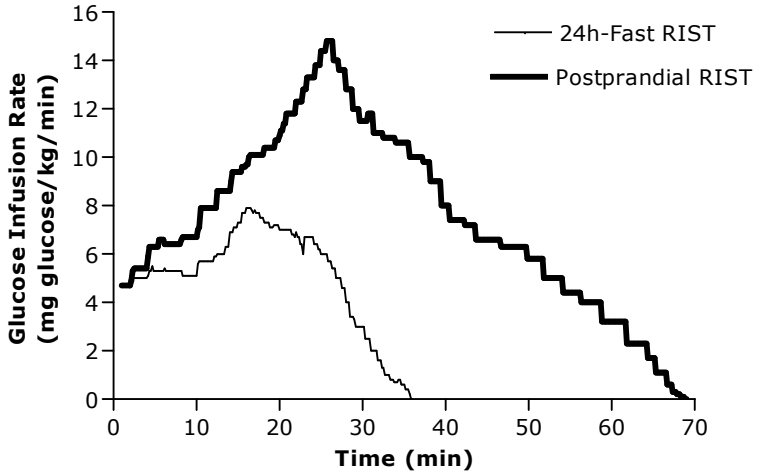


Figure 6.2 – Mean dynamic curves for the Rapid Insulin Sensitivity Test (RIST) performed on 24h-fast (simple line) and postprandial (bold line) states, in overweight subjects, n=4. The mean dynamic RIST curves were obtained by averaging glucose infusion rates at 0.1min intervals throughout the test.

As stated before for the lean subjects, in the postprandial group all the dynamic parameters (action peak magnitude, peak time, and action curve duration) were higher in comparison to the 24h-fast group.

Based on animal studies, the difference between the 2 curves from Figure 6.2, was plotted to show the dynamic action of the HISS-dependent component of insulin action, in overweight subjects (Figure 6.3 and Table XV).

[25] Based on animal studies, the HISS-dependent component can be calculated by subtracting each 24h-fast dynamic curve from the corresponding fed dynamic curve (Lautt *et al.*, 2001).

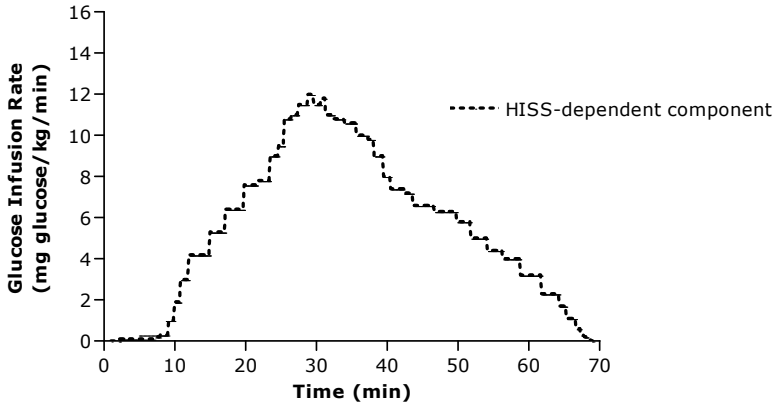


Figure 6.3 – Mean dynamic profile curve for the HISS-dependent component of insulin action, calculated from the difference between the curves in Figure 6.2 in overweight subjects. HISS action began at 9.5±4.0min after the onset of insulin administration, n=4.

The mean characteristics of the dynamic profiles of the RISTs obtained, in lean and overweight subjects, both in the fasted and in the fed states are summarized in Table XV.

Table XV - Dynamic profile characteristics for the 24h-fast RIST, postprandial RIST and the HISS-dependent component of insulin action, for both lean (n=7) and overweight subjects (n=4). Values are means±SEM. $^{\#,\S}$=p<0.01 between fasted state, fed state and HISS-dependent component. $^{\$}$=p<0.05 between fasted state, fed state and HISS-dependent component. Repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test, within the overweight group.

		Lean	**Overweight**
24h-Fast RIST	Peak (mg glucose/kg/min)	8.0 ± 0.8	7.8 ± 1.4$^{\$}$
	Peak time (min)	16.1 ± 2.2	16.4 ± 4.6
	Duration (min)	41.9 ± 2.9	34.0 ± 4.3$^{\#,\S}$
Postprandial RIST	Peak (mg glucose/kg/min)	13.3 ± 0.7	14.8 ± 2.6$^{\$}$
	Peak time (min)	28.4 ± 3.0	26.3 ± 3.1
	Duration (min)	90.5 ± 5.0	68.5 ± 11.4$^{\#}$
HISS-dependent component	Onset (min)	6.3 ± 2.3	9.5 ± 4.0
	Peak (mg glucose/kg/min)	10.4 ± 0.8	11.5 ± 1.7
	Peak time (min)	40.8 ± 4.7	27.5 ± 4.1
	Duration (min)	90.4 ± 5.0	68.4 ± 11.4§

As shown in Table XV there are no differences in the mean dynamic parameters (action peak magnitude, peak time, and action curve duration) for the 24h-fast and fed insulin action between the lean and overweight groups.

6.3.1.3. Effect of fasting and feeding on insulin sensitivity, in lean and overweight subjects

The area under each dynamic curve, from the results abovementioned (Figure 6.2 and Figure 5.1, Chapter 5) provides us the RIST index for each prandial state.

In lean and overweight subjects, after the 24h fasting period, the RIST index was 215.5±20.8 and 177.8±40.5mg glucose/kg bw, respectively. Thus, the insulin action *per se* was not different in lean and overweight fasted subjects. After feeding the standardized test meal, the RIST index increased to 681.2±60.9 and 388.8±107.3mg glucose/kg bw ($p<0.05$), in lean and overweight subjects, respectively (Figure 6.4). The increase in insulin sensitivity in lean subjects was 232.1±46.3% while in overweight subjects was only 174.0±67.9%, corresponding to the percentage of potentiation of the insulin action induced by the meal.

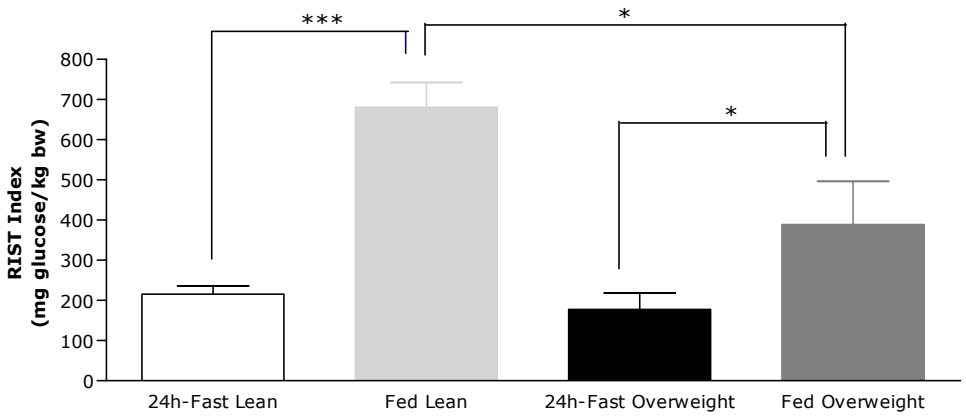


Figure 6.4 – Standardized test meal increases insulin sensitivity both in lean (n=7) and overweight (n=4) subjects. However, the increase in insulin sensitivity is much more marked in the lean subjects. Results are means±SEM. ***=$p<0.001$ and *=$p<0.05$. Paired t-test between the same groups of subjects and unpaired t-test between lean and overweight group.

The HISS-dependent component of total insulin action, calculated by subtracting the 24h-fast RIST index from the corresponding fed RIST index, is lower in the overweight subjects than in the lean (465.7±57.8 vs 233.1±72.6mg glucose/kg bw, respectively, $p<0.05$, Figure 6.5).

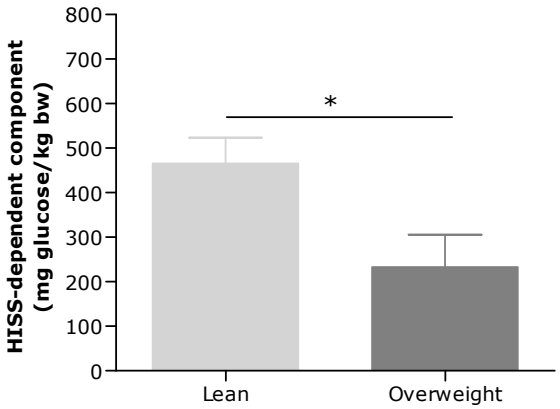


Figure 6.5 - The contribution of the HISS-dependent component of total insulin action is lower in overweight (n=4) than in lean (n=7) subjects. Results are means±SEM. *=$p<0.05$. Unpaired t-test.

6.3.1.4. Plasma insulin and C-Peptide levels during the fasted and fed RIST, in lean and overweight subjects

The evaluation of plasma insulin and C-peptide profiles during the RIST was done in fasted and fed state, in both lean (n=7) and overweight subjects (n=4). The fasted and fed insulinemia and C-peptide profiles in lean and overweight subjects were super-imposable and not significantly different at any time point (Figure 6.6 and Figure 6.7).

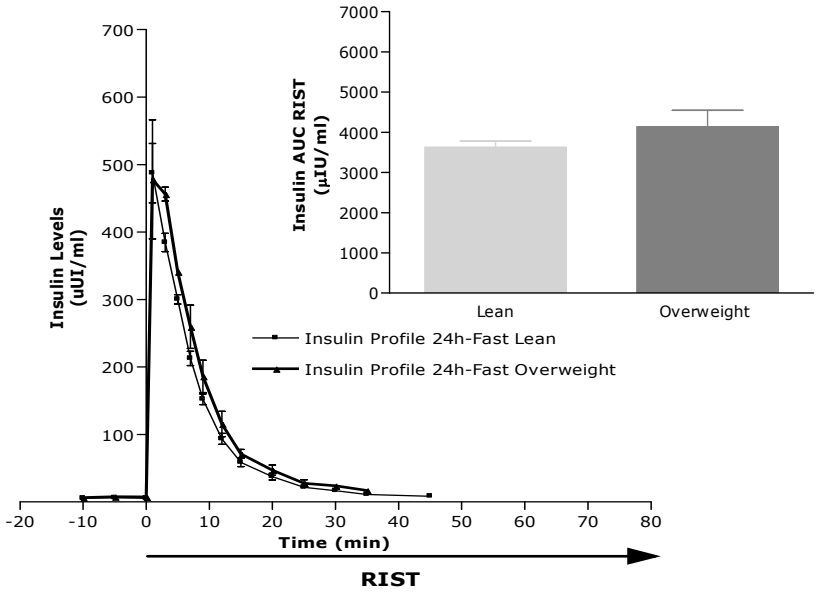
Fasted basal insulinemia and C-peptide levels both in lean and overweight subjects were similar (5.4±0.6 and 7.7±1.7μIU/ml, and 1.5±0.2 and 2.3±0.5ng/ml, respectively) and 1min after the insulin bolus injection of 50mU/kg the insulin levels spiked to similar concentration (487.3±44.0μIU/ml, in the lean group and 478.0±88.3μIU/ml, in the overweight group). At the end of the fasted RIST, approximately 42min for the lean group and 30min for the overweight group, plasma insulin levels returned to basal levels. The C-peptide levels remained stable and not statistically different from basal levels throughout the 24h-fast RIST, for both groups. The mean area under the curve (AUC) for insulin measured during the 24h-fast RIST in the lean and overweight subjects was similar (3633±156.7 and 4138±406.7μIU/ml, respectively) and the AUC for C-peptide was also not different between the lean and overweight group (15.8±4.2 and 10.8±1.7ng/ml, respectively) (Figure 6.6 (A) and Figure 6.7 (A)). In addition, the molar ratio of C-peptide to insulin during the 24h-fast

RIST was not significantly different between the lean and overweight groups (0.0044±0.0012 and 0.0026±0.0002, respectively), suggesting that hepatic extraction of insulin was not different between the two groups, during the 24h-fast RIST.

Both lean and overweight subjects were fed a standardized test meal and 100min after (the time required to achieve stable glycemia), the basal plasma insulin and C-peptide levels were similar in the two groups (39.5±6.4 and 39.9±8.1μIU/ml, and 9.1±1.0 and 9.5±0.6ng/ml, respectively). Immediately after the bolus of insulin (1min after starting the RIST), insulinemia reached a value of 501.6±30.2 (lean group) and 470.5±56.3μIU/ml (overweight group).

At the end of the fed RIST (≈70min), plasma insulin levels returned to basal plasma levels (lean subjects: 40.5±5.5 and overweight subjects: 33.9±3.1μIU/ml). As noticed already in the 24h-fast RIST, the C-peptide levels remained stable and not statistically different from basal levels throughout the fed RIST, for both groups. The mean AUC for insulin measured during the postprandial RIST in the lean and overweight subjects was similar (5926±919.3 and 4265±796.6μIU/ml, respectively) and the AUC for C-peptide were also not different between the lean and overweight group (136.3±27.1 and 102.5±36.5ng/ml, respectively) (Figure 6.6 (B) and Figure 6.7 (B)). In addition, the molar ratio of C-peptide to insulin during the postprandial RIST was not significantly different between the lean and overweight groups (0.0248±0.0052 and 0.0372±0.0235, respectively), suggesting that hepatic extraction of insulin was not different between the two groups, during the fed RIST. The plasma insulin and C-peptide profiles obtained during the 24h-fast and fed RIST, in both lean and overweight subjects are represented in Figure 6.6 and Figure 6.7, respectively.

(A)



(B)

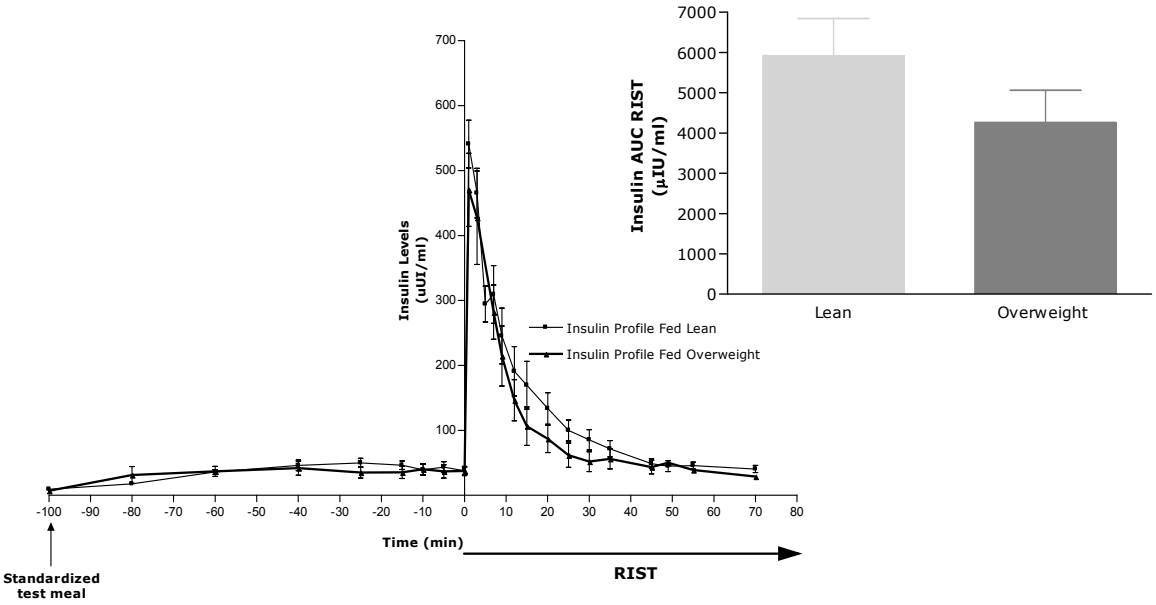


Figure 6.6 - Plasma insulin profiles obtained during the 24h-fast (A) and fed RIST (B), in both lean (n=7) and overweight (n=4) subjects. The regular line represents the insulin level profile obtained for the lean 24h-fast and fed RIST and the bold line represents the insulin level profile obtained for the overweight 24h-fast and fed RIST. Statistically significant differences were not seen at any time point. Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for 24h-fast RIST in both lean and overweight subjects. Right insert: Insulin area under the curve (AUC) calculated by the trapezoid rule for fed RIST in both lean and overweight subjects. Unpaired t-test.

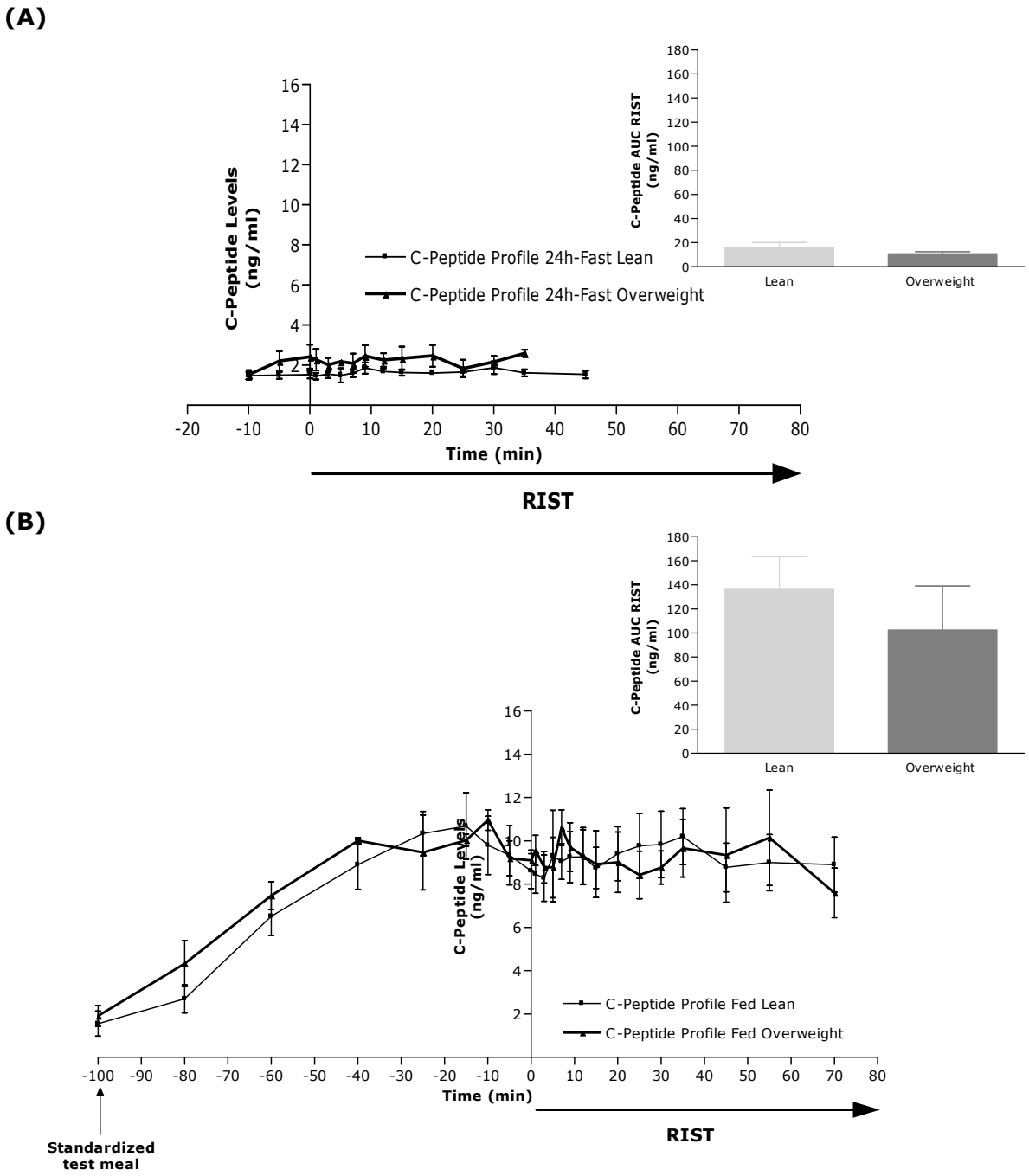


Figure 6.7 - Plasma C-peptide profiles obtained during the 24h-fast (A) and fed RIST (B), in both lean (n=7) and overweight (n=4) subjects. The regular line represents the C-peptide level profile obtained for the lean 24h-fast and fed RIST and the bold line represents the C-peptide level profile obtained for the overweight 24h-fast and fed RIST. Statistically significant differences were not seen at any time point. Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. Left insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for 24h-fast RIST in both lean and overweight subjects. Right insert: C-peptide area under the curve (AUC) calculated by the trapezoid rule for fed RIST in both lean and overweight subjects. Unpaired t-test.

6.4. DISCUSSION

The aim of the study described in this chapter was to characterize the meal-induced insulin sensitization (MIS) in overweight subjects, since it is well documented that adiposity is often associated with impaired glucose homeostasis, metabolic abnormalities, insulin resistance, type 2 diabetes, lipid disorders and cardiovascular disease (Astrup *et al.*, 2000; Kahn *et al.*, 2006; LeRoith, 2007; Weyer *et al.*, 1999b).

The meal-induced insulin sensitization (MIS), in lean and overweight subjects

When lean and overweight subjects were submitted to a 24h fasting period, when HISS is absent, the insulin action *per se*, is similar in both lean and overweight subjects (Figure 6.4). However, after a meal, the increase in insulin sensitivity was observed in the two groups, but was much more evident in the lean subjects. The lower MIS observed in the overweight subjects is associated with an impairment of the HISS-dependent component, since the contribution of the HISS-dependent component of total insulin action is lower in overweight than in lean subjects (Figure 6.5).

Our data are consistent with the hypothesis that insulin resistance observed in the fasted state is reversed by a meal, in both lean and overweight subjects. Furthermore, the MIS is much more pronounced in lean subjects (232.1±46.3%) in comparison to the overweight subjects (174.0±67.9%), suggesting that even mild overweight is already associated with a postprandial metabolic dysfunction.

The results of a clinical study performed by Petersen and colleagues are consistent with the HISS hypothesis. They showed that, in lean insulin-resistant subjects, skeletal muscle insulin resistance predates hepatic insulin resistance (Petersen *et al.*, 2007). The mechanism of the initiating skeletal muscle insulin resistance was not suggested, but is compatible with the postprandial (HISS-dependent insulin resistance) HDIR state.

The question of the causal relationship between obesity and HDIR cannot be directly dealt within our study, but the theory that absence of MIS leads to adiposity suggests that mild adiposity may show significant HDIR. If adiposity causes HDIR, the impairment in HISS action would not be expected to be large in mildly overweight subjects.

The incremental changes in glucose, insulin and C-peptide after feeding the standardized test meal, in lean and overweight subjects

The 24h-fast and fed basal values of glucose, insulin and C-peptide were not different between lean and overweight subjects (Table XIII). Since the overweight subjects recruited to this study were mildly overweight (BMI 27.7±0.4kg/m^2), one would not expect severe differences within basal values.

Following the ingestion of the meal, there was a rise in the glycemia, insulin and C-peptide levels which was expected due to food absorption and assimilation. As shown in Figure 6.1 and Table XIV, the augment of glucose, insulin and C-peptide levels was identical in both lean and overweight subjects. The area under the curve (AUC) calculated during 100min after the meal ingestion for glucose, insulin and C-peptide, as well as the molar ratio of C-peptide:insulin, were not statistically different when compared between the lean and overweight group (Figure 6.1, right insert). However, there was a trend toward an increase in the insulin and C-peptide AUCs which are in accordance with the results from Teff *et al.* (Teff *et al.*, 1999b). The peripheral C-peptide:insulin molar ratio have been assumed to reflect changes in hepatic insulin extraction both in steady-state and non-steady state conditions (Bonora *et al.*, 1983; Polonsky *et al.*, 1984). The C-peptide:insulin molar ratio is dependent upon the interaction of many factors related to the secretion, metabolism, distribution and half-lives of the C-peptide and insulin[26].

Since the overweight subjects admitted in this study were mildly overweight and they were not hyperinsulinemic, one would not expect to observe major differences between the C-peptide:insulin molar ratio, suggesting the hepatic insulin extraction was not different between the lean and overweight group, after the meal ingestion.

Insulin action and secretion, in lean and overweight subjects

Besides the differences observed in basal levels of insulin and C-peptide, between fasted and fed states in both lean and overweight subjects, the insulin and C-peptide profiles during the RIST at both prandial states, had similar patterns. These profiles indicate that the differences observed in the postprandial insulin sensitivity between the two groups studied are not related to the insulin action *per se* nor endogenous insulin secretion.

[26] To assume that changes in the C-peptide:insulin molar ratio reflect changes in hepatic insulin extraction is a simplistic approach to a complex metabolic problem (Ahrén *et al.*, 2003; Polonsky *et al.*, 1984).

In this study, we showed that even with the low degree of insulin sensitivity at the fasted state in both lean and overweight subjects, the increase of the insulin sensitivity after a meal should not be due to the insulin action *per se* (Figure 6.4), since the insulin profiles were super-imposable and the AUC of insulin was not statistically different in the two groups studied (Figure 6.6). Assuming this similarity between the insulin profiles, the differences between the postprandial insulin sensitivity among lean and overweight subjects could not be due to insulin action *per se*, suggesting that HISS action has an additive insulin-like effect. These results also point out the advantage for the use of the RIST as a method to evaluate the degree of insulin resistance/insulin sensitivity in the postprandial state.

Although in the recent past, evaluation of glycemic control has leaned heavily on assessments done in the fasted state, the importance of alterations in the postprandial periods has become increasingly recognized (Ceriello, 2005; Ceriello *et al.*, 2008a; Ceriello *et al.*, 2004; Hanefeld *et al.*, 2002; Woerle *et al.*, 2007).

Postprandial glucose excursions are among the most important risk factors contributing to type 2 diabetes, micro and macrovascular complications (Del Prato, 2002; Meier *et al.*, 2009). Alterations in real-life glucose metabolism can only be fully understood if both fasted and fed states are considered. As frank diabetes develops, postmeal glucose excursions continue to worsen (Monnier *et al.*, 2007).

Based on evidence to date, the International Diabetes Federation guidelines recommends implementing a comprehensive management programme that targets both fasting and postmeal glucose, which should be initiated simultaneously at any HbA_{1c} level to improve outcome in diabetes (Ceriello *et al.*, 2008a).

Some studies have also proposed that, loss of early insulin response to glucose and poor suppression of hepatic glucose output, are primarily responsible for postprandial hyperglycemia associated with impaired glucose tolerance (Mitrakou *et al.*, 1992; Pratley *et al.*, 2001). In contrast, under experimental conditions, defective insulin action was shown to contribute more to postprandial hyperglycemia than defective insulin secretion (Tripathy *et al.*, 2000).

HISS and Prediabetes

HISS action accounts for the increased response to insulin seen following a meal and the most straightforward method to quantitate MIS is to compare insulin sensitivity in the fasted state with the same determinations in the fed state. Most of the published studies evaluate insulin sensitivity in a fasted state, ignoring the fact that the first alterations of insulin sensitivity seem to occur in the postprandial state, in which the role of the HISS-dependent component of insulin action is crucial (Lautt, 2004).

Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are intermediate states in glucose metabolism that exist between normal glucose tolerance and overt diabetes. Insulin resistance and impaired β-cell function, the primary defects observed in type 2 diabetes, both can be detected in subjects with IGT and IFG. However, clinical studies suggest that the site of insulin resistance varies between the two disorders. While subjects with IGT have marked muscle insulin resistance with only mild hepatic insulin resistance, subjects with IFG have severe hepatic insulin resistance with normal or near-normal muscle insulin sensitivity. Both IFG and IGT are characterized by a reduction in early-phase insulin secretion, subjects with IGT also have impaired late-phase insulin secretion (Abdul-Ghani *et al.*, 2006).

The associated risk factors should benefit from efforts directed to early detection of abnormal MIS and to therapy directed toward the prevention or reversal of insulin resistance (Lautt et al., 2008).

Within this study, we show that overweight subjects have a decrease in insulin action due to an impairment of MIS process, associated with a decrease in HISS action. A decrease in insulin sensitivity in the overweight subjects was only detected in the fed state, thus demonstrating an insulin resistance state seen already with mild adiposity. Within our results, these mild overweight subjects at the fasted state were considered healthy, but when insulin sensitivity was assessed in the fed state they revealed impaired insulin sensitivity.

To summarize, the absence of HISS in the postprandial state might be the first metabolic defect that occurs in the prediabetic state. In this setting HISS dysfunction may contribute decisively to the progression of metabolic defects towards type 2 diabetes.

Addressing the several possible aetiologies for prediabetes will provide clues for an earlier diagnosis, efficient treatment of these conditions and possibilities of slowing the deterioration towards type 2 diabetes.

7. THE RELEVANCE OF GLUCAGON ON HISS-DEPENDENT INSULIN SENSITIVITY

7. THE RELEVANCE OF GLUCAGON ON HISS-DEPENDENT INSULIN SENSITIVITY

7.1. INTRODUCTION AND AIMS

We have recently proposed that the regulation of insulin action by the prandial status is dependent on hepatic glutathione (GSH) content, which is known to be strongly related to the nutritional status (Guarino *et al.*, 2003; Tateishi *et al.*, 1977). This hypothesis was highlighted by the observation that hepatic GSH depletion produced by administration of the γ-glutamylcysteine synthase inhibitor, L-buthionine-[S,R]-sulfoximine (BSO), produced insulin resistance in a degree similar to that observed after hepatic nitric oxide synthase (NOS) inhibition (Guarino *et al.*, 2003). Therefore, both GSH depletion and NOS antagonism inhibit the insulin-sensitizing signal in the liver by blocking Hepatic Insulin Sensitizing Substance (HISS) action. Moreover, exogenous nitric oxide (NO) was not able to restore insulin action in BSO-treated rats in contrast to sham rats, which suggests that both GSH and NO are required in the liver to allow full peripheral insulin action (Guarino *et al.*, 2003). Also, the reduced insulin sensitivity observed in the fasting state is only reversed after co-administration of GSH and NO into the liver (Guarino *et al.*, 2006).

Considering that hepatic GSH is a crucial factor for HISS action, it becomes imperative to understand the mechanisms involved in the regulation of hepatic GSH levels. An important regulator of hepatic GSH levels is the cAMP pathway, since it has been shown that a rise in hepatic cAMP levels is associated with a decrease in hepatic GSH levels (Goss *et al.*, 1994; Higashi *et al.*, 1976; Lauterburg *et al.*, 1981; Lu *et al.*, 1990; Lu *et al.*, 1991; Tateishi *et al.*, 1974).

Glucagon is a pancreatic hormone, released in the fasted state in order to maintain an adequate level of blood glucose. According, to Lu and colleagues, glucagon's effects, mediated via cAMP, decrease hepatic GSH levels (which are reduced in the fasted state and raised in the postprandial state) due to an inhibition of the enzyme γ-glutamylcysteine synthase, which plays a key role in GSH synthesis (Lu *et al.*, 1990; Lu *et al.*, 1991).

In healthy subjects, plasma glucagon levels increase with fasting and decrease in the immediate postprandial state (Ahrén, 2006; Butler *et al.*, 1991), which is the inverse of GSH pattern (Leeuwenburgh *et al.*, 1996; Vogt *et al.*, 1993). However, in type 2 diabetes patients, plasma levels of glucagon are often abnormally high, even after ingestion of a meal and may contribute to impaired glucose tolerance and other metabolic changes, suggesting that the cAMP signaling pathway is upregulated in insulin-resistant individuals (Butler *et al.*,

1991; Dunning *et al.*, 2007; Meier *et al.*, 2006). These observations lead us to speculate about a possible role of glucagon on hepatic GSH depletion, which can be related with postprandial insulin resistance and decreased insulin sensitivity in the fasted state.

Glucagon has also been shown to be a hormone associated with hyperglycemia and hyperinsulinemia. This hyperinsulinemia is not able by itself to compensate for the observed hyperglycemia, leading to the question of which mechanism is thus responsible for this insulin resistance (Lechin *et al.*, 2006).

Therefore, the main objective of the present study was to test the hypothesis that glucagon modulates the hepatic GSH content, through the activation of the cAMP pathway, resulting in a state of HISS-dependent insulin resistance (HDIR).

7.2. PROTOCOLS

In this chapter, 9-weeks old male Sprague-Dawley (SD) rats (319.4±7.6g) were used in all experimental protocols. All animals were fasted for 8h, and then allowed *ad libitum* access to food for 2h to ensure postprandial state, as described in chapter 3, section 3.3.2.

7.2.1. Hepatic effect of DBcAMP, a cAMP analogue, on insulin sensitivity

The main goal of these experiments was to evaluate the hepatic effect of DBcAMP on insulin sensitivity.

After a control RIST in the fed, DBcAMP was infused ipv at different doses ranging from 0.01 to 1.0mg/kg, for 10min (t=-10min) at an infusion rate of 0.04ml/min. The second RIST was carried out after DBcAMP ipv infusion. During the experiment, blood samples were taken at specific time points, for insulin analysis. The detailed experimental protocol is described in chapter 3, Section 3.3.2.1.

7.2.2. Hepatic effect of glucagon, on HISS-dependent insulin sensitivity

The major objective of these experiments was to evaluate the hepatic effect of glucagon on HISS-dependent sensitivity. This protocol was divided into 2 different series.

In the **first series**, after a control RIST, glucagon was infused ipv at different doses ranging from 0.5ng/kg to 20μg/kg, for 10min (t=-10min) at an infusion rate of 0.04ml/min. The second RIST was carried out after glucagon infusion. The detailed experimental protocol is described in chapter 3, Section 3.3.2.2.

In the **second series**, after a control RIST, L-NMMA was infused ipv at a dose of 0.73mg/kg for 10min at an infusion rate of 0.04ml/min. The second RIST was carried out after L-NMMA ipv infusion. Then, glucagon was infused ipv at 200ng/kg for 10min at an infusion rate of 0.04ml/min and a third RIST was carried out. The detailed experimental protocol is described in chapter 3, Section 3.3.2.2.

7.3. RESULTS

7.3.1. Hepatic effect of DBcAMP, a cAMP analogue, on insulin sensitivity

7.3.1.1. Effect of DBcAMP on mean arterial pressure and arterial glycemia

Preliminary experiments were performed to analyze the ipv DBcAMP infusion effect on arterial glycemia. Different doses of DBcAMP (0.01, 0.1 and 1mg/kg) were tested to achieve the dose that did not interfere with the glycemia during the assessment of the insulin sensitivity (Figure 7.1). The DBcAMP was infused during 10min; at minute 0, the glycemic profile was started.

The values of mean arterial pressure were not significantly altered by the DBcAMP doses ipv infused (DBcAMP 0.01mg/kg: from 89.5±8.0 to 90.8±8.0mmHg; DBcAMP 0.01mg/kg: from 113.0±4.0 to 114.7±2.2mmHg; DBcAMP 1mg/kg: from 93.0±4.0 to 98.0±13.0mmHg).

Basal glycemia levels, in the fed state, measured before DBcAMP ipv infusion (t=-10min), did not change after DBcAMP 0.01mg/kg infusion (t=0min) (from 119.3±5.8 to 122.03±7.2mg/dl). The DBcAMP 0.1 and 1mg/kg doses increased glycemia levels (DBcAMP 0.1mg/kg: from116.3±10.5 to 126.7±14.2mg/dl, $p<0.05$; DBcAMP 1mg/kg: from122.6±6.1 to 187.8±25.3, $p<0.05$mg/dl).

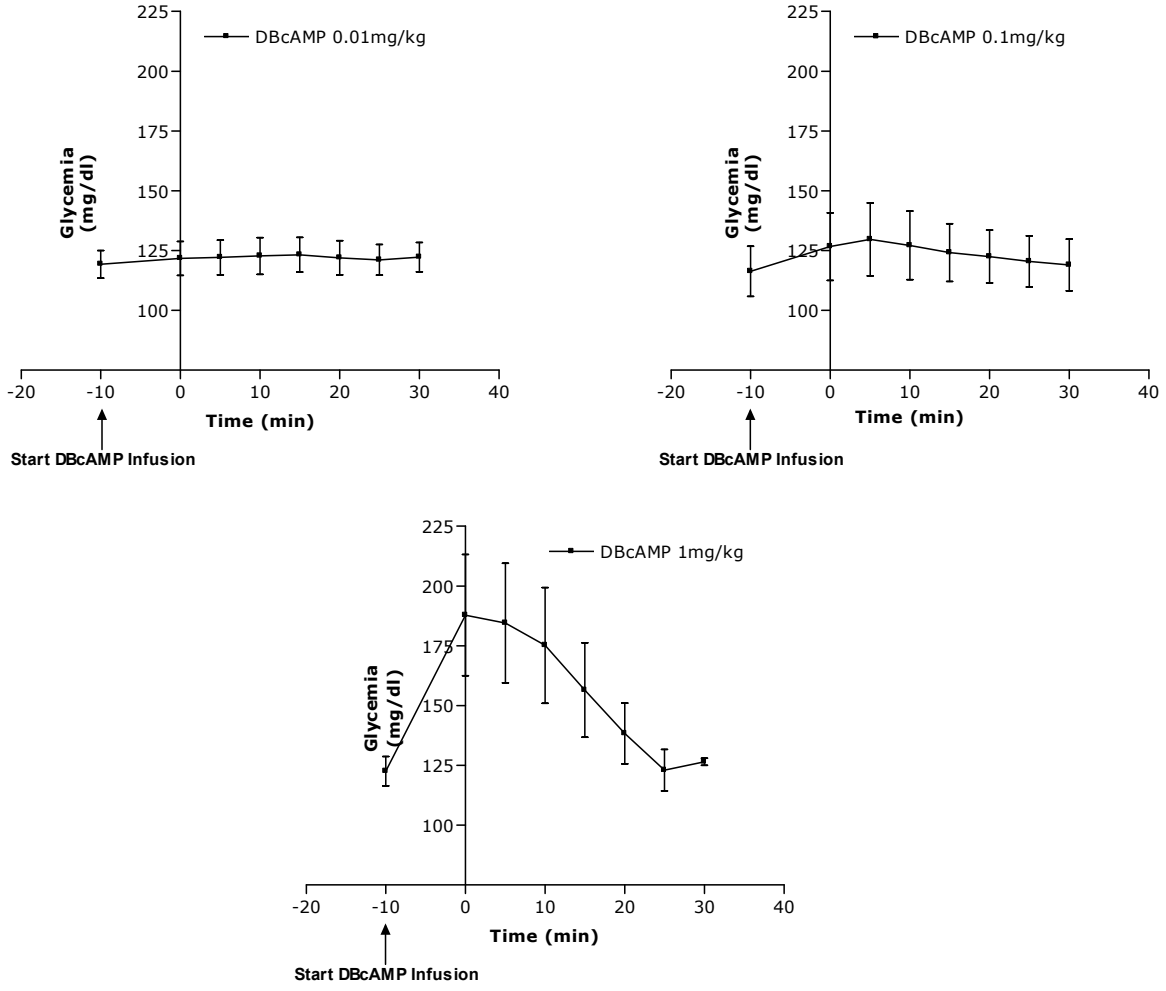


Figure 7.1 - Glycemic profile at specific time points determined after DBcAMP 0.01, 0.1 and 1mg/kg ipv infusion. Results are means±SEM, n=10.

From these results, the ipv DBcAMP 0.01mg/kg dose was the dose that not has an effect on the glycemia levels. Even though DBcAMP doses of 1 and 0.1mg/kg had an initial effect on glycemia, but by the time insulin sensitivity was assessed (post-DBcAMP RIST, ≈30min after DBcAMP infusion), the glucose levels were similar to controls.

7.3.1.2. Effect of DBcAMP on insulin sensitivity

In this protocol, we evaluated ipv DBcAMP effect on insulin sensitivity and also if it was able to produce a similar degree of insulin resistance to that obtained with other pharmacological tools, previously used to evaluate the HISS-dependent insulin sensitivity. The results are summarized in Figure 7.2 and Figure 7.3.

The control RIST index was 172.3±6.3mg glucose/kg bw and decreased to 125.7±8.3mg glucose/kg bw after ipv DBcAMP infusion (n=3, $p<0.01$), corresponding to a 27.2±2.1% inhibition of insulin sensitivity (Figure 7.2 and Figure 7.3).

When the ipv DBcAMP administration was increased to 0.1mg/kg, the RIST index changed from 165.7±26.2 mg glucose/kg bw (control RIST index) to 77.0±7.5mg glucose/kg bw (post-DBcAMP 0.1mg/kg RIST index, n=2, $p<0.05$), corresponding to an insulin sensitivity inhibition of 51.6±12.2% (Figure 7.2 and Figure 7.3).

With the higher ipv DBcAMP dose tested (1mg/kg), the control RIST index was 173.2±24.0mg glucose/kg bw and decreased to 98.1±38.0mg glucose/kg bw after ipv DBcAMP infusion (n=3, $p<0.05$), corresponding to a 47.2±14.1% inhibition of insulin sensitivity (Figure 7.2 and Figure 7.3).

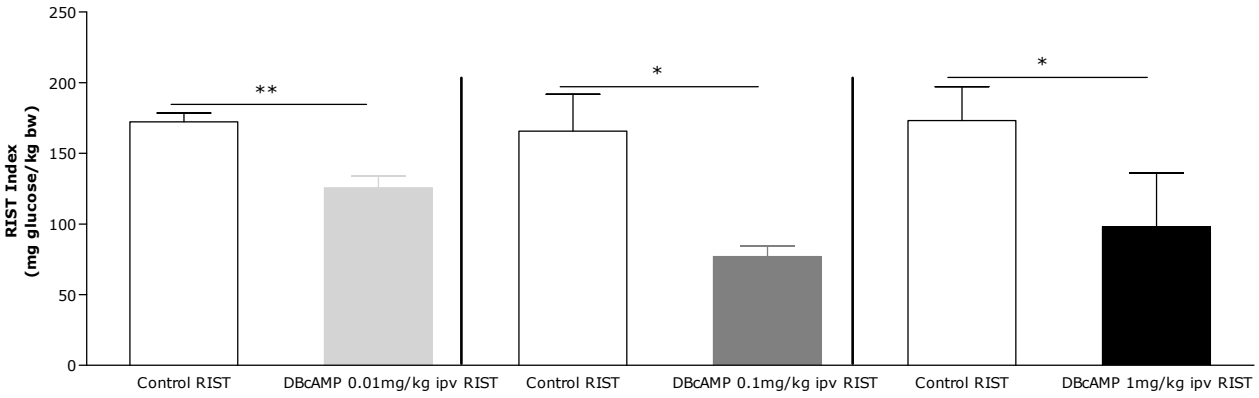


Figure 7.2 - Insulin sensitivity decreases after DBcAMP 0.01, 0.1 and 1mg/kg ipv infusion. Results are means±SEM. Paired t-test. **=p<0.01, *=p<0.05.

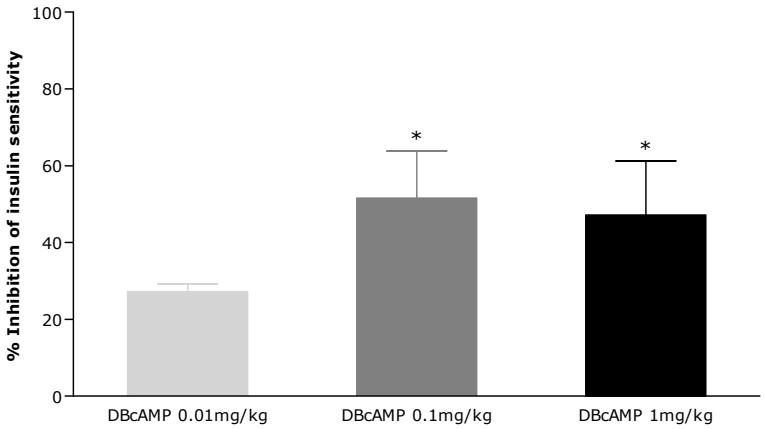


Figure 7.3 - Insulin sensitivity decreases after DBcAMP 0.01mg/kg infusion by 27.2±2.1%, after DBcAMP 0.1mg/kg infusion by 51.6±12.2% and after DBcAMP 1mg/kg infusion by 47.2±14.1%. Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test. *=p<0.05 *vs* DBcAMP 0.01mg/kg.

From the results of the 3 different doses of DBcAMP infused, the doses of 1 and 0.1mg/kg were able to show a similar degree of insulin resistance to that obtained with other pharmacological tools (e.g., atropine, L-NMMA) that were previously used to evaluate the HISS-dependent insulin sensitivity.

7.3.1.3. Effect of DBcAMP on insulin levels

In all the protocols, the different doses of DBcAMP did not affect insulin levels measured before the control RIST, after the DBcAMP infusion nor at the end of the DBcAMP RIST (Figure 7.4).

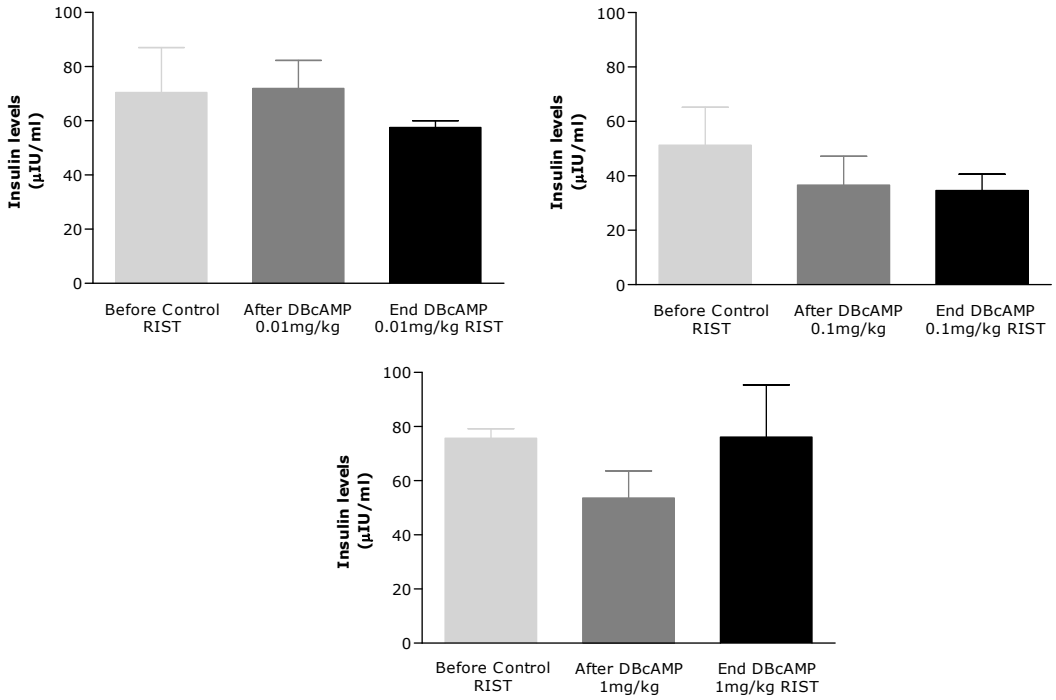


Figure 7.4 – Effect of DBcAMP on plasma insulin levels. Insulin levels were not altered by the 3 doses of ipv DBcAMP infusion. Results are means±SEM. One-way ANOVA, followed by the Tukey-Kramer multiple-comparison test.

7.3.2. Hepatic effect of glucagon, on HISS-dependent insulin sensitivity

7.3.2.1. Effect of glucagon on arterial glycemia

Preliminary experiments were performed to analyze the ipv glucagon infusion on arterial glycemia. In the fed state, different doses of glucagon (0.5, 1, 2.5, 5, 10, 200ng/kg, 2 and 20µg/kg) were infused to evaluate the glycemic profile after each dose infusion (Figure 7.5). The glucagon was infused during 10min; at minute 0, the glycemic profile was started.

The values of mean arterial pressure were not significantly altered by the glucagon doses ipv infused (Table XVI).

Table XVI – Effect of ipv glucagon infusion on arterial pressure (n=14).

Glucagon dose	Arterial pressure before glucagon infusion (mmHg)	Arterial pressure after glucagon infusion (mmHg)
0.5ng/kg	69.0	85.0
1ng/kg	103.3±7.8	94.5±14.2
2.5ng/kg	90.0	94.0
5ng/kg	108.0	107.0
10ng/kg	106.5±10.5	117.5±3.5
200ng/kg	118.3±9.0	115.0±6.8
2μg/kg	111	96
20μg/kg	104	93

Basal glycemia levels, in the fed state, before glucagon ipv infusion (t=-10min), did not change after glucagon 0.01mg/kg infusion (t=0min). The glucagon 200ng/kg, 2 and 20μg/kg doses increased glycemia levels (Table XVII).

Table XVII - Effect of ipv glucagon infusion on arterial glycemia (n=14).

Glucagon dose	Glycemia before glucagon infusion (mg/dl)	Glycemia after glucagon infusion (mg/dl)
0.5ng/kg	104.5	102.5
1ng/kg	109.0±3.7	111.3±4.1
2.5ng/kg	113.1	115.5
5ng/kg	102.5	104.5
10ng/kg	113.4±0.9	118.0±0.0
200ng/kg	104.1±1.2	116.3±3.6
2μg/kg	128.0	189.0
20μg/kg	104.0	176.5

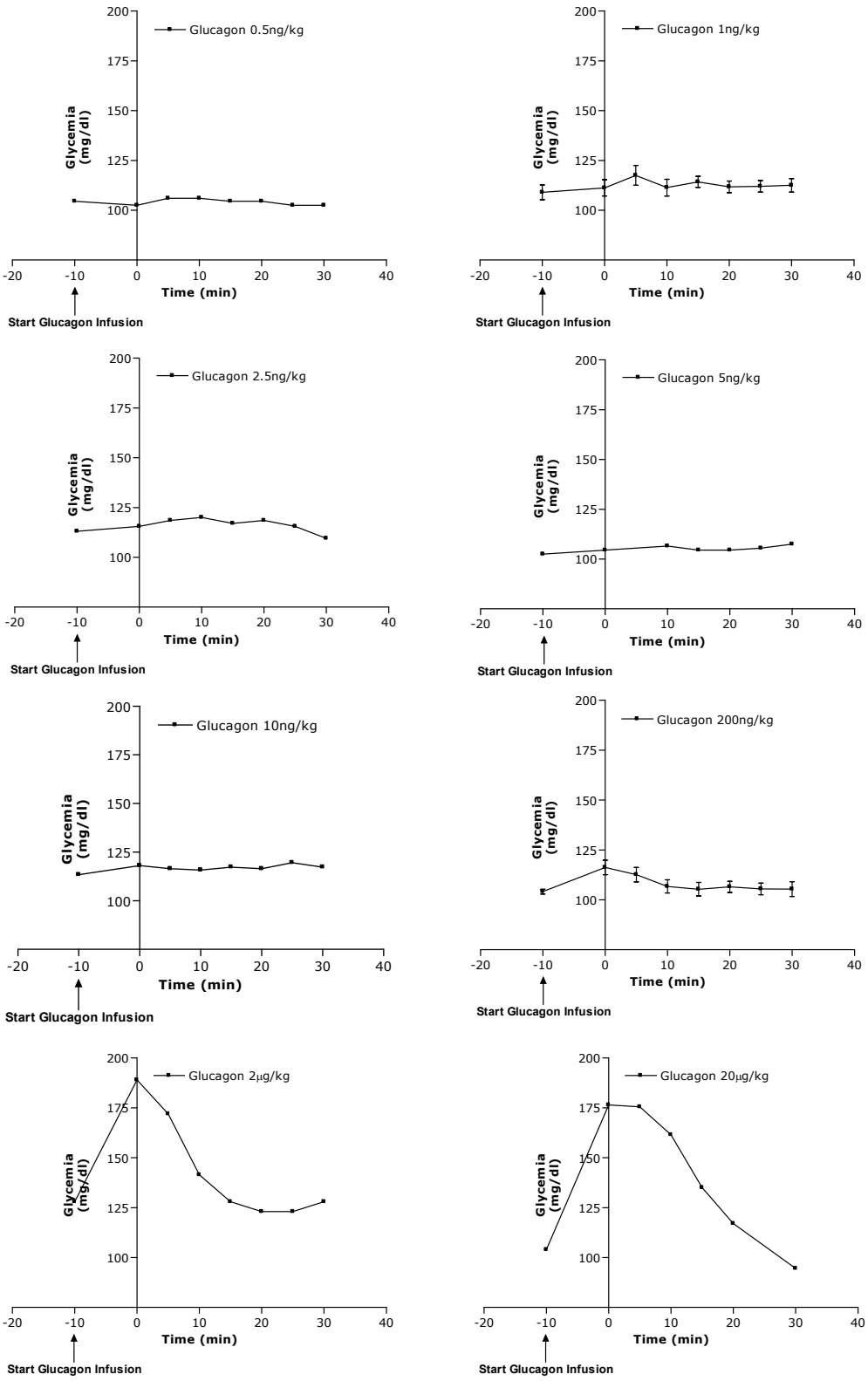


Figure 7.5 – Glycemic profile at specific time points determined after glucagon 0.5, 1, 2.5, 5, 10, 200ng/kg, 2 and 20μg/kg ipv infusion. Results are means±SEM, n=14.

As shown in Figure 7.5, the higher ipv doses of glucagon tested (20 and 2μg/kg) induced a significant increase in glucose levels after its infusion. However, by the time that insulin sensitivity was assessed (post-glucagon RIST, ≈30min after glucagon infusion), the glycemia was already returned to the pre-established baseline glycemia before glucagon infusion.

7.3.2.2. Effect of glucagon on insulin sensitivity

Given the results of the first glycemic profile experiments (Section 7.3.2.1.), the next step was to evaluate the ipv glucagon effect on insulin sensitivity and determined if it is able to produce a similar degree of insulin resistance to that obtained with other pharmacological tools (e.g., L-buthionine-[*S,R*]-sulfoximine, BSO), which were previously used to evaluate the HISS-dependent insulin sensitivity.

As shown in Figure 7.6 and, Table XVIII all the different doses of glucagon tested that were previously ipv administered produced a decrease in insulin sensitivity in a dose-dependent manner.

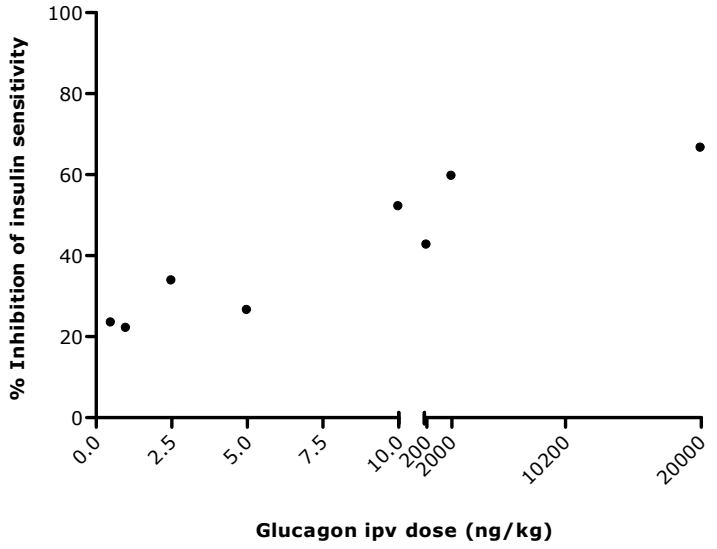


Figure 7.6 - Insulin sensitivity decreases after glucagon in a dose-dependent manner, for the ipv glucagon doses tested. Results are means±SEM, n=14.

Table XVIII - Percentage of inhibition of insulin sensitivity after ipv glucagon infusion (n=14).

Glucagon dose (ng/kg)	% inhibition of insulin sensitivity
20000	≈67
2000	≈60
200	≈43
10	≈52
5	≈27
2.5	≈34
1	≈22
0.5	≈23

7.3.2.3. Effect of glucagon on HISS-dependent insulin sensitivity

Further studies were performed with a glucagon dose of 200ng/kg. This dose produced an hyperglycemia at the first minute after ipv infusion, but stabilized immediately at the basal levels. Simultaneously, it was able to decrease insulin sensitivity to the same level obtained with other pharmacological or surgical tools previously used to evaluate the HISS-dependent insulin sensitivity. The HISS pathway can be inhibited with several procedures such as, hepatic surgical denervation, blockade of hepatic muscarinic cholinergic receptors, hepatic NOS blockade, and depletion of hepatic glutathione levels.

With the 200ng/kg ipv glucagon dose, the control RIST index was 173.0±5.3mg glucose/kg bw and decreased to 96.1±9.7mg glucose/kg bw after ipv glucagon 200ng/kg infusion (n=4, $p<0.05$), corresponding to a 42.6±6.5% inhibition of insulin sensitivity (Figure 7.7).

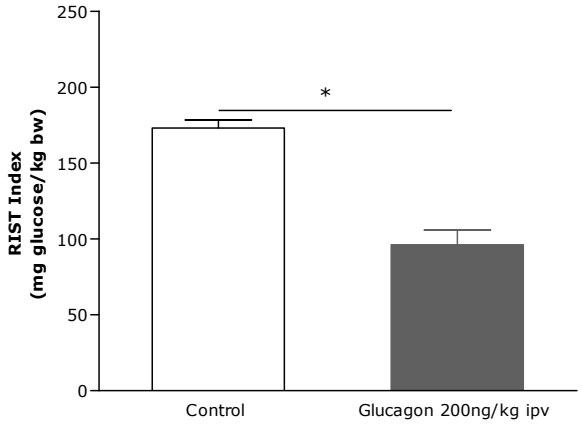


Figure 7.7 - Insulin sensitivity decreases after glucagon 200ng/kg ipv infusion. Results are means±SEM, n=4. Paired t-test. *=p<0.05.

To ascertain the role of glucagon on the HISS pathway, the pathway was inhibited with L-NMMA (a NOS specific inhibitor), in order to decrease HISS-dependent insulin sensitivity, and after, glucagon was ipv infused.

In this series of experiments, the control fed RIST index was 177.1±1.6mg glucose/kg bw and after ipv L-NMMA infusion the RIST index decreased to 81.0±8.3mg glucose/kg bw (n=5, p<0.001). Glucagon infusion did not alter the RIST index (80.4±5.9mg glucose/kg bw, n=5, Figure 7.8). Indeed, the percentages of inhibition of insulin sensitivity after administration of L-NMMA and glucagon were 54.2±4.9 and 54.6±3.5%, respectively. These results suggest that in HISS-impaired animals, glucagon does not produce an additional insulin resistance.

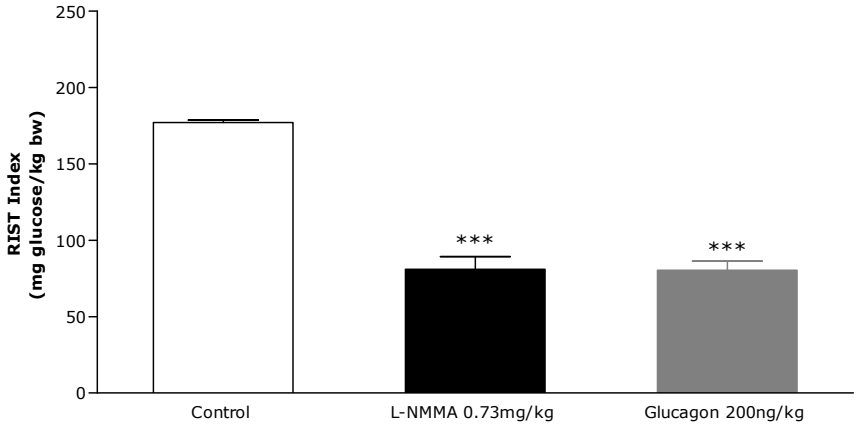


Figure 7.8 – Effect of L-NMMA and glucagon on insulin sensitivity. The insulin sensitivity decreased after ipv L-NMMA 0.73mg/kg infusion and did not change after ipv glucagon 200ng/kg infusion. Results are means±SEM, n=5. Repeated measures ANOVA, followed by the Tukey-Kramer multiple-comparison test. ***=p<0.001 Control *vs* L-NMMA 0.73mg/kg and Control *vs* Glucagon 200ng/kg.

7.4. DISCUSSION

The study in this chapter aimed to understand the highly regulated pathway involved in the glutathione (GSH) synthesis, controlled by 3',5'-cyclic adenosine 5'-monophosphate (cAMP) and/or glucagon action, and its repercussions on HISS-dependent insulin resistance (HDIR). Since it has been reported that glucagon, which increases intracellular cAMP concentration, decreases hepatic GSH content due to a decrease in enzymatic activity of γ-glutamylcysteine synthase (Lu *et al.*, 1991), and GSH is an important factor for HISS synthesis/release (Guarino *et al.*, 2003; Guarino *et al.*, 2006), it becomes important to understand the relationship between HISS action and glucagon.

Our data show that both DBcAMP (N^6,2′-O-dibutyryladenosine 3',5'-cyclic monophosphate), a cAMP analog, and glucagon produce a decrease of insulin sensitivity in a dose-dependent manner.

The HDIR produced by N-monomethyl-L-arginine (L-NMMA) was not aggravated by glucagon, suggesting that glucagon, leads to reduced insulin sensitivity through a decrease of HISS action and not via some other indirect action.

The two observations together are in support of our initial hypothesis that glucagon acts via cAMP pathway to decrease hepatic GSH levels, leading to an impairment of HISS synthesis/release resulting in an insulin resistance state responsible for the development of hyperglycemia and type 2 diabetes.

Hepatic cAMP and HISS-dependent insulin resistance

The effect of glucose on insulin secretion can be amplified by signaling pathways involving inositol trisphosphate and diacylglycerol derived from activation of phospholipase C (Gilon *et al.*, 2001; Howell *et al.*, 1994) and by cAMP following activation of adenylate cyclase (Howell *et al.*, 1994).

Glucose itself has long been known to elevate the pancreatic islet β-cell cAMP content (Grill *et al.*, 1973). This cyclic nucleotide is generally accepted as an important amplifier of glucose-induced insulin release (Holz *et al.*, 1992), particularly when its levels are increased by glucose itself (Harndähl *et al.*, 2002). It has also been suggested that cAMP is a competence factor for normal islet β-cell responsiveness to glucose (Huypens *et al.*, 2000; Schuit, 1996). Numerous hormones that stimulate insulin secretion increase islet β-cell cAMP, including glucagon (Huypens *et al.*, 2000).

Previous studies on rat β-cells *in vitro* have suggested that insulin release is synergistically regulated by signaling molecules derived from glucose metabolism on the one hand and adenylate cyclase stimulation by glucagon or related peptides on the other (Schuit *et al.*, 1985). In rodent β-cells, regulation of the cAMP-dependent signaling pathway has been shown to depend on expression of specific receptors for glucagon (Unson *et al.*, 1989).

From our results, both ipv DBcAMP and glucagon infusion at different doses decreased insulin sensitivity (Figure 7.2Figure 7.2 and Figure 7.6). The insulin sensitivity results were not affected by the insulin levels, since insulinemias were not different before and after each RIST (Figure 7.4). Therefore, the main DBcAMP effect is not on insulin levels, but in its action instead. Indeed, it is proposed that increased cAMP level leads to a decrease of GSH and consequently to a state of insulin resistance.

Based on our glucagon results, after blocking HISS-dependent insulin sensitivity with L-NMMA, the subsequent ipv infusion of glucagon did not aggravate the insulin resistance in fed animals (Figure 7.8). These results showed that glucagon caused a state of HDIR. The glucagon infusion in the fed state may mimic the fasted state with respect to glucagon, hepatic cAMP and GSH levels, since glucagon is an hormone responsible for the regulation of GSH levels through the cAMP pathway (Lu *et al.*, 1991).

Intracellular cAMP levels depend on the balance between its formation through the activity of adenylate cyclase and its destruction by cAMP-degrading enzymes known as phosphodiesterases (PDEs). The activity of two of these cAMP-PDEs (PDE2A and PDE3B)[27] in

[27] PDE2A is activated by cGMP, leading to cAMP hydrolysis. In contrast, PDE3B is inhibited by cGMP allowing cAMP intracellular accumulation (Houslay, 1995).

the liver is dependent on cGMP levels which are released through NO action[28] (Beavo, 1995). Our group reported that hepatic NO/cGMP is essential for HISS action (Guarino *et al.*, 2004), which strongly suggests that cGMP-dependent cAMP-PDEs may represent fine regulators of the HISS pathway, by controlling GSH levels through a cAMP/cGMP crosstalk (Conti, 2000; Houslay, 1995).

In type 2 diabetes, the postprandial high glucagon levels, could compromise HISS secretion/release, due to a decrease of hepatic GSH levels. This impairment on HISS secretion/release will aggravate the insulin resistance state, leading to an increase of glucose excursions. This is in agreement with an imbalance of the insulin:glucagon molar ratio, since this ratio mainly affects hepatic glucose production (Unger, 1971). Because of the reduction of the insulin:glucagon molar ratio, basal endogenous glucose concentration will be higher causing fasting hyperglycemia, while the hepatic glucose output will not be efficiently suppressed after the ingestion of a meal, contributing to excessive postprandial glucose rise. The defect in insulin secretion is coupled with inappropriate secretion of glucagon. This results in significant changes in the portal insulin:glucagon molar ratio causing impaired regulation of glucose handling at the level of the liver (Del Prato *et al.*, 2004).

Hyperglucagonemia and Diabetes

When glucagon interacts with its receptor on liver cells, adenylate cyclase is activated and intracellular cAMP concentrations rise. The increase in cytosolic cAMP activates the all-important PKA which, by phosphorylating certain key enzymes, induces the major hepatic actions of glucagon (stimulation of glycogenolysis, gluconeogenesis and, ketogenesis and inhibition of glycogen synthesis, glycolysis and lipogenesis) (Unger, 1971). Glycogenolysis is thereby increased and glycogenesis inhibited. Insulin opposes this action largely by reducing PKA activity (Gabbay *et al.*, 1984). When insulin is not present, the unopposed action of glucagon greatly increases cAMP and PKA, and initiates the catabolic cascade (Unger, 1985). But, if glucagon is not present, cAMP and PKA are low and a major site of insulin action on hepatic fuel metabolism is eliminated. This explains why the excessive hepatic fuel production that characterizes the insulin-deficient state does not occur in the total absence of glucagon (Unger, 1985).

[28] Binding of NO to guanylate cyclase leads to generation of the second messenger cGMP (Beavo, 1995).

More than 30 years ago, Unger and Orci proposed the bihormonal hypothesis to explain the pathophysiology of diabetes (Unger *et al.*, 1975). According to this hypothesis, this metabolic disease is the result of an insulin deficiency or resistance along with an absolute or relative excess of glucagon, which can cause a higher rate of hepatic glucose production than glucose utilization, favouring hyperglycemia. The rate of hepatic glucose output has been correlated with the hyperglycemia found in animal models of diabetes, as well as, in human diabetes, and the maintenance of this abnormality has also been associated with hyperglucagonemia (Dunning *et al.*, 2007; Gastaldelli *et al.*, 2000; Li *et al.*, 2008).

In type 2 diabetes, the impairment of insulin release and development of insulin resistance is often accompanied by absolute or relative increased levels of glucagon in the fasting and postprandial states (Butler *et al.*, 1991; Larsson *et al.*, 2000b). In this situation, insulin is not effective as a negative feedback for hepatic glucose output, while glucagon potentiates glucose mobilization from the liver, thus contributing to hyperglycemia.

Another malfunction reported in diabetic patients is the lack of suppression of glucagon release in hyperglycemic conditions, which would contribute further to postprandial hyperglycemia, in type 2 diabetes (Dinneen *et al.*, 1995; Henkel *et al.*, 2005; Shah *et al.*, 2000). However, this irregular α-cell behaviour does not occur when insulin levels are adequate, suggesting that abnormalities in glucagon release are relevant for hyperglycemia in the context of diabetes or impairment of insulin secretion or action (Shah *et al.*, 1999). All these problems in the glucagon secretory response observed in diabetes have been attributed to several defects in α-cell regulation including defective glucose sensing, loss of β-cell function, insulin resistance or autonomic malfunction (Quesada *et al.*, 2008).

However, one could speculate about the involvement of glucagon on HISS pathway, and their implication on insulin sensitivity regulation. The sustained high levels of glucagon, usually seen in type 2 diabetes, could be due to a decrease in hepatic GSH levels that leads to a decrease in HISS secretion/action, culminating in a state of insulin resistance.

Since insulin resistance anticipates diabetes onset by 15 years, all studies that contribute to its characterization and pharmacological control should be considered a priority. The studies in this chapter suggest new directions to understand the highly regulated pathway evolved in the GSH formation controlled by hormones that affect the cAMP pathway and its repercussions in insulin resistance.

8. GENERAL DISCUSSION

8. GENERAL DISCUSSION

The results presented in this PhD thesis regard both human and animal studies. The human experiments were designed to develop a new technique to evaluate insulin sensitivity in humans, both in the fasted and fed state, and also to characterize the role of a novel neurohumoral regulatory mechanism - the HISS hypothesis - that controls peripheral insulin sensitivity. Furthermore, the involvement of the cholinergic system in the HISS pathway was evaluated and observed to be inhibited by a cholinergic antagonist, in the fed state.

The HISS pathway was likewise evaluated in lean and overweight subjects. This study is consistent with data from several other species, where the hepatic parasympathetic nerves also played a role in the HISS synthesis/release.

Glucagon is a counter-regulatory hormone which levels are generally higher in type 2 diabetic patients, particularly after a meal. The involvement of glucagon on HISS pathway and their implication on insulin sensitivity regulation was further observed. HISS-dependent insulin sensitivity can be affected by glucagon and this might be due to a decrease in the hepatic cAMP/GSH pathway. The constant hyperglucagonemia, usually seen in type 2 diabetics, could be a result of a decrease in hepatic GSH levels, which is known to decrease HISS secretion/action, culminating in a state of insulin resistance.

8.1. METHODOLOGICAL CONSIDERATIONS IN THE ASSESSMENT OF HUMAN WHOLE-BODY INSULIN SENSITIVITY

The studies described in Chapter 4, suggest that the Rapid Insulin Sensitivity Test (RIST), is a valid technique to evaluate whole-body insulin sensitivity *in vivo* in humans, with the ability to be used in both fasted and fed state, with high sensitivity and reproducibility. The decision to develop the RIST in humans was based on the several advantages that this method presents regarding to other methods of insulin sensitivity assessment, as described below.

In animals, the RIST, which is a modified euglycemic clamp, was already described as being a powerful research tool to assess the glucose utilization action of an insulin bolus in fasted and fed states, both evaluated in the same day.

The RIST has been only described in laboratory animals, such as rats (Lautt *et al.*, 1998a), cats (Xie *et al.*, 1996c) and mice (Latour *et al.*, 2002a). In animals, it offers advantages over other forms of insulin sensitivity testing for a number of reasons. As many as four consecutive RIST's can be carried out in the same anaesthetized animal on the same

day. Moreover, glucose, insulin, glucagon and catecholamine levels remain unchanged between tests (Afonso *et al.*, 2007b; Xie *et al.*, 1995a). The RIST is extremely sensitive and can be shown to generate very clear dose-response relationships to insulin and muscarinic receptor antagonism. This insulin sensitivity test, showed that insulin sensitivity is highest in the immediately postprandial state and lowest in the fasted state, when insulin action is not useful or desirable (Lautt *et al.*, 2001). The RIST index (parameter to evaluate insulin sensitivity) is physiologically relevant; it does not only need to be carried out in the fasted state, and reveals that insulin sensitivity is normally extremely low in the conditions required for other tests.

Several other studies have been tried to evaluate postprandial insulin action, like the oral glucose tolerance test (OGTT) and the meal tolerance test (MTT). Regarding to the OGTT, it was shown by Sadri *et al.* that glucose *per se* was not able to activate the HISS pathway, and therefore does not induce a maximal insulin action (Sadri *et al.*, 2006). Thus, the OGTT is only sensitive to changes related to the loss of insulin action *per se* (independent of HISS). Moreover, the MTT has been found to be a more potent stimulator of insulin action than glucose alone (Berthiaume *et al.*, 2002), it is able to better mimic the conditions of lifestyle nutritional conditions, it is more capable to discriminate among non-diabetic, prediabetic and type 2 diabetic subjects than the OGTT, and it is a more consistent and reproducible method than the OGTT (Wolever *et al.*, 1998). So, the MTT is much more physiological and accurate method to evaluate postprandial insulin action than the OGTT.

The RIST was specifically designed to avoid the counter-regulatory responses to hypoglycemia that follows an insulin bolus, as administered in an insulin tolerance test (ITT) and also to prevent the non-physiological approach of the maintained continuous hyperinsulinemia throughout the hyperinsulinemic euglycemic clamp (HIEC), since these were the most commonly used tests to evaluate insulin sensitivity.

The RIST allows us to quantify HISS-dependent and HISS-independent insulin action in a series of studies that suggest that this novel pathway is of major physiological, pathological, and therapeutic importance.

While the ITT is equally able to detect HISS action, the ensuing hypoglycemia requires a longer time to reach baseline, has the danger of unpleasant effects from the hypoglycemia, and has the complication of interference by counter-regulatory mechanisms to restore euglycemia. This latter complication prevents full dynamic estimation of HISS action over the entire duration of the test (Reid *et al.*, 2002).

In contrast with the RIST and ITT, the HIEC is only able to detect HISS action over the first few minutes of insulin infusion, but by the end of the test, HISS action cannot be detected and post-HIEC tests, using either the RIST or ITT in rats, show that the HIEC produces blockade of HISS action (Reid *et al.*, 2002)[29]. So, HIEC may introduce some artifacts in the measurement of postprandial insulin sensitivity, which result from the prolonged continuous infusion of insulin (Lautt, 2003a).

Therefore, for any study attempting to evaluate HISS action, the chosen method must first show HISS action detection under the specific and appropriate test conditions.

In summary, the RIST is a new insulin sensitivity test that has decreased time duration; is able to be performed not only in the fasted state, but also in the postprandial state, on the same day with the same volunteer; it is reproducible, that is, can be performed more than one RIST per day without significant differences; permits pharmacological manipulation, so one can infuse more than one drug and evaluate the drugs' effect on insulin sensitivity, allowing us to design paired experiments; and the RIST does not cause discomfort, because the RIST is an euglycemic test and does not trigger any counter-regulatory hormonal response.

The traditional focus on insulin sensitivity and blood levels of markers of risk determined in the fasted state, is inconsistent with the large volume of recent data that indicates that the metabolic defect, in the prediabetic and diabetic condition, relates more strongly to postprandial deficiency than to the fasting state.

Growing awareness of the importance of postprandial plasma glucose (PPG) levels in the overall control of glycemia has led to the suggestion that PPG monitoring be integrated into routine diabetes care, particularly because patients' diabetes may not be adequately controlled if monitoring is based on glycosylated hemoglobin (HbA_{1C}) and fasting plasma glucose (FPG) data alone. Given the strong correlation between PPG levels and glycemic status and the emergence of cardiovascular complications in patients with type 2 diabetes, it is reasonable to suggest that PPG levels be tracked along with the conventionally monitored FPG and HbA_{1C} parameters (Bastyr *et al.*, 2000). Inclusion of PPG measurement in a patient home monitoring plan, will provide comprehensive information on the total glycemic status of the patient that may be used as a basis for altering the treatment regimen, to better control postprandial excursions (Fonseca, 2003).

[29] The blockade of HISS action by HIEC may happen due to the alterations produced by the constant infusion of insulin, which physiologically has a pulsatile behaviour, and it is known that changes in insulin secretion pattern leads to glucose intolerance (Zarkovic *et al.*, 1999)

More recently, emphasis has been put on the years that precede the development of type 2 diabetes, characterized by a progressive decline in both insulin action, and defects in the early phase of insulin secretion. As impaired glucose tolerance is worldwide acknowledged as a prediabetic stage, it becomes clear that loss of postprandial glucose control occurs before deterioration of the fasting glucose concentration (Monnier *et al.*, 2007).

Based on all these assumptions and since insulin action varies with the prandial state, it become essential to evaluate whole-body insulin sensitivity in the fed state. Therefore, we developed the RIST methodology in humans due to the perceived limitations of other the methods of assessing insulin action. The RIST was found to be a useful, reliable, and reproducible tool, both in the fasted and fed state.

8.2. MEAL-INDUCED INSULIN SENSITIZATION IN HUMANS AND ITS PARASYMPATHETIC REGULATION

The "HISS hypothesis", first proposed in 1997, appears to explain a range of metabolic responses both in health and disease and, represents a paradigm shift from the classic understanding of what is insulin resistance. The most pioneering aspect of this new mechanism is the finding that 50-60% of the hypoglycemic action of insulin is the responsible for an humoral factor, secreted by the liver, which acts on skeletal muscle and in the postprandial state.

It is well known that, in response to a meal, the glucose disposal response to insulin more than doubles. However, insulin sensitivity determinations made in the fasted state only represent the direct action on insulin, and disregards the HISS-dependent component of insulin action. The evaluation of the insulin sensitivity only in the fasted state leads to a late detection and an underestimation of the insulin resistance condition.

The results presented and discussed in Chapters 5 and 6 showed that the meal-induced insulin sensitization (MIS) process occurs and, HISS secretion/action is able to be blocked through the cholinergic pathway.

MIS is a readily identified phenomenon that has been only recently quantified in animals (Sadri *et al.*, 2006). MIS is illustrated by the dramatic increase in the glucose disposal response to insulin immediately following a meal. The phenomenon of MIS is derived from the observation that the dynamic response to insulin-stimulated glucose disposal determined after a 24h fast, is at least doubled when tested after administration of a mixed meal. The process of MIS is a result of HISS action.

The MIS process was evaluated both in lean (Chapter 5) and overweight (Chapter 6) subjects. It was shown that in the fasted state the insulin sensitivity, evaluated using the RIST, was the same in both groups; however, the postprandial state insulin sensitivity was lower in the overweight than in the lean subjects, suggesting that in the overweight subjects HISS secretion/action is decreased, which is responsible for the impaired insulin sensitivity. These results strongly suggest the importance of assessing the sensitivity in the postprandial state.

Several studies have shown that, by considering only the fasting glucose levels, a large proportion of subjects with impaired glucose tolerance (IGT) are overlooked (de Vegt et al., 1998; Larsson et al., 1998). There is considerable controversy regarding the relative importance of insulin resistance and abnormal insulin secretion in the pathogenesis of IGT (Dinneen, 1997). Some studies have proposed that loss of early insulin response to glucose and poor suppression of hepatic glucose output, are primarily responsible for postprandial hyperglycemia associated with IGT (Mitrakou et al., 1992). In contrast, under experimental conditions, defective insulin action was shown to contribute more to postprandial hyperglycemia than defective insulin secretion (Basu *et al.*, 1996).

The pattern of insulin secretion differs between IGT and impaired fasting glucose (IFG). People with isolated IFG have a decrease in first-phase (0–10min) insulin secretory response to iv glucose and a reduced early phase (first 30min) insulin response to oral glucose. However, the late-phase (60–120min) plasma insulin response during the OGTT is normal in isolated IFG. Isolated IGT also has a defect in early-phase insulin secretion in response to an oral glucose load, and in addition has a severe deficit in late phase insulin secretion. The combination of hepatic insulin resistance and defective insulin secretion in isolated IFG, results in excessive fasting hepatic glucose production accounting for fasting hyperglycemia. The impairment in early insulin response in combination with hepatic insulin resistance results in the excessive early rise of plasma glucose, in the first hour of the OGTT. However, the preservation of late insulin secretion combined with normal muscle insulin sensitivity allows glucose levels to return to the preload value in isolated IFG. In contrast, in isolated IGT the defective late insulin secretion, combined with muscle and hepatic insulin resistance, results in prolonged hyperglycemia after a glucose load (Nathan *et al.*, 2007).

The strong association between diabetes and obesity suggests that the first priority is the maintenance of healthy weight and obesity prevention. All individuals who are overweight or obese, regardless of their blood glucose value, should be intensively counselled to lose weight and to exercise (Nathan *et al.*, 2007).

The association of obesity, insulin resistance, and the onset of type 2 diabetes is well documented. However, the mechanisms underlying such relationship remain unclear, and can vary according to the way obesity is induced (Cornier *et al.*, 2006; Kahn *et al.*, 2006). Obesity is not only a problem of too much fat, *per se*, but also a much more far-reaching dysregulation of metabolism that affects adipose tissue and other organs, such as the liver, muscle, and pancreas (Hansen *et al.*, 2006). To further blur the relationship between obesity and insulin resistance, most of the published studies evaluate insulin sensitivity in a fasted state, ignoring the fact that the first alterations of insulin sensitivity seem to occur in the postprandial state, in which the role of the HISS-dependent component of insulin action is crucial.

From the results presented in Chapter 6, one could consider that overweight subjects have a decreased in insulin action due to an impairment of HISS release/action. This decrease in insulin action was only noticed in the fed state, the state where HISS is present.

Another main conclusion from these human studies is consistent with the hypothesis that iv atropine administration suppresses HISS (Chapter 5), in a dose-dependent manner, once it has been allowed to develop. The partial blockade of HISS by atropine is consistent with the hypothesis that a hepatic parasympathetic "feeding signal" is necessary in order for insulin to cause the release of HISS from the liver.

From the human studies, we observed that even in the fed state, the whole-body insulin sensitivity evaluation after atropine infusion was decreased and the plasma insulin profiles during the control fed and atropine fed RIST were superimposable. Taken these results together, we can postulate that some factor other than insulin *per se* is responsible for the difference in the dynamic insulin action seen (Figure 8.1).

Atropine has previously been demonstrated in numerous publications to result in impairment of the HISS-dependent response with the post-atropine RIST index in fed animals being similar to that in fasted animals. However, in humans the HISS-independent component of insulin action obtained after iv infusion of atropine was not completely abolished, since the RIST index after atropine 0.5 and 0.75mg infusion was not the same as the one obtained after a 24h-fast RIST; these doses of atropine used were the highest therapeutic doses ethically permissible and that had minor side effects. Based on animal atropine results, we estimate that an atropine dose of 4mg in humans should produce a full block of HISS release.

Atropine does not impact on the HISS-independent component of insulin action (Lautt *et al.*, 2001). Similar dependence on the hepatic parasympathetic signal was demonstrated in animals where surgical denervation was carried out prior to administration of the meal. These data confirm that, in humans, the hepatic parasympathetic signal is essential for HISS-mediated MIS and that absence of the signal can prevent MIS from occurring and eliminate it once it has occurred.

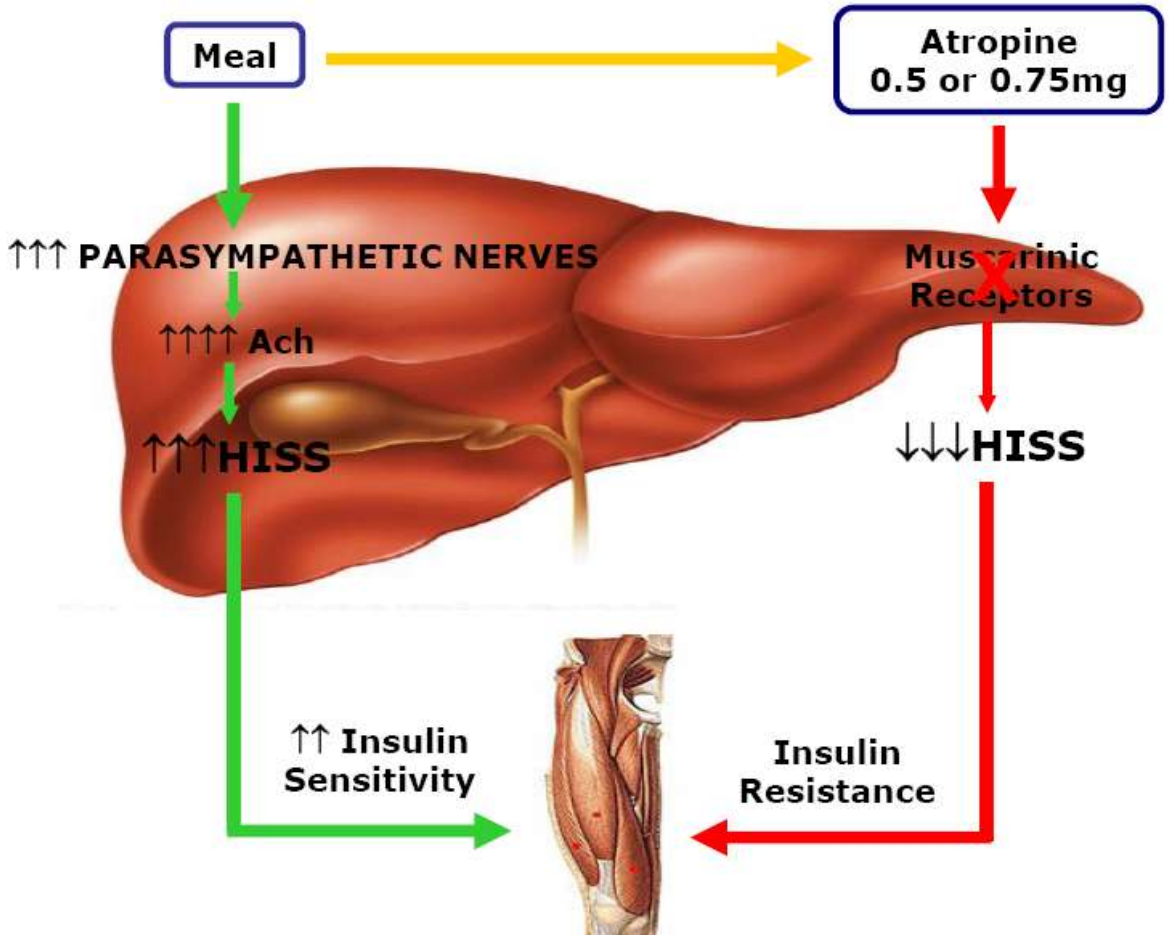


Figure 8.1 - Atropine infusion results in a HISS-dependent decreased insulin sensitivity due to blockade of the muscarinic receptors. After a meal, insulin sensitivity increases in the skeletal muscle. On the other hand, in the postprandial state, after atropine infusion, we partially block the muscarinic receptors and subsequently decrease the release of HISS, leading to a state of insulin resistance at the skeletal muscle.

According to the HISS hypothesis, loss of MIS occurs as a result of lack of HISS release, thereby resulting in postprandial hyperglycemia and compensatory hyperinsulinemia. One could speculate that the elimination of HISS action on skeletal muscle, results in postprandial hyperinsulinemia maintaining glycemic control in the prediabetic state, by increased glucose uptake in insulin-sensitive tissues, primarily liver and adipose tissue.

In the scope of understanding pathophysiology it is clear that fasting glucose represents a different pathophysiological process from the 2h-glucose, and that however diabetes is defined in the future, there will always be a value in studying these different processes alongside measures of insulin resistance and β-cell function in order to understand disease mechanisms. The progression from prediabetes to type 2 diabetes occurs over many years and strong evidence exists to support intervention to delay the progression from prediabetes to diabetes (Aroda *et al.*, 2008).

With the increasing recognition that the prediabetic state represents mainly a postprandial defect and the ability of the HISS hypothesis to account for selective skeletal muscle insulin resistance observed in impaired glucose tolerant subjects, obesity, metabolic syndrome and full blown diabetes, one can suggest that the HISS hypothesis and the MIS phenomenon may represent the "missing link" between lifestyle issues and insulin resistance in prediabetes, diabetes and obesity. In the fasted state, overweight subjects showed the same degree of insulin resistance as lean subjects; however, in the fed state, the insulin sensitivity in the overweight subjects was lower than lean subjects. In summary, if overweight subjects were only evaluated in the fasted state, they were normal subjects in terms of insulin resistance, but from postprandial insulin sensitivity results, they were insulin resistant subjects (Figure 8.2).

According to our hypothesis, an impairment of HISS would be the first event in the development of insulin resistance; progression to obesity and to frank diabetes, would only be detectable in the fed state.

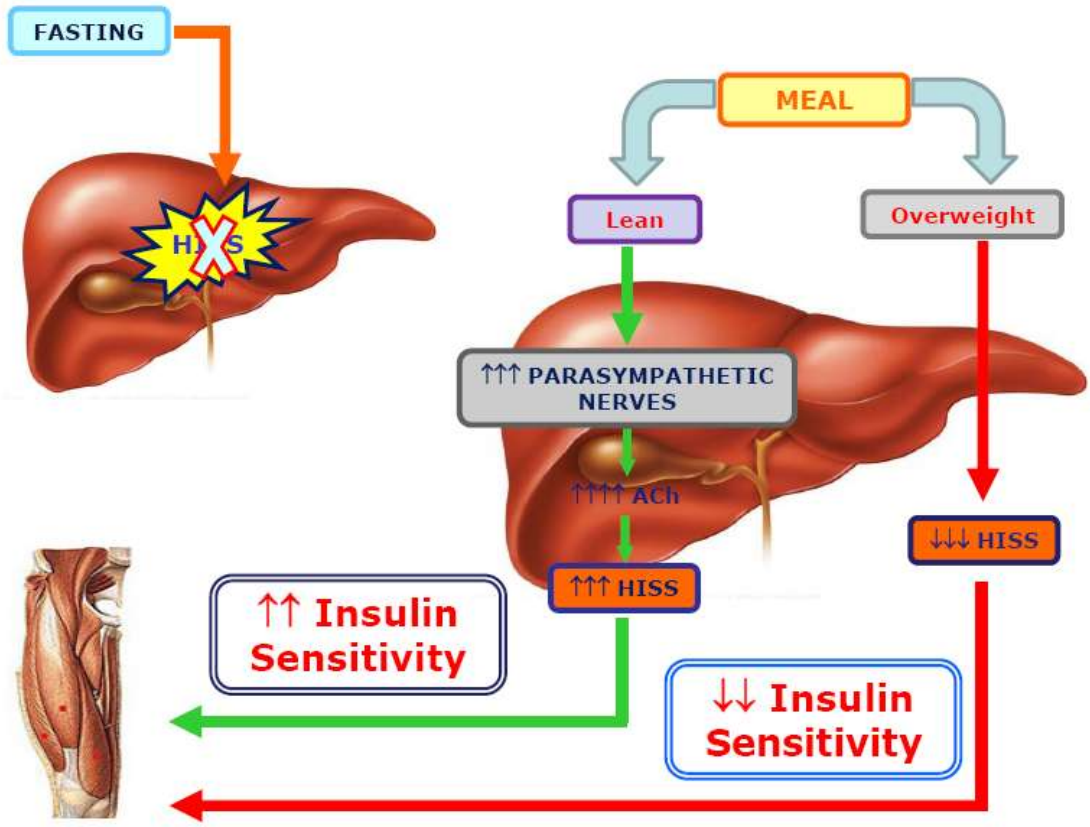


Figure 8.2 – Overweight subjects have impaired insulin sensitivity due to a compromise of HISS-dependent component. Fasting is a state of insulin resistance and HISS release is inhibited, both in lean and overweight subjects. However, in lean subjects, one can reverse this HISS inhibition with a provision of a meal, which will increase insulin sensitivity, at the skeletal muscle. On the other hand, in overweight subjects, even after a meal, a decrease of HISS release is observed, leading to a decrease of insulin sensitivity. This decrease in insulin sensitivity, in overweight subjects, could be important in the detection postprandial insulin resistance.

8.3. THE RELEVANCE OF GLUCAGON ON HISS-DEPENDENT INSULIN SENSITIVITY

Glucagon is a pancreatic hormone, which is released in the fasted state in order to maintain glucose levels in a steady-state. After a meal, in healthy subjects glucagon levels decrease and increase with the duration of fasting, which have the inverse pattern of the hepatic GSH content (Tateishi *et al.*, 1974). In the fasted state, type 2 diabetic patients have continuously higher levels of glucagon than healthy subjects which do not change even after a meal (Butler *et al.*, 1991). In addition, in the fasted state, glucagon through adenylate cyclase activation promotes an increase of the cAMP levels. Studies performed by Lu and colleagues, showed that high levels of cAMP leads to a decrease of the hepatic GSH synthesis, due to a alteration in the γ-glutamilcysteine synthase enzyme, one of the key enzymes involved in GSH synthesis (Lu *et al.*, 1991). Since the GSH molecule is a crucial molecule to HISS synthesis and release, it becomes imperative to understand the highly regulated pathway involved in the GSH formation controlled by either hormones or enzymes that affect the cAMP pathway, and its repercussions in HISS-dependent insulin resistance.

The results presented in chapter 7 indicated that ipv administration of DBcAMP (N^6,$2'$-O-dibutyryladenosine 3',5'-cyclic monophosphate), a cAMP analog, and glucagon produce a decrease of insulin sensitivity. Moreover, ipv administration of glucagon cause a decrease in insulin sensitivity and, after inhibition of the HISS pathway, with a specific inhibitor of NOS, L-NMMA, glucagon infusion did not alter insulin sensitivity, suggesting that glucagon inhibits HISS-dependent insulin sensitivity (Figure 8.3).

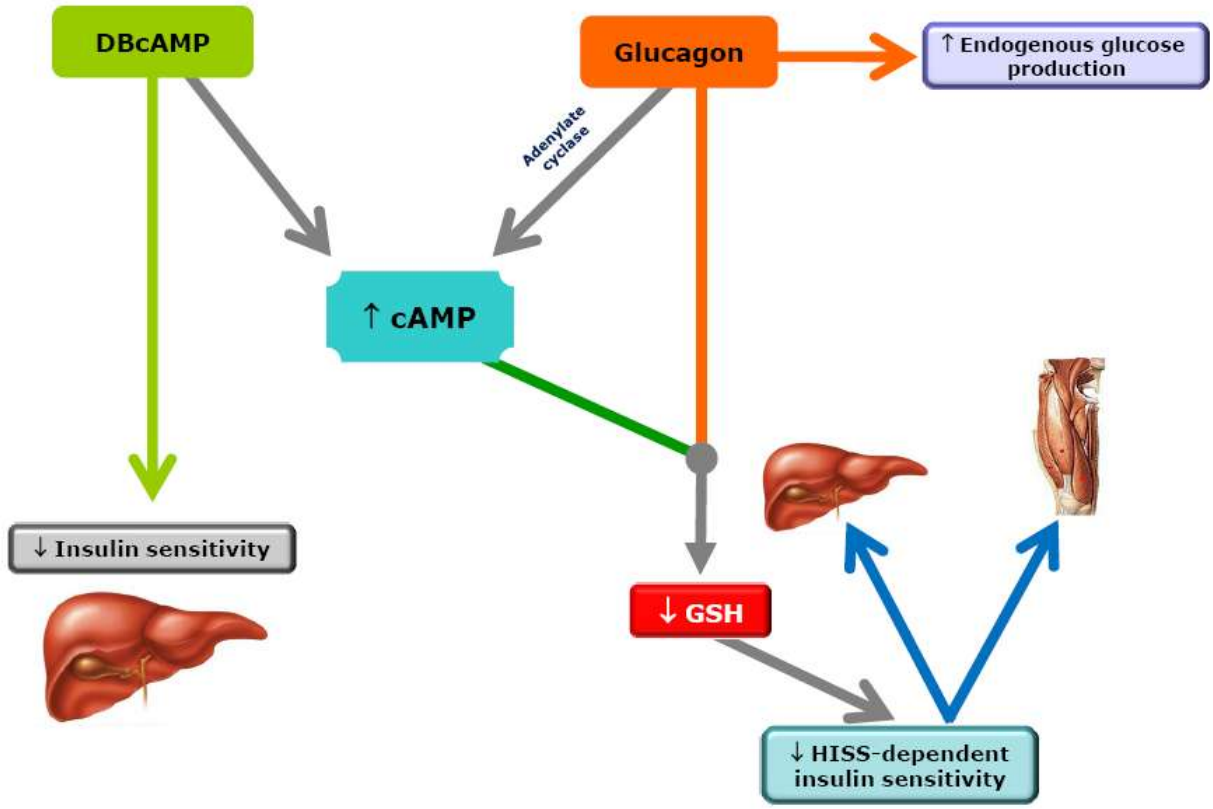


Figure 8.3 - DBcAMP and glucagon produces a decrease of insulin sensitivity. The cAMP analog and glucagon promote an increase in hepatic cAMP levels that are related to the decrease of hepatic GSH synthesis, leading to an impairment of HISS synthesis/release and resulting in an insulin resistance state responsible for the development of hyperglycemia and type 2 diabetes. DBcAMP: N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate; cAMP: 3′,5′-cyclic adenosine 5′-monophosphate; NO, nitric oxide; GSH, gluthatione

Glucagon should be an important parameter to consider in relation to glucose intolerance in prediabetic subjects, and may be a novel risk factor for diabetes development.

As mention earlier, glucose regulates pancreatic hormone secretion, that is, insulin and glucagon in an opposite manner and it is the molar ratio between insulin and glucagon, that determines in a qualitative and quantitative, the amount of glucose that circulates across the liver. Although glucose triggers insulin secretion through its metabolism by β-cells, the regulation of glucagon secretion is more complex and depends on several control mechanisms (Gerich *et al.*, 1976).

Pancreatic α-cells have an intrinsic capacity to monitor glucose levels, but are also under the control of insulin secretion by the β-cells, which inhibits glucagon secretion. There is ample evidence that the α-cell secretory activity is strongly regulated by both, the sympathetic and parasympathetic nervous systems, implying that glucose and hypoglycemia detecting cells, control the activity of the autonomic nervous system (Lechin *et al.*, 2006; Teff, 2008).

Therefore, based on our hypothesis, an autonomic nervous dysfunction observed in impaired glucose tolerance states, leads to an abnormal hyperglucagonemia, leading to an impairment of HISS secretion/release.

Eventhough, one can observed an hyperinsulinemia in a prediabetic state, the shut down of HISS secretion/release by the increased glucagon levels, results in a postprandial insulin resistant state (Figure 8.4).

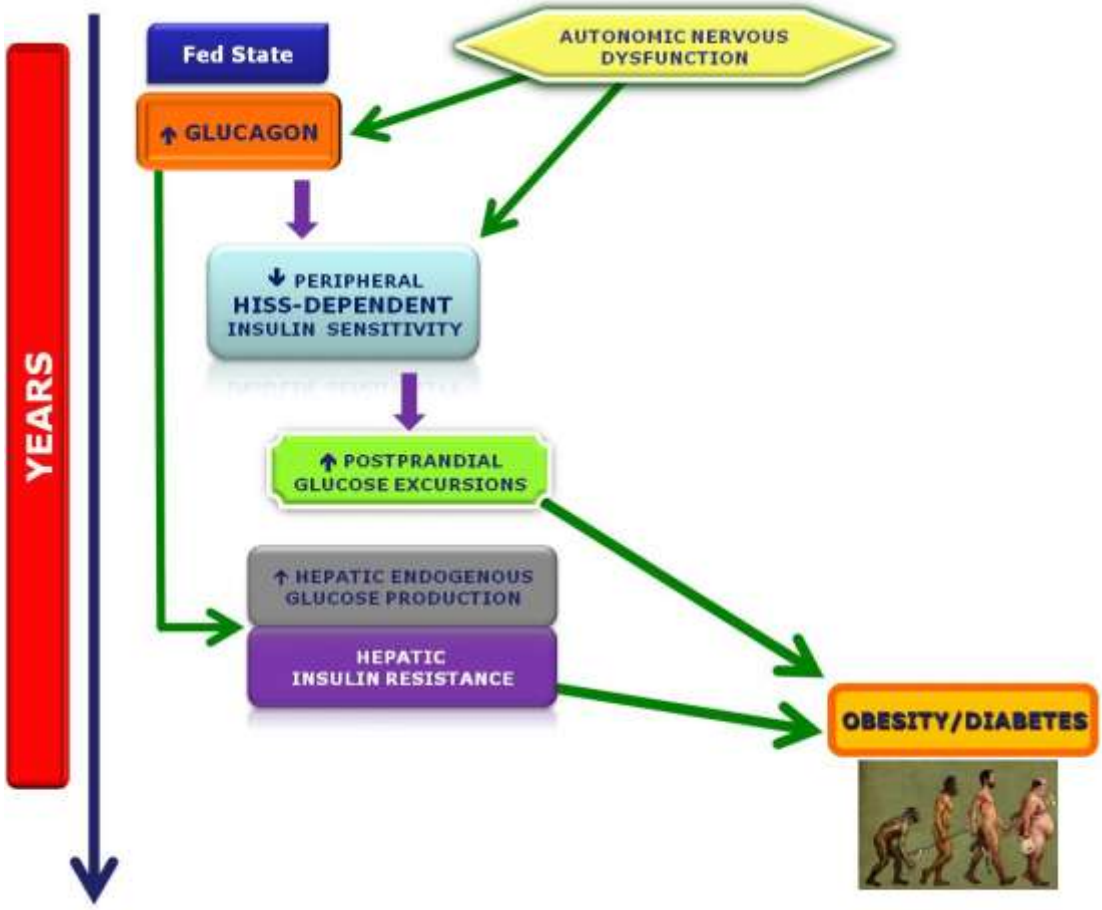


Figure 8.4 – The hyperglucagonemia hypothesis. In the fed state, at an early stage, the autonomic nervous dysfunction, results in increased levels of glucagon, leading to a decrease of HISS-dependent insulin sensitivity, at the skeletal muscle. On the other hand, the increase of postprandial glucose excursions results in obesity. Afterwards, hepatic insulin resistance state will be present, due to an abnormal postptandial increase of the endogenous glucose production. The transition from prediabetes to overt diabetes seems to result from the sum of deficiencies in HISS secretion/action, which was caused by high levels of glucagon, of insulin action *per se* (HISS-independent) and, later of pancreatic insulin secretion.

8.4. FUTURE DIRECTIONS

In light of the present PhD thesis and as is the prerogative of any research project, the observations associated with the development of the work itself raise new questions which future work may respond.

Although the studies presented in this dissertation provided further knowledge about the HISS hypothesis in lean and overweight subjects, additional work is required to clarify not only the mechanisms involved in the regulation of HISS secretion/action, but also its relevance in human insulin resistance syndromes, like hypertension, obesity and type 2 diabetes. Considering what is known about the HISS mechanism, in terms of physiology and pathophysiology, it will be very important to understand the mechanism behind human insulin resistance diseases.

In the future, it will be essential to perform the nutritional characterization of the physiological stimulus that activates the mechanism of insulin action sensitization after a meal. In this regard, preliminary experiments performed by our group in collaboration with the Lautt's group, confirmed that a liquid mixed-meal is capable of triggering the MIS, rather than carbohydrates (glucose and sucrose). However, it is still not known either which nutrients or the exact nature of the feeding signal that are involved in this MIS. Whether these nutrients act upon absorption or stimulate a neural reflex is not yet known and it constitutes one of the future objectives. In this future studies, the recognition together with the disclosure of the trigger of the MIS mechanism in humans will be the first steps for better understand its impact on both, prediabetic and diabetic states.

Another important perspective of this thesis was the role of glucagon on the HISS pathway, and its relationship with the hepatic GSH levels, which are crucial to HISS release. More studies need to be developed in order to bring insight into the mechanisms that lead to HISS-dependent insulin resistance and type 2 diabetes, through the hepatic cAMP pathway.

It is reasonable to assume that antagonists of glucagon will suppress the actions of endogenous circulating glucagon, and will provide evidence that glucagon is a contributing factor to diabetes mellitus. Most of the effects of glucagon are mediated by its interaction with specific receptors in the liver to increase the intracellular cAMP level. Due to the relationship between glucagon and cAMP levels, hepatic GSH and HISS-dependent insulin sensitivity, it will be relevant to study the effect of the administration of a glucagon receptor antagonist on HISS-dependent mechanism.

Our group reported that hepatic NO/cGMP is also essential for HISS action which strongly suggests that cGMP-dependent cAMP-PDEs may represent fine regulators of the HISS pathway, by controlling GSH levels through a cAMP/cGMP cross-talk.

Furthermore, we expect that pharmacological blockade of the cAMP pathway in the liver will enhance hepatic GSH levels, and HISS-dependent insulin sensitivity in animal models of insulin resistance, an approach that can be used to improve insulin sensitivity in humans.

Since insulin resistance can anticipate diabetes onset by 15 years, all studies that contribute to its characterization and pharmacological control should be considered a priority. The understanding of the regulation of the cAMP pathway from the early-stage of insulin resistance to frank diabetes can provide a solid platform for the development of new forms of therapy foreseeing the prevention and control of the epidemic of diabetes.

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APPENDIX

APPENDIX

INSTITUTO PORTUGUÊS DE ONCOLOGIA FRANCISCO GENTIL

RESEARCH STUDY: "THE RELEVANCE OF THE HEPATIC INSULIN SENSITIZING SUBSTANCE (HISS) ON DISEASES ASSOCIATED WITH INSULIN RESISTANCE"

SUBJECTS STUDY INFORMATION

You are invited to participate in a research study conducted by an international research team consisting of scientists, physicians, and pharmacists from Canada and Portugal. The study involves the testing of a new method to assess the ability of insulin to act in the body.

Insulin is normally released from the body in response to glucose (sugar) that appears in the blood following a meal. Most types of diabetes are caused by a decreased ability of insulin to stimulate the removal of glucose from the blood and storage for later use. These individuals are said to be insulin resistant. Current methods used to assess insulin resistance are of limited value. The researchers of this project developed a new method to evaluate insulin sensitivity called by Rapid Insulin Sensitivity Test (RIST). The RIST that will be tested in participants of this study has been fully validated in animal studies and is shown to be an excellent test that can clearly identify insulin resistance and that can also be used to determine the type of therapy that should be used to restore insulin sensitivity to normal levels.

These studies will be carried out in several phases. If you are a healthy you could participate in phase 1a, 1b or 1c, or if you want in two phases in or all of them. The phase 1a is to determine the dose of most accurate dose of to perform the RIST. The phase 1b want to check if the RIST is influenced by ingestion of food, and in this case, the volunteers will perform an initial RIST; after the volunteer fed 16 cookies and drink 0.5l of mineral water, and a second RIST will take place 100min after the meal. In the phase 1c, the RIST will be performed before and after intravenous administration of atropine (0.5mg), in order to block the parasympathetic nervous system.

The test will involve administration of a small dose of insulin through one catheter and administration of glucose through the other catheter in order to maintain your blood glucose level absolutely constant. The fasted RIST has duration of approximately 20-50min and the fed RIST will take around 45-90min. In phases 1a and 1b, the test will be carried out in fast

and the last meal was the breakfast (ham and cheese sandwich, orange juice, croissant with cheese or ham) on the day before the test, which should occur between 10:00 and 10:15am. In stage 1c, the RIST's will be done after the standardized test meal. The test will begin at 10am.

The rapid insulin sensitivity test (RIST) consists of placement of an intravenous catheter into each arm. One catheter will be used for blood sampling and the other will be used for the administration of insulin and glucose (sugar). The arm from which blood will be sampled will be warmed throughout the test. The entire blood volume removed during the testing will be less than half of that which is normally taken for a routine blood donation. Samples will be taken from the blood throughout the test period to determine the blood glucose level. The blood removed by this method will be stored and made available to measure a variety of substances. You should experience no discomfort since your blood glucose level will not be allowed to fluctuate during the test. The dose of atropine administered is not usually associated with any symptoms, but may cause a slight decrease in heart rate, dryness of mouth and some decrease in sweating. An electrocardiogram will be performed before the study and your heart rate will be monitored during the test.

It is important that you read and understand the following general principles which apply to all participants in our studies:

1. Your participation is entirely voluntary;

2. There is no immediate personal benefit by participating in this study;

3. Withdrawal from the study may be done at any time without jeopardy or prejudice;

4. The information derived from this study will become part of several scientific publications and presentations but your identity will remain completely confidential;

5. If you request, you will be provided with full scientific details of the completed studies. You are free to inquire the researchers about any aspect of the study;

6. Allows the researchers to perform the determination, in the blood that will be sampled during the test, for all substances that will be considered necessary to performed an accurate project;

7. This study does not involve any cost from the financial point of view;

7. The study was analyzed and approved by the Ethics Committee of the IPO, Lisboa, Portugal.

Thank you very much.

The project researchers.

INSTITUTO PORTUGUÊS DE ONCOLOGIA FRANCISCO GENTIL

RESEARCH STUDY: "THE RELEVANCE OF THE HEPATIC INSULIN SENSITIZING SUBSTANCE (HISS) ON DISEASES ASSOCIATED WITH INSULIN RESISTANCE"

SUBJECT CONSENT FORM

I,_____, born in _____, at ___/____/____, agree to take part in phase ___ of the study "The relevance of the Hepatic Insulin Sensitizing Substance (HISS) on diseases associated with insulin resistance"

I understand that my participation in this study is voluntary and the doctor gave me all the information about the study participation, from who I received a written information sheet. I declare that I have read and understood this page and I can clarify any doubts with the researcher. More declare that I give the authorization to the researchers to use the blood that was collected during this study in subsequent research projects, if authorized by the Ethics Committee of the Francisco Gentil Portuguese Institute of Oncology.

Signature (Volunteer)_____ Date_____

Signature (Investigator) _____ Date_____

INSTITUTO PORTUGUÊS DE ONCOLOGIA FRANCISCO GENTIL

ESTUDO: "IMPORTÂNCIA DA SUBSTÂNCIA HEPÁTICA SENSIBILIZADORA DA ACÇÃO DA INSULINA EM DOENÇAS ASSOCIADAS A INSULINO-RESISTÊNCIA"

FOLHA DE INFORMAÇÃO AO VOLUNTÁRIO

É convidado a participar num estudo de investigação conduzido por uma equipa internacional que envolve cientistas Canadianos e Portugueses e que tem como objectivo o desenvolvimento nos humanos de um novo teste de sensibilidade à insulina.

A insulina é normalmente libertada em resposta a um aumento de glucose (açúcar) no sangue que ocorre após as refeições. Na maioria das situações de diabetes mellitus existe uma diminuição da capacidade da insulina em remover a glucose do sangue para a armazenar nos tecidos do organismo. Os indivíduos em que a insulina não é capaz de remover a glucose do sangue de forma eficiente são denominados resistentes à insulina. Os métodos actualmente usados para a avaliação da resistência à insulina têm várias limitações. Os investigadores deste projecto desenvolveram um novo método de avaliação da resistência à insulina, designado por teste rápido de sensibilidade à insulina (RIST), que tem demonstrado ser mais reprodutível que outros métodos em vigor e que já foi testado em diferentes espécies animais.

Este estudo decorrerá em fases distintas. Se for um voluntário normal poderá participar na fase 1a, 1b ou 1c, ou caso o deseje em duas fases ou em todas elas. A fase 1a tem como objectivo determinar a dose de insulina mais adequada para o RIST. A fase 1b pretende verificar se o RIST é influenciado pela ingestão de alimentos, e neste caso, os voluntários farão um RIST inicial que será seguido da ingestão de 16 bolachas integrais e de 0.5l de água mineral, sendo um segundo RIST efectuado 100min após esta refeição. Na fase 1c o RIST será realizado antes e após a administração de atropina (0.5mg) por via endovenosa, de modo a bloquear o sistema nervoso parassimpático.

O RIST consiste na administração de uma dose de insulina seguida de uma perfusão de glucose de forma a manter as concentrações de glucose no sangue em valores estáveis. O RIST em jejum tem a duração aproximadamente entre 20-50min e o RIST pós-prandial tem a duração aproximadamente entre 45-90min. Nas fases 1a e 1b, o teste será realizado em jejum sendo a última refeição o pequeno-almoço (tosta mista, sumo de laranja, croissant com queijo ou fiambre) do dia anterior ao do teste, que deverá ocorrer entre as 10h00m e

as 10h15m. Na fase 1c, os RIST'S serão realizados após a refeição standard. O teste terá início às 10h da manhã.

Para realizar este teste é necessário colocar um catéter intravenoso em cada braço. Um dos catéteres será utilizado para retirar sangue para a avaliação da glicémia (concentração de glucose no sangue) e o outro para a administração de glucose e insulina. O braço usado para avaliação da glicémia no sangue será mantido aquecido durante todo o procedimento. A quantidade de sangue retirada durante o teste será menos de metade da quantidade normalmente retirada quando se faz uma doação de sangue. O sangue retirado será armazenado e posteriormente utilizado para a determinação de várias substâncias. Não deverá sentir qualquer tipo de desconforto durante o teste dado que não haverá flutuações nos níveis de glicémia. A dose de atropina administrada não está geralmente associada a qualquer sintoma, embora possa provocar uma ligeira diminuição da frequência cardíaca, alguma secura de boca e diminuição da sudação. Fará um electrocardiograma prévio ao estudo e a sua frequência cardíaca será monitorizada durante o teste.

É importante que leia e compreenda os seguintes princípios gerais que se aplicam a todos os participantes neste estudo:

1. A sua participação no estudo é totalmente voluntária;
2. Não existe beneficio pessoal imediato ao participar neste estudo;
3. Em qualquer altura pode desistir de participar no estudo, sem ter de dar explicações e sem que a qualidade dos cuidados médicos seja afectada;
4. A informação obtida com este estudo poderá originar algumas publicações científicas, mas a sua identidade será sempre mantida confidencial;
5. Se assim o desejar, ser-lhe-á fornecido detalhe científico sobre o estudo em questão. Será livre de fazer perguntas aos investigadores sobre o projecto;
6. Autoriza os investigadores a realizar a determinação, no sangue que lhe vai ser retirado durante o teste, de todas as substâncias que forem consideradas necessárias para uma eficaz realização do projecto;
7. Este estudo não lhe acarreta qualquer custo do ponto de vista financeiro;
8. O estudo foi analisado e aprovado pela Comissão de Ética do IPO.

Muito obrigado.

Os investigadores do projecto.

INSTITUTO PORTUGUÊS ONCOLOGIA FRANCISCO GENTIL

ESTUDO: "IMPORTÂNCIA DA SUBSTÂNCIA HEPÁTICA SENSIBILIZADORA DA ACÇÃO DA INSULINA EM DOENÇAS ASSOCIADAS A INSULINO-RESISTÊNCIA"

CONSENTIMENTO INFORMADO DO VOLUNTÁRIO

Eu,_____, natural de _____, em ___/____/____, concordo em participar na fase ___ do estudo "IMPORTÂNCIA DA SUBSTÂNCIA HEPÁTICA SENSIBILIZADORA DA ACÇÃO DA INSULINA EM DOENÇAS ASSOCIADAS A INSULINO-RESISTÊNCIA".

Estou ciente de que a minha participação neste estudo é voluntária, tendo sido informado pelo meu médico sobre o estudo em que participo, do qual recebi uma folha informativa escrita. Declaro que li e compreendi esta folha e que pude esclarecer todas as dúvidas com o investigador. Mais declaro que autorizo os investigadores a utilizar o sangue que me foi retirado durante o estudo em ulteriores projectos de investigação, distintos do que agora me é proposto, desde que autorizados pela Comissão de Ética do Instituto Português de Oncologia.

Assinatura (Voluntário)_____ Data_____

Assinatura (Investigador)_____ Data_____