Fátima Patrícia da Costa Oliveira Martins

BRAIN-LIVER NITRIC OXIDE AXIS: A MAJOR REGULATORY PLAYER IN INSULIN CLEARANCE AND ACTION

Tese de Doutoramento em Biologia Experimental e Biomedicina, Ramo de Biologia Molecular, Celular e do Desenvolvimento orientada por Dr. John Grifth Jones e apresentada ao Instituto de Investigação Interdisciplinar da Universidade de Coimbra (IIIUC)

Setembro, 2014

Universidade de Coimbra

INSTITUTO DE INVESTIGAÇÃO INTERDISCIPLINAR (III-UC)

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Tese apresentada à Universidade de Coimbra (III-UC) para prestação de provas de Doutoramento em Biologia Experimental e Biomedicina, Ramo de Biologia Molecular, Celular e do Desenvolvimento. Este trabalho foi realizado no Centro de Neurociências e Biologia Celular (CNC) da Universidade de Coimbra sob orientação do Doutor John Griffith Jones. O trabalho foi efectuado ao abrigo de uma bolsa de doutoramento atribuída pelo programa doutoral em Biologia Experimental e Biomedicina do Centro de Neurociências e Biologia Celular da Universidade de Coimbra, financiada pela Fundação para a Ciência e Tecnologia (SFRH/BD/51194/2010).

Agradecimentos

First of all I have to start to acknowledge to Doctor John Jones for the support, guide and knowledge that gave to me. It was a great pleasure to share these 4 years (and the others before) with you in the lab and outside, where we had very good moments of fellowship. Thank you for the trust and for everything.

Agradeço também a todos os meus colegas do laboratório em Coimbra pela ajuda e companheirismo mesmo que à distância. Obrigada à Cristina, Filipa, João Rito, João Silva, Ivan, Chica, Teresa.

Agradeço imenso à Doutora Paula Macedo pelos ensinamentos e oportunidades que me ajudou a ter ao longo deste trajecto. Muitos foram os momentos que partilhamos e em todos eles senti que tinha o seu apoio, confiança e incentivo para seguir em frente. Agradeço-lhe de verdade a amizade, paciência e convívio ao longo destes anos.

Agradeço aos meus colegas de laboratório de Lisboa com quem partilhei a maioria dos dias destes últimos 4 anos. Agradeço à Rita (por manter o "barco" à tona), à Inês L., Inês C., Joana V., Margarida, Ricardo, Rogério. Todos vocês foram essenciais em diferentes momentos desta caminhada, e sem cada um o trajecto teria sido mais difícil.

Agradeço à Joana Gaspar pelo imenso tempo que convivemos no laboratório e fora dele, por todos os seus conselhos, companheirismo e verdadeira amizade. Agradeço-lhe todas as aventuras que partilhou comigo e que me fizeram crescer pessoal e profissionalmente. Tenho a certeza que estes 4 anos teriam sido muito menos divertidos sem ti ao meu lado.

Agradeço ao Professor Rui de Carvalho. Tenho de o considerar o meu mentor na ciência e muito aprendi com ele. Se os nossos caminhos se separaram na ciência, não se terão separado seguramente na vida. Sei que posso e que irei contar sempre consigo. Muito obrigada por tudo.

No me puedo olvidar de todas las personas que me ayudaron en Santiago de Compostela. Casi un año he pasado allí y muchas fueron las personas que estuvieron conmigo en el laboratorio y en la bella ciudad de Santiago. Agradezco al Doctor Carlos Diéguez el haberme abierto las puertas de su grupo. Tengo también que agradecer a Sulay el haberme recibido en el laboratorio y haberme enseñado todo lo mejor que hay en Santiago. Agradezco a todos los

compañeros de laboratorio, y en especial a Sonia, por la ayuda en todo y la amistad verdadera y a David por la ayuda en todos mis experimentos. Muchas gracias a todos.

Agradeço a todos os meus amigos fora do laboratório em Coimbra e Lisboa pela amizade, companheirismo e momentos fantásticos.

Agradeço à Lisbeth por ter sido uma excelente companheira e uma verdadeira amiga durante a minha estadia em Santiago.

Agradeço ao Manel por ter feito parte da minha vida na última metade deste trajecto, precisamente a altura em que o seu apoio foi crucial para ultrapassar os momentos menos fáceis desta etapa. Muito obrigada.

Por fim, agradeço da forma mais especial possível à minha mãe, ao meu pai, aos meus irmãos e aos meus sobrinhos que me tornaram na pessoa que sou hoje e sem os quais nada faria sentido. Eles ensinaram-me que a única coisa que é certa na vida é que os terei sempre comigo; tudo o resto bem com trabalho e persistência.

Agradeço ao Centro de Neurociências e Biologia Celular pela atribuição da bolsa do programa doutoral em Biologia Experimental e Biomedicina. Agradeço ao Centro de Estudos de Doenças Crónicas da Universidade Nova de Lisboa pelo acolhimento e pela disponibilização das condições para a realização de grande parte deste trabalho. Agradezco al Centro Singular de Investigación en Medicina Molecular e Enfermedades Crónicas (CIMUS) de Santiago de Compostela por me haber acollido por casi un año y me haber permitido hacer parte de mios experimentos. Agradeço à Fundação para a Ciência e Tecnologia pelo financiamento do meu trabalho (SFRH/BD/51194/2010).

Publications

Martins F. O., Rito J., Jarak I., Viegas I., Pardal M. A., Macedo M. P, Jones J. G.; Disposition of [U-$^{2}H_{7}$] glucose into hepatic glycogen in rat and in seabass; Comparative Biochemistry and Physiology-Part A, 166, 316-322, 2013

Martins F. O., Mari A., Natali A., Jones J. G., Macedo M. P.; Regulation of insulin bioavailability by hepatic clearance: the role of nitric oxide (Subbmited to Diabetes)

Martins F. O., Delgado T. C., Viegas J., Gaspar J., Scott D. K., O'Doherty R., Macedo M. P., Jones J. G.; Mechanisms by which thiazolidinediones protect against sucrose-induced fatty liver and hyperinsulinemia (Subbmited to PLoS One)

Note: The results presented in this document in Chapter 4 are formatted according to the style of the journal where the papers were published or submitted with minor modifications.

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Abbreviations list

^{1}H	Proton
^{2}H	Deuterium
$^{2}H_2O$	Deuterated Water
Ach	Acetylcholine
AD	Alzheimer's Disease
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
ARC	Arcuate Nucleus of Hypothalamus
ATP	Adenosine Triphosphate
Aβ	Amyloid β
BH4	Tetrahydrobiopterin
Ca^{2+}/CaM	Calcium/Calmodulin
cAMP/PKA	cyclic Adenosine Monophosphate/Protein Kinase A
Cbl/CAP	Cbl Associated Protein
CD26	Lymphocyte Cell Surface Protein
cNOS	constitutive Nitric Oxide Synthase
CNS	Central Nervous System
DNL	*de novo* Lipogenesis
DPP-4	Dipeptidyl Peptidase-4
eNOS/NOS3	endothelial Nitric Oxide Synthase/Nitric Oxide Synthase 3
ER	Endoplasmic Reticulum
F-6-P	Fructose-6-Phosphate
FAD	Flavin Adenine Dinucleotide
FFA	Free Fatty Acid
FMN	Flavin Mononucleotide
G-1-P	Glucose-1-Phosphate
G-6-P	Glucose-6-Phosphate
G-6-Pase	Glucose-6-Phosphatase
GIP	Gastric Inhibitory Polypeptide
GLP1	Glucagon-Like Peptide-1
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GRB2/MAPK	Growth Factor Receptor-Bound Protein 2/Mitogen-Activated Protein Kinase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
HIV	Human Immunodeficiency Virus
HSD	High Sucrose Diet
i.v.	Intravenously
icv	Intracerebroventricular

IDE	Insulin Degrading Enzyme
iNOS/NOS2	inducible Nitric Oxide Synthase/Nitric Oxide Synthase 2
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate 1
IRS-2	Insulin Receptor Substrate 2
LAH	Lateral Hypothalamus
L-NMMA	N^G-Monomethyl-L-Arginine
MAG	Monoacetone Glucose
MTP	Microsomal Triglyceride Transfer Protein
N_2	Nitrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-Alcoholic Fatty Liver Disease
NEFA	Non Esterified Fatty Acid
NMR	Nuclear Magnetic Resonance
nNOS/NOS1	neuronal Nitric Oxide Synthase/Nitric Oxide Synthase 1
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NPY	Neuro Peptide Y
OGTT	Oral Glucose Tolerance Test
PC	Pyruvate Carboxylase
PDI	Protein Disulfide Isomerase
PEPCK	Phosphoenolpyruvate Carboxykinase
PI3K	Phosphatidylinositol-3-Kinase
PPARγ	Peroxisome Proliferator-Activated Receptor γ
PVN	Paraventricular Nucleus of Hypothalamus
s.c.	Subcutaneous
STZ	Streptozotocin
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TA	Transaldolase
TCA	Tricarboxylic Acid Cycle
TG	Triglyceride
Triose-P	Triose Phosphate
TZD	Thiazolidinedione
UDPG	Uridinediphosphoglucose
VMH	Ventromedial Nucleus of Hypothalamus

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Resumo

A incapacidade de regular o metabolism glucídico e lipídico está na base das epidemias mundiais obesidade e diabetes tipo 2 (T2D). O eixo intestino-cérebro-fígado tem um papel fundamental na regulação do metabolismo e a insulina é um componente central nas conexões desta tríade. Assim, alterações na cinética da insulina estão implicadas na desregulação metabólica destas patologias.

Os níveis plasmáticos de insulina dependem das suas taxas de secreção e clearance. Enquanto a secreção tem sido alvo de estudo para perceber a patogénese da diabetes, o estudo dos mecanismos da clearance de insulina têm sido negligenciados. Recentemente, a desregulação da clearance de insulina tem sido descrita como uma das primeiras disfunções na progressão da T2D, demonstrando a necessidade de melhorar o conhecimento acerca dos mecanismos de clearance da insulina.

Estudos *in vitro* têm relacionado o óxido nítrico (NO) com a regulação da clearance de insulina, pela inibição da actividade da enzima degradadora de insulina (IDE). Em humanos, os níveis plasmáticos de NO correlacionam-se com alterações na clearance de insulina e no metabolismo glucídico. Assim, pretendemos avaliar, *in vivo*, o papel regulatório do NO na taxa de clearance da insulina e os seus efeitos na actividade da IDE.

Usamos modelos de roedores para investigar o efeito de perturbações farmacológicas e nutricionais nos níveis de NO e na clearance da insulina. Esses tratamentos incluíram a inibição sistémica aguda ou crónica da actividade da sintase do óxido nítrico (NOS), atingindo desta forma todo o eixo intestino-cérebro-fígado, assim como a inibição específica em diferentes regiões do cérebro, com especial incidência sobre o hipotálamo. Para estudar perturbações nutricionais, os animais foram submetidos a uma dieta rica em sacarose, que já se sabe induzir alterações na ação da insulina e no metabolismo glucídico e lipídico que se assemelham às da T2D. Este modelo permitiu determinar se as tiazolidinedionas (TZDs) - fármacos desenvolvidos para tratar alterações metabólicas envolvidas nos estadios iniciais da T2D ao alterarem o metabolismo lipídico - também têm efeitos na clearance da insulina.

Testamos a hipótese que, *in vivo*, o aumento pós-prandial dos níveis de NO inibe a IDE hepática e a clearance da insulina levando a um aumento da biodisponibilidade de insulina. O raciocínio por detrás deste mecanismo baseia-se em que em coordenação com a secreção de insulina, providencia um aumento da regulação dos níveis sistémicos de glucose mediada pela insulina no estado pós-prandial. Mostramos uma relação inversa entre os níveis de NO e a actividade da IDE/clearance da insulina tanto em jejum como no estado pós-prandial.

Adicionalmente, a depleção aguda do NO promoveu uma diminuição na biodisponibilidade da insulina, sem alterações na secreção, e um aumento na sinalização de insulina e na expressão dos transportadores de glucose. Contudo, o aumento da secreção observado nos animais com depleção crónica de NO resultou em níveis de insulina plasmática e sinalização de insulina similares aos controlos. Assim, concluímos que o NO é um regulador fisiológico da clearance de insulina pela inibição da actividade da IDE, levando a um aumento da biodisponibilidade de insulina.

Considerando este papel do NO na regulação da clearance de insulina fomos avaliar as alterações na clearance de insulina resultantes da inibição do NO central. Estabelecemos a hipótese que o NO produzido no cérebro modula a clearance de insulina e a sua biodisponibilidade periférica. Mostramos que o núcleo paraventricular (PVN) do hipotálamo é importante mas não um regulador exclusivo da clearance de insulina via NO. Para além disso, a produção hipotalamica de NO está envolvida na regulação da ação hepática da insulina e no metabolismo lipídico.

Tínhamos também como objectivo estudar o possível papel da alteração da clearance de insulina no desenvolvimento de resistência à insulina e os mecanismos de ação de uma TZD. Estabelecemos a hipótese que a TZD tem efeitos benéficos sobre a esteatose e a hiperinsulinémia. Confirmamos a hipótese e mostramos dever-se à redução da lipogénese e ao aumento da clearance de insulina pela elevação da actividade da IDE, o que estaria correlacionado com a diminuição nos níveis hepáticos de NO observados.

Alterações no metabolismo glucídico e lipídico são efeitos chave da resistência à insulina e da T2D. Métodos directamente translacionáveis de modelos animais para humanos que permitam quantificar essas vias metabólicas são essenciais. O método da água deuterada (^2H_2O) para a quantificação das fontes da síntese de glucose e glicogénio é altamente translacionável, mas tem sido baseado em suposições que apenas têm sido testadas em humanos e não em modelos animais. Detectámos diferenças entre espécies na troca de deutério ao nível da glucose-6-fosfato e triose fosfato hepáticas e com base nisto, factores de correcção que permitem determinações mais precisas dos fluxos da síntese de glucose e glicogénio em ratos foram obtidos.

Concluindo, o NO hepático inibe a IDE *in vivo*, levando á diminuição da clearance de insulina no estado pós-prandial e consequente aumento da biodisponibilidade de insulina. Para além disso, o NO produzido no hipotálamo também regula a clearance de insulina, por um mecanismo que necessita de ser clarificado, mas que parece estar relacionado com alteração do sistema nervoso autónomo (ANS). O tratamento com TZDs pode também beneficiar dos resultados aqui apresentados, uma vez que mostramos que este fármaco altera

a clearance de insulina, com repercussões no metabolismo lipídico. Por fim, os nossos resultados facilitam os estudos translacionais acerca do metabolismo glucídico e lipídico: fatores chave na patogénese e terapia da T2D.

Abstract

The inability to regulate systemic glucose and lipid metabolism is the basis of the worldwide epidemic of obesity and type 2 diabetes (T2D). The gut-brain-liver axis is of central importance in metabolic fuel regulation and insulin is a central component for interconnections within this triad. Hence, alterations in insulin kinetics are highly implicated in the metabolic deregulation of these pathologies.

Plasma insulin levels are dependent on the rates of insulin secretion and insulin clearance. While β-cell insulin secretion has long been a target for understanding diabetes pathogenesis, the study of insulin clearance mechanisms has been neglected. Recently however, insulin clearance deregulation has been described as one of the primary defects in the progression of T2D, thereby demonstrating the need for increasing our knowledge about insulin clearance mechanisms.

In vitro studies have implicated nitric oxide (NO) as a regulator of insulin clearance, by inhibiting the activity of insulin degrading enzyme (IDE). In humans, plasma NO levels correlate with alterations in insulin clearance and glucose metabolism. Therefore, we examined the *in vivo* regulatory role of NO on the rate of insulin clearance and its effects on IDE activity.

We used rat models for investigating pharmacological and nutritional perturbations of NO levels and insulin clearance enzymes. Those treatments comprised acute or chronic inhibition of systemic nitric oxide synthase (NOS) activity, thereby targeting the entire gut-liver-brain axis, as well as specific NOS inhibition in different regions of the brain, with special emphasis on the hypothalamus. To study nutritional perturbations, rats were placed on high-sucrose diet that is well known to induce changes in insulin actions as well as in hepatic carbohydrate and lipid metabolism that resemble the onset of T2D. This model also provided the opportunity to determine if thiazolidinediones (TZDs) - drugs that were developed to counteract metabolic decompensation in the early stages of T2D by targeting lipid metabolism - also had effects on insulin clearance.

We tested the hypothesis that *in vivo*, increased postprandial levels of NO inhibits hepatic IDE and insulin clearance leading to a temporary increase in insulin bioavailability. The rationale behind this mechanism is that in coordination with β-cell secretion, it provides increased responsiveness for insulin-mediated regulation of systemic glucose levels during the

postprandial state. We showed an inverse relationship between NO levels and both IDE activity and insulin clearance in fasted as well as postprandial states. Moreover, acute NO depletion led to a decrease in insulin bioavailability with unchanged insulin secretion; and an increase in insulin signaling and glucose transporters expression. However, the increased insulin secretion observed in the chronic NO-depleted animals results in normalized plasma insulin levels and signaling activities. We conclude that NO is a physiological regulator of insulin clearance by inhibiting hepatic IDE activity, thus promoting increased insulin bioavailability in the post-prandial state.

Considering the newly discovered role of NO in the regulation of hepatic insulin clearance we looked for the alterations in insulin clearance as a result of central NOS inhibition. We hypothesized that brain NO production modulates hepatic insulin clearance and peripheral insulin bioavailability. We found that paraventricular nucleus (PVN) of the hypothalamus is important but not an exclusive regulator of insulin clearance by NO signaling. Additionally, NO production in the hypothalamus is involved in the regulation of hepatic insulin actions and lipid metabolism.

We aimed to study the possible role of altered insulin clearance on the development of insulin resistance and we described the mechanisms of action of a TZD. We hypothesized that TZD ameliorates steatosis and hyperinsulinemia. We confirmed the hypothesis and showed that TZD reduced *de novo* lipogenesis and increased insulin clearance by the elevation of IDE activity and this was correlated with a decrease in hepatic NO levels.

Changes in hepatic glucose and glycogen metabolism are key early outcomes of insulin resistance and T2D and methodologies to quantify these pathways that are directly translatable from animal models to humans are essential. The deuterated water (^2H_2O) method for quantifying sources of hepatic glucose and glycogen synthesis is highly translatable, but to date has been based on assumptions about metabolite deuterium exchanges that have been tested in humans, but not in animal models. We found important species-specific differences in deuterium exchange at the level of hepatic glucose-6-phosphate and triose phosphate and based on this information, correction factors that allow more accurate determination of glucose and glycogen synthesis fluxes in rats were obtained.

In conclusion, hepatic NO inhibits IDE activity *in vivo*, leading to a decrease in insulin clearance in the post-prandial state and consequent increase on insulin bioavailability, when plasma glucose levels are higher. Moreover, NO produced in hypothalamus also regulates insulin clearance, by a mechanism that needs to be further clarified, but that seems to be

related to ANS alteration. Knowledge about treatment with TZDs can benefit from the results of this study, since we confirmed that this drug targets insulin clearance, with impact on lipid metabolism. Finally, our studies have enabled better translational studies of hepatic glucose and glycogen metabolism: key outcomes in both T2D pathogenesis and therapy.

CHAPTER 1 – INTRODUCTION

1. Introduction

The process of living requires energy and living organisms have the capacity and the requirement to precisely match its energy demands to available energy sources. Higher organisms, including humans, must maintain constant levels of blood glucose in order to sustain the function of the central nervous system since the brain is primarily dependent on glucose as an energy source. However, other tissues require different nutrients in different quantities, for example skeletal muscle and heart derive most of their energy from fat oxidation. The ingestion of a mixed meal supports these demands by providing all the needed nutrients. How an organism couples these ingested nutrients to the energy demands of constituent tissues is a fundamental cornerstone of metabolic regulation.

The gut is crucial for nutrient digestion and absorption, and through regulation of these processes it is a major participant in nutrient homeostasis. Downstream of the gut, the liver is a central player in nutrient homeostasis by rectifying the periodic surge of nutrients from feeding into constant circulating levels for peripheral tissue metabolism. Coordination of gut and liver nutrient handling via the central nervous system (CNS) and integration of these functions with the CNS nutrient sensing circuits constitutes an efficient and responsive system for whole body nutrient homeostasis. The evolution of this system, involving gut, liver and brain, has occurred in order to deal with a limited and intermittent supply of food and to: a) always maintain threshold levels of oxidizable substrates such as glucose under all conditions and b) efficiently store nutrients during the few times when they were is excess. However, over the last decades in Western societies, with the chronic excess intake of food and the consumption of high caloric diets this system has been challenged in a completely opposite direction to its normal evolutionary pressures. Therefore, its inability to adjust to this new state of overnutrition is on the basis of many of the major diseases in modern societies, including Type 2 Diabetes (T2D) and Non-Alcoholic Fatty Liver Disease (NAFLD).

1.1. Gut to Brain to Liver Axis

As already mentioned, the sensing of nutrient and energy status of any organism is based on communication of the brain with both gut and liver. The brain is the key organ for finding the food, the gut is the main site for digesting the food and liver is the main organ involved on portioning the food into oxidation or storage and for maintaining constant circulating nutrient levels.

After meal ingestion, among the first communication components of this axis are the nutrients themselves. Nutrient digestion and/or absorption trigger responses in other tissues, such as the pancreas and liver. These include synthesis and secretion of hormones in the gut (e.g. incretins), pancreas (e.g. insulin) and adipocytes (e.g. leptin), which in turn mediate a wide range of metabolic flux responses in splanchnic as well as in more peripheral tissues. Enteric stimulation as well as hormone secretion also activates central nervous system, which in turn influences the metabolic activity of key regulatory organs such as the liver. Finally, other kinds of metabolic products have an important role in the gut-brain-liver axis. They are messenger metabolites, such as nitric oxide (NO) and superoxide, with a short half-life, which are generated as a result of hormonal or nerve actions and in turn promote local physiological and metabolic changes. The synchronized function of the liver-gut-brain axis in matching nutrient availability to demand and maintaining constant levels of blood glucose depends on the integrated actions of these components.

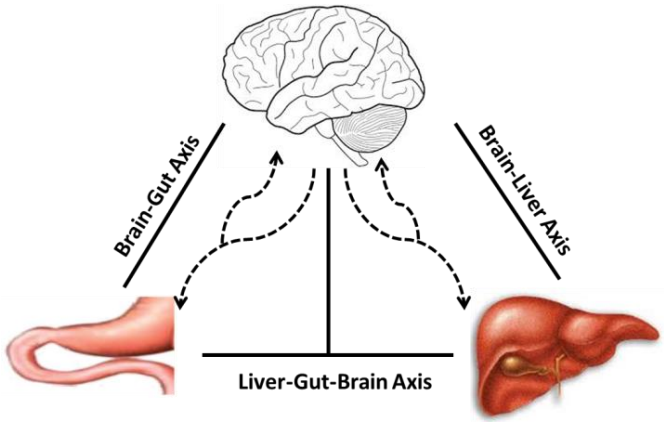

Figure 1: Representation of the interconnections between gut, brain and liver: the synchronization of the connections between liver, gut and brain is crucial on matching nutrient availability to demand and maintaining constant levels of blood glucose. There are afferent and efferent ramifications which will be responsible for the regulation of coupled organs.

There are many examples of known interactions involving hormones, nerves and messenger metabolites. It is now known that enteric cells can sense alterations in glucose levels and releases incretins, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). These incretins will be responsible for potentiating insulin secretion above and beyond that mediated by beta-cell glucose sensing. It has also been described that enteric glucose sensors transmit endocrine and neuronal signals to peripheral tissues. GLP-1 is a candidate regulator of the neuronal network that controls glucose homeostasis. The GLP-1 signal that reaches the hypothalamus activates NPY-positive cells and by inhibition of AMP-activated protein kinase (AMPK) pathway and consequent increase of malonyl-CoA, stimulates glucose uptake by muscle (Knauf, Cani et al. 2008).

The brain receives those signals from the intestine by vagal afferent nerves, and nutrient-regulating hormones such leptin, insulin, ghrelin, etc. contribute to the regulation of metabolism through responses of the brain to these intestinal signals. The hypothalamus has been demonstrated to regulate hepatic glucose production, which can be abolished by hepatic vagotomy (Beraza and Trautwein 2008). Duparc demonstrated that the hypothalamus needs peripherally-derived factors such as glucose and/or insulin to enable NO-mediated responses to the stimulation of glycolysis and inhibition of oxidative phosphorylation (Duparc, Colom et al. 2011). Conversely, the direct infusion of glucose into the brain produces a different hypothalamic signal that inhibits muscle glucose uptake (Knauf, Cani et al. 2008). Other studies suggest that the increase in glucose utilization, after GLP-1 receptor activation is due to the recruitment of muscle microvasculature. This consequently results in an increased insulin delivery that occurs via a NO-dependent mechanism previously described in the work of Chai and colleagues (Chai, Dong et al. 2012). In this work, the effects of GLP-1 infusion over microvasculature recruitment were accompanied by increased NO levels and more interestingly, was also followed by an increase in muscle insulin clearance. Therefore, it seems that NO generation via GLP-1 receptor signaling is tightly correlated with glucose utilization and metabolism of insulin.

These regulatory systems involving the gut-brain-liver axis can be disrupted by excessive nutrient intake and/or genetic polymorphisms and alterations. Humans and other living organisms are not biologically adapted to an excess availability of nutrients. Moreover, the effect of polymorphisms may only be revealed when under such a nutritional challenge.

For example, animals maintained in high-fat diet showed impaired enteric glucose signaling, but it is not known if the dysfunction occurs in the transmission of enteric glucose sensor signals to the hypothalamus or in the transmission of signals from the hypothalamus to peripheral tissues (Knauf, Cani et al. 2008). What is known is that in conditions of impaired muscle glucose utilization (obesity, diabetes, etc.) the stimulation of enteric glucose sensors failed to increase hypothalamic NO release (Duparc, Colom et al. 2011).

Therefore, it is important to resolve the hepatic and enteric signals that are involved in the burst of central NO production in response to a glucose bolus and how these signals function under pathologic conditions induced by overnutrition, such as diabetes and obesity.

1.1.1. Hypothalamus

The hypothalamus is the gray matter flanking the third ventricle below the thalamus. It is a critical regulator of hepatic energy metabolism and endocrine functions. The hypothalamus is likely to be a key player in central glucometabolic regulation since it contains specialized neurons whose activity is regulated by changes in glucose levels (Uyama, Geerts et al. 2004).

It is known that the autonomic nerves that enervate the liver are derived from the hypothalamus (Uyama, Geerts et al. 2004). The autonomic nervous system (ANS) comprises the nerves from the sympathetic and the parasympathetic system, and various regions of the brain are described to be involved in autonomic regulation (Figure 2).

The hypothalamus has been divided in different areas, depending on its neuronal type, content, or activity. The lateral hypothalamus (LAH) is able to send parasympathetic signals to peripheral tissues that result in local modulation of metabolic activity that promote nutrient storage, such as glycogen synthesis and lipogenesis, as well as contributing to the control of appetite and food intake. The ventromedial hypothalamic nucleus (VMH) sends sympathetic signals that are antagonistic to those from LAH promoting catabolic responses like gluconeogenesis, lipolysis, appetite gain, etc. The arcuate nucleus (ARC), which is also involved in the regulation of glucose and lipid metabolism, is able to send signals to the paraventricular nucleus (PVN) and other nuclei in the hypothalamus. The PVN region is insulin, leptin and glucose sensitive, and can also be considered as an integrative station by receiving information from other parts of the hypothalamus. It is also responsible for transmitting information to autonomic nerves and the pituitary gland. The PVN contains a high density of insulin receptors

and there is evidence that insulin-dependent increase of sympathetic activity is a consequence of their activation (Dampney 2011). The actions of PVN on peripheral tissue metabolism seem to be also dependent on vagus activity (Zhang and Fogel 2002). The PVN region is formed by two types of cells: one type controls the ANS and regulates the sympathetic and parasympathetic outflow to visceral organs, including liver, pancreas and adrenal glands; while the other type consists of neurosecretory neurons, which communicate with the pituitary gland and control the secretion of various hormones. Thus, the PVN regulates a wide range of conditions by stimulation of the ANS and release of various hormones from the pituitary gland (Uyama, Geerts et al. 2004; Zheng, Mayhan et al. 2006).

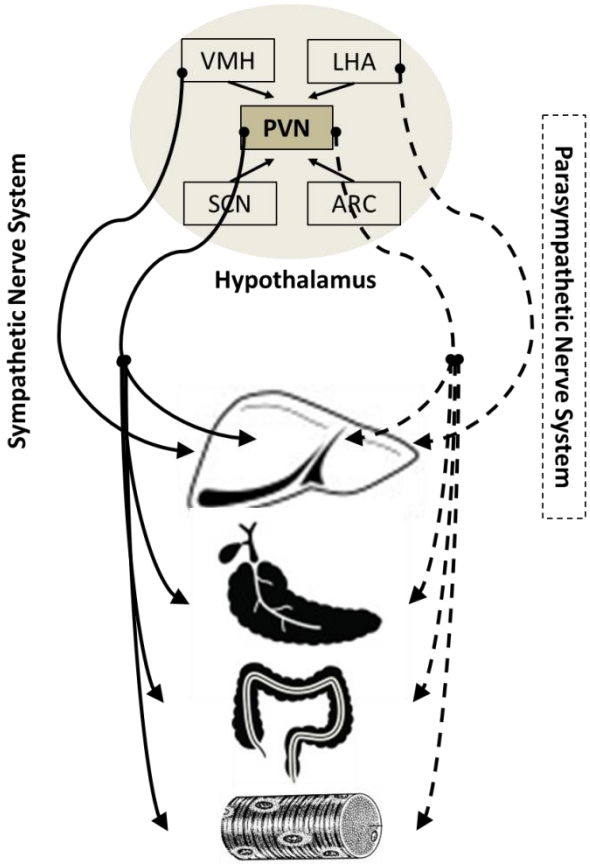

Figure 2: Autonomic nervous system, hypothalamus regions interconnections and peripheral tissues enervations: the ANS comprises nerves from sympathetic and parasympathetic nervous system; various regions of the hypothalamus sends ANS signals for peripheral tissues, with the PVN acting as a central integrator of converging signals from the other hypothalamic regions.

Nitric oxide has been recognized as an important second messenger in brain and the stimulation of NO levels in the hypothalamus and consequent control of glucose homeostasis has been studied in detail. NO production seems to be dependent on the activation of the phosphatidylinositol 3 kinase (PI3K) pathway, which can increase nitric oxide synthase (NOS) activity in the brain either directly via Akt or indirectly by inactivation of the AMPK pathway (Canabal, Song et al. 2007). Moreover, in addition to the autonomic signals sent from the hypothalamus, centrally produced NO can affect key peripheral tissues, including pancreas, liver and muscle (Cabou, Campistron et al. 2008). Krukoff and Patel revealed that there were significant increases in neuronal activity within the PVN region in streptozotocin (STZ)-induced diabetic rats, suggesting that neurons in the PVN region are activated during diabetes and may contribute to the autonomic dysfunctions (Krukoff and Patel 1990). The microinjection of a NO donor or the inhibition of NO production in the PVN region also demonstrated that NO in this site has an inhibitory effect on sympathetic neuronal activity (Zhang, Mayhan et al. 1997). Therefore, altered NO dynamics in the PVN region may contribute to the increased sympathetic nerve activity during diabetes.

1.1.2. Liver

The liver has a central role in the disposal of a meal due to its anatomic location and its ability to process a large variety of nutrients (Figure 3). It is responsible for the removal of ≈30% of absorbed glucose after meal ingestion. In the fasted state, when there is no glucose being absorbed from digestion, the liver is a net producer of glucose by promoting gluconeogenesis, enabling the constant demand by the central nervous system and other glucose-dependent cells and tissues to be sustained. It is able to rapidly downregulate this activity following meal ingestion and store excess dietary carbohydrate as glycogen or convert it to lipid (Commerford, Bizeau et al. 2001).

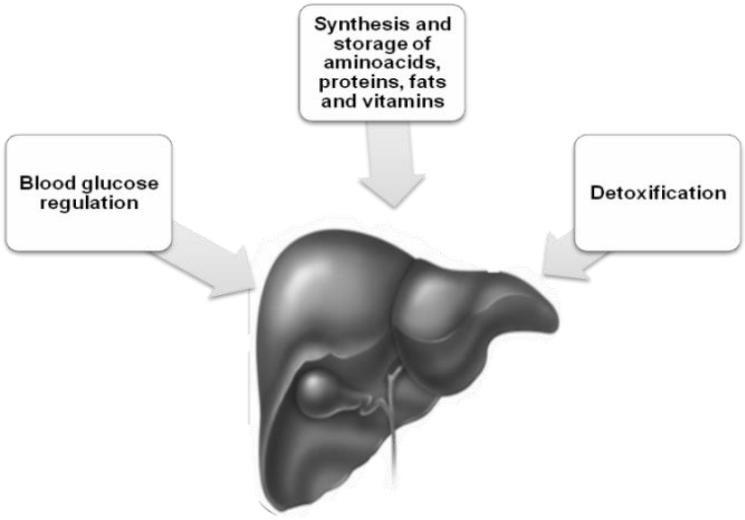

Figure 3: The liver as an active organ in many metabolic pathways: glycogenesis, glycogenolysis, gluconeogenesis, fatty acids oxidation, lipogenesis, amino acid synthesis and catabolism.

In the setting of glucose homeostasis, alterations in net hepatic glycogen synthesis rates or gluconeogenesis can reveal hormonal or cell signaling defects in glycemic control, such as impaired insulin signaling in T2D. Hepatic glycogen can be synthesized from glucose via the so called "direct pathway" or from gluconeogenic precursors by the indirect pathway , as depicted in Figure 4. Indirect pathway precursors can be derived from glucose metabolism (i.e. lactate) or from other sources, for example glycerol from triglyceride hydrolysis or dietary gluconeogenic amino acids such as alanine.

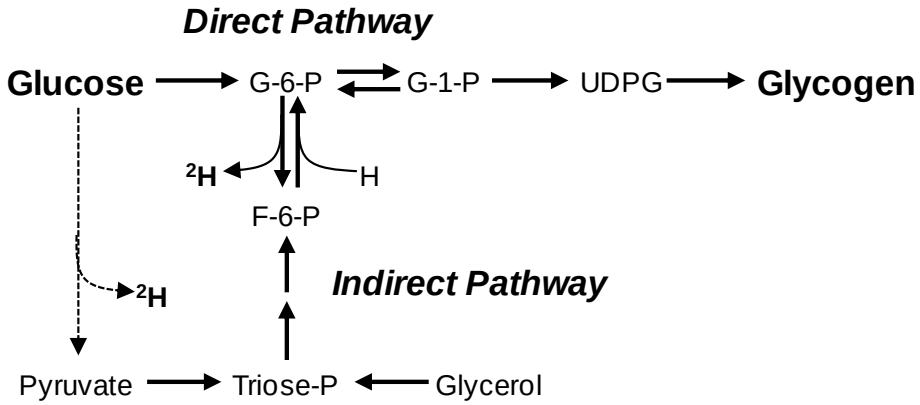

Figure 4: Depiction of direct and indirect pathways during conversion of glucose to glycogen. Some metabolic intermediates are omitted for clarity. F-6-P = fructose-6-phosphate, G-6-P = glucose-6-phosphate, G-1-P = glucose-1-phosphate, Triose-P = triose phosphates, UDPG = uridinediphosphoglucose [adapted from (Martins, Rito et al. 2013)].

Humans and rodents typically synthesize the majority of glycogen via the direct pathway reflecting a higher proportion of dietary carbohydrate (Jones, Fagulha et al. 2006; Delgado, Silva et al. 2009; Soares, Viega et al. 2009; Soares, Carvalho et al. 2012). Alterations in direct and indirect pathway activities can reflect changes in hepatic insulin actions, such as in insulin-dependent diabetes as well as adaptation to different dietary regimes (Obeid, Bittar et al. 2005; Obeid, Boukarim et al. 2006).

In addition to glycogen synthesis, excess glucose can be metabolized through glycolysis and *de novo* lipogenesis (DNL) to be stored in the form of lipids. Glucose is converted into pyruvate through glycolysis in the cytoplasm, and pyruvate is subsequently oxidized in the mitochondria to generate adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. So, DNL plays a crucial role by converting excess of non-lipid substrates, mainly carbohydrates, to free fatty acids (FFAs) and triglycerides (TGs), to overcome the relatively limited capacity to store energy as carbohydrate .

Although many different tissues possess the enzymes for DNL, the primary site of lipid biosynthesis is the liver. Moreover, the liver synthesizes the lipoprotein vehicles for TGs loading and export to the blood for delivery, utilization and storage by peripheral tissues. Hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or DNL) and lipid disposal (via FFA oxidation or TG-rich lipoprotein secretion to be deposited in fat) leading to lipoperoxidative stress and hepatic injury.

1.2. Insulin Metabolism

1.2.1. Insulin Structure

Insulin is a small hormone secreted by the pancreatic beta cells and is a primary regulator of glucose and lipid metabolism under physiological feeding-fasting conditions. Insulin circulates in a range of 8–11 μU/mL (57–79 pmol/L) between meals. It is composed of 51 aminoacids divided in two polypeptide chains: chain A and chain B. The assembly of insulin starts with the translation of an inactive protein called preproinsulin. This protein undergoes post-translational modifications with cleavage of the amino terminal portion not needed in the bioactive hormone. In the endoplasmic reticulum (ER) the signal sequence of preproinsulin is proteolytically removed to form proinsulin. This molecule, stabilized by three disulfide bonds, is then cleaved by specific peptidases. The final products are the mature bioactive insulin, now

with two disulfide bonds between the two chains, and the connecting peptide (C-peptide) (Figure 5). Finally, insulin is packaged and stored in secretory granules, which accumulate in the cytoplasm, until release is triggered. C-peptide is also released to the circulation but little is known about it biological activity. Insulin is secreted in response to increased blood glucose and amino acids after ingestion of a meal and it is a key anabolic hormone.

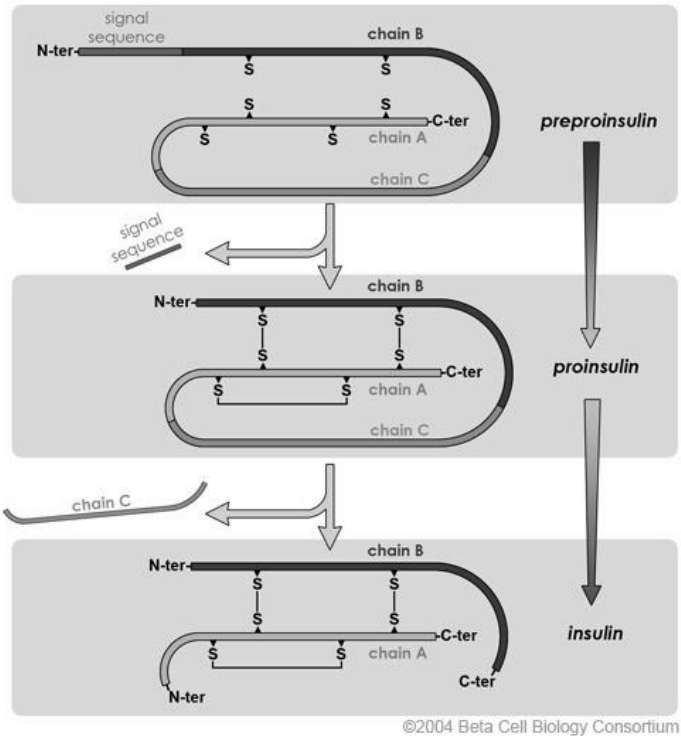

Figure 5: Schematic representation of post-translational modifications for the production of mature and bioactive insulin (chain C represents c-peptide) (adapted from Beta cell Biology Consortium, 2004).

1.2.2. Insulin Secretion Pathway

Insulin is stored in pancreatic beta cells inside of large dense secretory granules and is secreted in response to glucose or other nutrient secretagogues. Beta cells has both high-capacity and high-sensitivity properties. This means that beta cells exhibit absolute rates of insulin release and rapid and robust response to glucose increments (Ferrannini 2010). Beta cell integrates multiple inputs, both stimulatory and inhibitory to produce the secretory response. The final consequence must guarantee that the insulin release will be sufficient in quantity to promote the effects at peripheral tissues but also has to guarantee that insulin secretion will be appropriate in timing because delayed or early secretion is associated to hyper- or hypoglycemia, respectively. Thus, insulin secretion must be coupled to glucose levels through a steep dose-response relationship, rate sensitive by reading the rate of change in glucose levels and capable of potentiation by some form of glucose memory (Henquin, Dufrane et al. 2006).

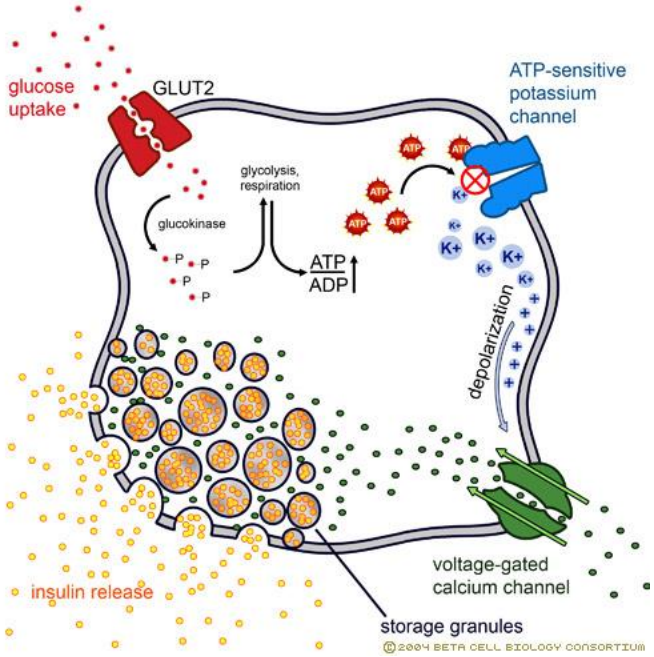

Figure 6: Representation of alterations in beta cells due to increase in plasma glucose levels and consequent secretion of insulin: GLUT2 in beta cells allow entering of glucose into beta cells. The increase in glucose inside the cell stimulates glycolysis and consequently the increase in ATP/ADP ratio. This increase promotes the inactivation of potassium channels which depolarizes the cell membrane. The depolarization leads to the intake of calcium into beta cell that promotes the release of insulin stored in vesicles (adapted from Cartailler, J-P, Beta cell Biology Consortium, 2004).

Beta cells sense increases in blood glucose levels due to the presence of glucose transporters 2 (GLUT2), which mediates the entry of glucose into these cells. The glycolytic phosphorylation of glucose in the beta cell causes a rise in the ATP:ADP ratio. This rise inactivates the potassium channel that depolarizes the membrane, causing the calcium channel to open up allowing calcium ions to flow inward. The ensuing rise in levels of calcium leads to the exocytotic release of insulin from their storage granule (Figure 6) (Lang 1999).

In the post-prandial state, the secretion of insulin occurs in two phases: an initial rapid release of preformed insulin found in large dense core vesicles and a second phase of increased newly synthetized insulin and release after the encapsulation in the secretory vesicles, in response to blood glucose. Beta cells also have to regenerate the stores of insulin initially depleted in the fast response phase (Cartailler, J-P, Beta cell Biology Consortium, 2004).

Besides glucose, other nutrients promote the secretion of insulin. These include some amino acids (arginine, leucine and lysine especially), as well hormones such as the incretins GLP-1 and GIP, and also neurotransmitters acting on G-protein coupled receptors (Lang 1999).

1.2.3. Insulin Signaling

Insulin action starts with the binding of insulin to the alpha subunits of its specific receptor present in many cells including those of adipose, liver and muscle tissues. The receptor for insulin is embedded in the plasma membrane and is composed of two alpha subunits and two beta subunits linked by disulfide bonds (Figure 7). The alpha chains are entirely extracellular and house insulin binding domains, while the linked beta chains penetrate through the plasma membrane.

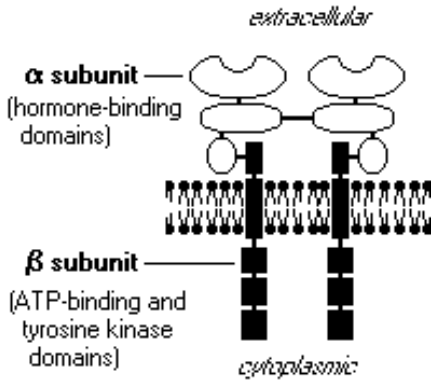

Figure 7: Representation of the structure of insulin receptor: composed of two extracellular alfa subunits and two cytoplasmic beta subunits; insulin binds to the alfa subunits outside the cells; the beta subunits contain the tyrosine kinase activity.

The binding of insulin to its receptor promotes the activation of glucose transporters (mainly GLUT4). The initial binding of insulin to its receptor and consequent autophosphorylation of the receptor initiates a signal cascade. This cascade involves numerous signaling entities and influences multiple metabolic pathways. The cascade endpoints can vary depending on the stimulus involved, the nutritional state of the organism, pathophysiology, etc. (Figure 8) indicating that insulin actions overlap and are integrated with other nutrient and energy sensing and transducing mechanisms. The canonical pathway involves PI3K/Akt, which are dependent on the phosphorylation of insulin receptor substrates (IRS) (principally IRS-1 and-2) and triggers the metabolic functions of insulin. IRS-1 appears to be linked to glucose homeostasis and IRS-2 to the regulation of lipid metabolism (Siddle 2011).

Additional signaling pathways have been linked with insulin-IR binding. The growth factor receptor-bound protein 2/mitogen-activated protein kinase (GRB2/MAPK) pathway involves also the IR autophosphorylation and leads to enhanced cell growth. Another cascade activated by insulin is the Cbl associated protein (Cbl/CAP) cascade, which mediates glucose transport through activation of the GTP-binding protein TC10 and remodeling of actin filaments (Meshkani and Adeli 2009; Gallagher, Leroith et al. 2010).

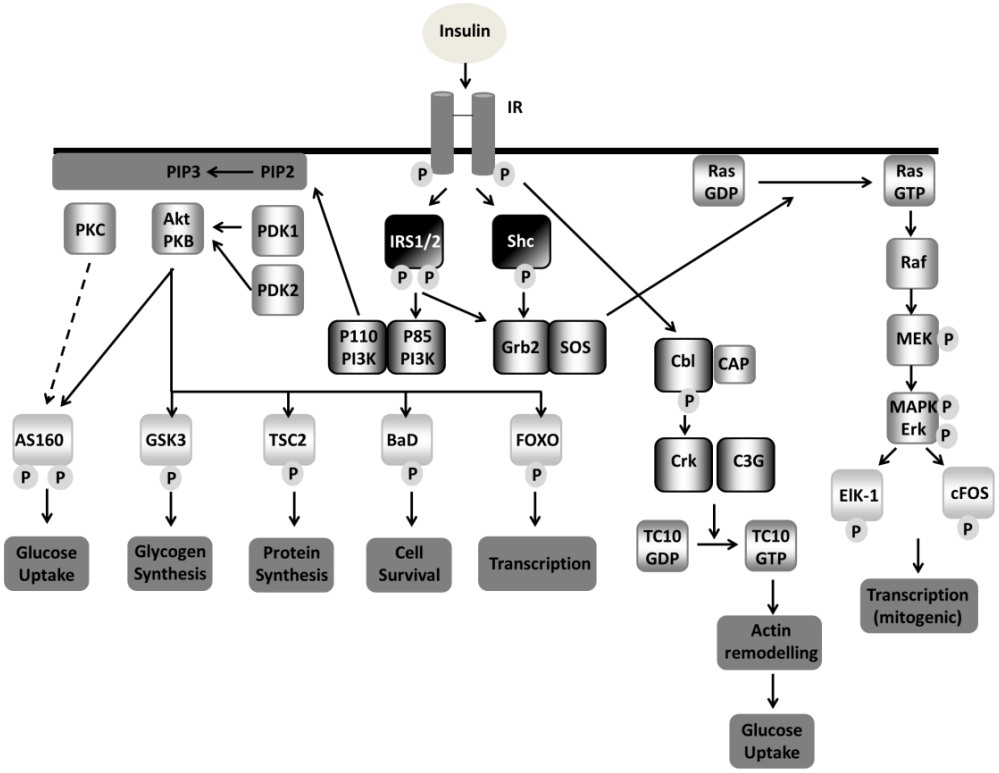

Figure 8: Insulin-Insulin receptor binding-dependent pathways and its metabolic effects: the canonical pathway involving PI3K molecule, GRB2/MAPK pathway and Cbl/CAP cascade.

The primary action of insulin is to stimulate glucose uptake from blood into tissues thereby lowering high levels of blood glucose, but it also has many other functions. In Table 1 the principal effects of insulin are summarized. As it can be seen insulin helps control blood glucose levels firstly by activating glucose uptake by peripheral tissues, of which skeletal muscle is quantitatively the most important (DCCT Research Group 1997; DeFronzo 2004). Insulin also acts on the liver to promote glycogenesis and, by inhibition of glucagon secretion from pancreatic alfa cells, to attenuate hepatic glucose production via gluconeogenesis and glycogenolysis. Moreover, insulin is a potent antilipolytic hormone that potentiates fat synthesis and triglyceride storage.

Table 1: Description of the primary actions of insulin over metabolism.

Effects of Insulin over metabolism
1. Increase of glucose uptake (mainly in skeletal muscle)
2. Decrease of hepatic glucose production: decrease of gluconeogenesis and glycogenolysis
3. Increase of glycogenesis
4. Decrease of lipolysis and increase of fat synthesis and storage in the form of TGs
5. Increase of protein synthesis

1.2.4. Insulin Clearance

After its secretion by the pancreas, insulin is released directly into the portal vein entering in the liver. During its first-pass through the liver, about 50% of this insulin is removed hence the concentration of circulating insulin seen by peripheral tissues is only one-half of portal vein levels (Figure 9) (Balks and Jungermann 1984; Duckworth, Bennett et al. 1998). The kidney is responsible for the degradation of ≈ 80% of the insulin that reaches periphery. The physiological rationale for this high clearance of insulin by the liver immediately after its secretion, the mechanisms involved in mediating and regulating this process and the role of altered insulin clearance in the loss of glucose homeostasis are poorly understood. Hence, the main goal of the work presented here is to contribute towards filling the knowledge gap about this overlooked feature of insulin kinetics.

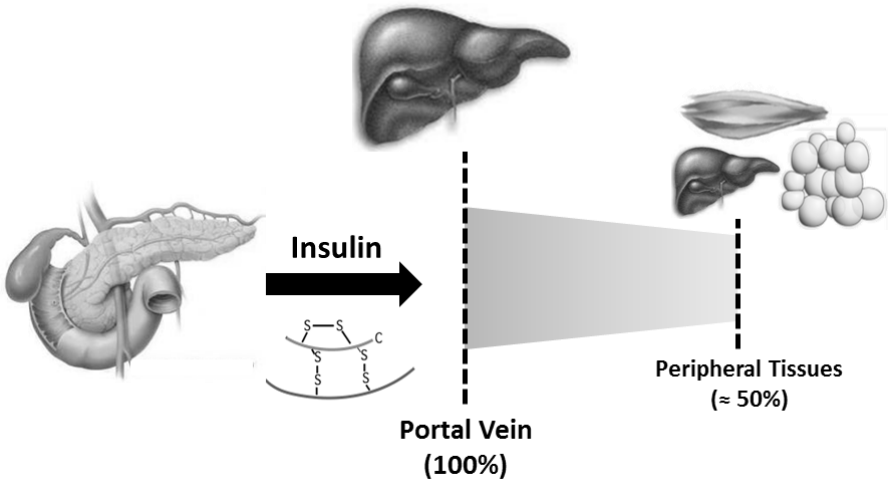

Figure 9: Insulin is secreted by the pancreas being released in portal vein and going direct to the liver. In this first-pass in the liver insulin is removed from circulation and only around half of the initial insulin reaches peripheral tissues.

Insulin clearance is largely mediated by binding to its receptor and only at very high concentration the non-receptor processes such as pinocytosis assume relevance. So, internalization of the bound insulin-receptor complex is critical for both insulin degradation and hepatic insulin actions. The 80% of the total insulin in the body that binds to liver receptors can be either fully degraded or be returned to the circulation either intact or partially degraded (Duckworth, Bennett et al. 1998). So, peripheral insulin levels and actions are therefore highly dependent on hepatic removal.

It is known that insulin degrading enzyme (IDE) is the primary enzyme involved in the proteolytic degradation and inactivation of insulin following endocytosis. However, the extent to which IDE participates in the degradation of internalized insulin remains controversial, and clarifying its role may be important for a better understanding of changes in insulin availability and activity (Duckworth, Bennett et al. 1998; Leissring, Malito et al. 2010).

The other enzyme involved in the process of insulin catabolism is protein disulfide isomerase (PDI), which catalyzes the formation, breakage and rearrangement of disulfide bonds (Duckworth, Bennett et al. 1998).

If insulin clearance is impaired, then the peripheral circulation will have excessive insulin levels for a given pancreatic secretory response. The role of diminished insulin clearance in

promoting systemic hyperinsulinemia is not clear, since establishment of high plasma insulin levels also reduces the number of insulin receptors in a negative feedback manner (Duckworth, Bennett et al. 1998). Glucose is also a modulator of insulin clearance as increasing doses of glucose (10g, 25g and 100g) result in increased insulin secretion (1.8U, 2.7U, and 7.2U) with concurrent decreases in hepatic extraction (67%, 53%, 42%) in physiologic conditions (Duckworth, Bennett et al. 1998). Insulin clearance impairment seems to be involved in the hyperinsulinemia associated with the establishment of insulin resistance in diabetes or obesity (Duckworth, Bennett et al. 1998). Some studies show that in the early stages of insulin resistance, the reduction in fractional insulin clearance contributes more to hyperinsulinemia than increased beta cell secretion rates (Erdmann, Pohnl et al. 2012). With the progression of insulin resistance there is eventually an increase in insulin secretion as glycemia becomes progressively less responsive to plasma insulin levels. However, the ability of the pancreas to secrete insulin reaches its maximum during the evolution of the insulin resistance state and further elevations of plasma insulin levels are only possible by a progressive reduction of insulin clearance (Erdmann, Mayr et al. 2009; Erdmann, Pohnl et al. 2012).

It is also well recognized that FFAs, emblematic metabolites of insulin resistance, also inhibit insulin degradation. Previous results suggest that FFAs interact with IDE (Hamel 2009). Moreover, in lean subjects an acute two-fold increase in plasma FFAs levels via intralipid/heparin infusion promoted a pronounced reduction in endogenous insulin clearance independently of glucose (Hennes, Dua et al. 1997).

Taken together, a decrease in hepatic insulin clearance seems to be a primary factor in the development of hyperinsulinemia and whole-body insulin resistance.

1.2.4.1. Insulin-Degrading Enzyme (IDE)

IDE, also known as insulysin and insulinase, is a ubiquitously expressed and soluble enzyme of ≈110-kDa, belonging to the superfamily of Zn^{2+}-metalloproteases (Figure 10) (Leissring, Malito et al. 2010; Abdul-Hay, Kang et al. 2011). It is found in the cytosol, peroxisomes, endosomes and cell surface and it is expressed mainly in the liver, followed by kidney, muscle cells, adipocytes and erythrocytes (Duckworth, Bennett et al. 1998).

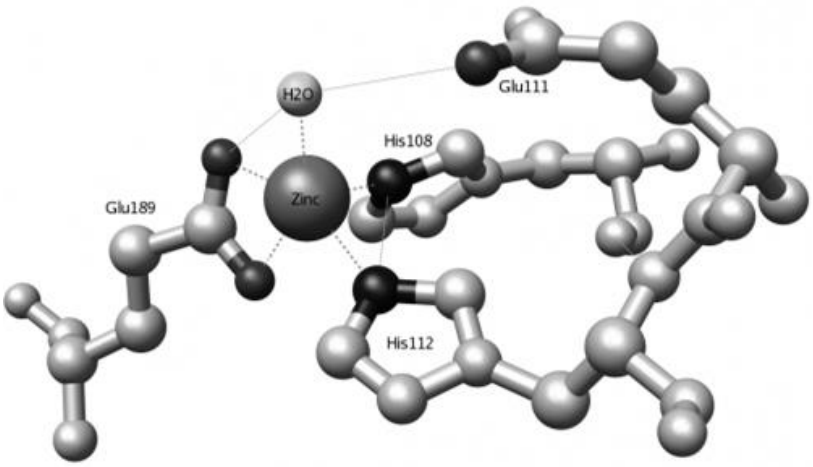

Figure 10: IDE3D structure: this enzyme is formed by two bowl shaped halves, linked by a 28 amino acids sequence. Zn ion is present in the catalytic active site and allows the enzyme to have the correct conformation and selectivity for its substrates (Shen, Joachimiak et al. 2006).

It consists of two bowl-shaped halves of 56-kDa, connected by a flexible linker of 28 amino acid residues, that can switch from open to closed states (Camberos, Perez et al. 2001; Leissring, Malito et al. 2010). In its closed state, IDE encapsulates its substrates and is remarkably specific to insulin. Hence, it is the primary protease involved in the degradation and inactivation of this hormone (Duckworth, Bennett et al. 1998; Shen, Joachimiak et al. 2006). IDE degrades several other peptides, with intermediate size (smaller than 70 residues), namely atrial natriuric peptide, glucagon, amyloid β-protein (Aβ), and many others. The principal difference from the degradation of these peptides and insulin is that unlike insulin, all the other mentioned peptides can also be degraded by other proteases (Farris, Mansourian et al. 2003). As well as proteolysis of insulin IDE also regulates the translocation of this hormone from the cytoplasm to the nucleus (Harada, Smith et al. 1993).

IDE seems to come into contact with insulin subsequent to the receptor-mediated internalization of the hormone (Baumeister, Muller et al. 1993). Within endosomes insulin detaches from its receptor, due to rapid acidification, which is than recycled to the cell surface. Intact or already partially degraded insulin from endosomes enter the cytosol and interacts again with cytosolic IDE to undergo further degradation (Duckworth, Bennett et al. 1998; Fawcett and Duckworth 2009). This partially degraded insulin has intact A chain, B chain with one or more cleavages and intact disulfide bonds. Subsequently, the disulfide bonds are cleaved and occurs the release of B chain fragments and intact A chain. This step of disulfide bonds cleavage was attributed to another enzyme, PDI, which will be discussed in detail later.

Afterwards, the intact A chain and B chain insulin fragments released to the cytosol can suffer further degradation by IDE (Figure 11) (Duckworth, Bennett et al. 1998).

There are several proposed mechanisms for the regulation of IDE activity, mostly involving the thiol sensitivity of the enzyme. IDE can be regulated by small molecules, like ATP and other nucleotide polyphosphates (Camberos, Perez et al. 2001; Song, Juliano et al. 2003), bradykinin (Malito, Ralat et al. 2008), insulin itself (Pivovarova, Gogebakan et al. 2009), reduced glutathione (GSH) (Cordes, Bennett et al. 2011), etc. as activators and many inhibitors like Iil (Leissring, Malito et al. 2010), bacitracin (Fawcett, Sang et al. 2010), glucose (Pivovarova, Gogebakan et al. 2009), insulin-binding inhibitors, non-esterified fatty acids (NEFAs) (Hamel 2009), NO (Cordes, Bennett et al. 2009), oxidized glutathione (GSSG) (Cordes, Bennett et al. 2011).

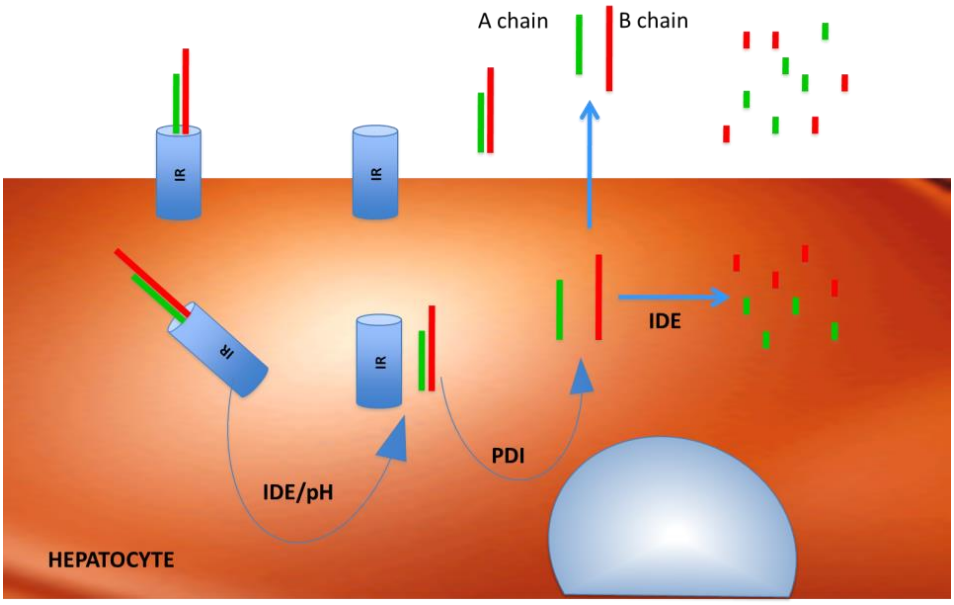

Figure 11: Steps sequence in the degradation of insulin by IDE: degradation starts with the binding of insulin to its receptor and endocytosis of the complex insulin-IR. Within endosomes pH decreases and insulin is detached from its receptor. Afterwards IDE, present both in endosomes and in cytoplasm cleaves insulin molecule in some specific points of the two chains.

The sensitivity of IDE activity to the intracellular redox state may be important in explaining altered IDE activity from fasting to feeding or in pathologic conditions such as diabetes or obesity. It is known that NO levels and GSSG/GSH ratios are also altered under these conditions. In situations where NO levels are high and GSSG/GSH is decreased, IDE can be nitrosylated and/or glutathionylated, and these modifications inhibit its activity (Cordes, Bennett et al. 2011). This can be an explanation for the alterations of insulin degradation via IDE inhibition in some studies, for example, the one from Fawcett and coworkers, in which they found that differences in the insulin degradation in adipocytes of pre-diabetic or diabetic overweight subjects were due to alterations in the IDE activity concomitant with increases of oxidative stress and inflammatory markers (Fawcett, Sang et al. 2010).

Moreover, IDE has been implicated in other diseases like Alzheimer's (AD). IDE activity has been described to be reduced in AD patients, promoting a decrease of Aβ degradation, leading to cognitive impairment (Zhao, Teter et al. 2004). Moreover, in aged mice IDE has been shown to be inactivated by alkylation (Caccamo, Oddo et al. 2005).

Recently some groups have reported an association of polymorphisms of the gene coding for the IDE with diabetes, with IDE genetic variants influencing insulin metabolism (Rudovich, Pivovarova et al. 2009; Pivovarova, Bernigau et al. 2013). Others have observed that the IDE gene alleles associated with diabetes also correlated with decreased pancreatic beta cell function, but the association with insulin clearance was not evaluated. These and other studies suggest that IDE plays a role in the pathogenesis of aging-related diseases, like T2D and AD, with a consequent involvement of insulin clearance alterations.

1.2.4.2. Protein Disulfide Isomerase (PDI)

PDI is a 57-kDa homodimeric ubiquitously expressed enzyme, with acidic nature and homology to thioredoxin activity (Lundstrom and Holmgren 1990). It locates abundantly in ER and it is secreted from a variety of cell types like hepatocytes, endothelial cells, pancreatic cells, platelets, etc.. It binds to the cell surface through electrostatic interactions (Terada, Manchikalapudi et al. 1995). It catalyzes the disulfide bond formation and cleavage of newly synthesized proteins (Figure 12). PDI has been reported to have different functions in different cells, namely in platelet activation/aggregation, protein adhesion, infection and pathology of many diseases like, human immunodeficiency virus (HIV), cancer and pathogenic infections,

etc. (Raturi and Mutus 2007). Moreover, PDI also mimics the subunits of prolyl-4-hydroxylase and the microsomal triglyceride transfer protein (MTP) and functions as a glutathione-insulin transhydrogenase, promoting the cleavage of disulfide bonds between the two chains of insulin (Varandani 1974).

Both the expression and catalytic activity of PDI was found to be predominantly located in liver and pancreas, and to a lesser extent in kidney, fat, heart, muscle and brain (Mikami, Genma et al. 1998). The expression of PDI has been shown to be influenced by the extent of protein biosynthesis and stress conditions. Its specific activity was found to be dependent on the ratio of [GSH]/ [GSSG], being attenuated in a more oxidizing environment (Raturi and Mutus 2007).

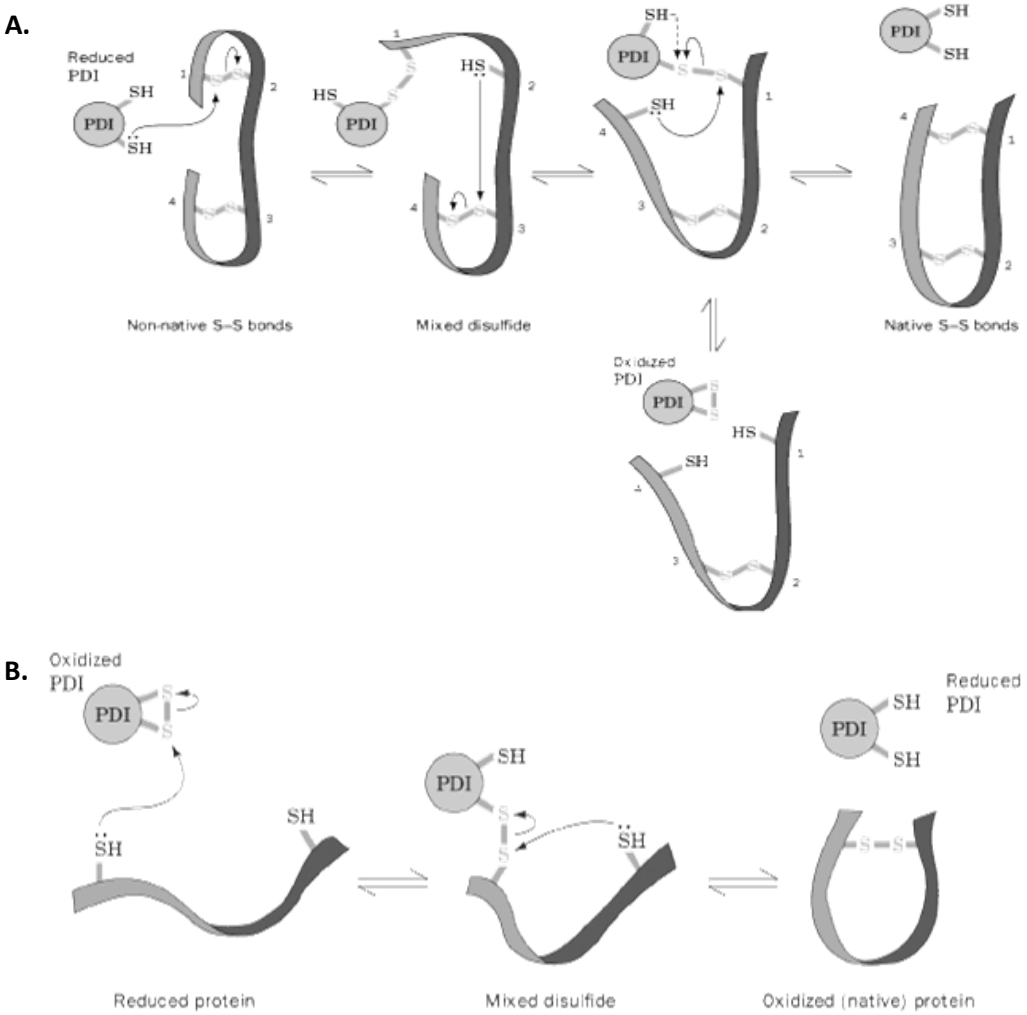

Figure 12: PDI activity: PDI cleaves and rearranges disulfide bonds (a); PDI forms new disulfide bonds during protein synthesis (b) (adapted from https://www3.nd.edu/~aseriann/CHAP9B.html/sld012.htm).

PDI is a plausible candidate for reactivity with NO because first, the active site of PDI comprises two cysteinyl residues, that are susceptible for nitrosylation by circulating NO; second, the reducing environment which is believed to be maintained at the PDI active site is crucial for the formation of an S-NO bond; third, PDI is involved in thiol-disulfide exchange reactions which are similar to thiol-S-nitrosothiol exchanges reactions; and fourth, transnitrosation mechanisms promoted by PDI may explain the biological effects of S-nitrosothiols (Zai, Rudd et al. 1999; Root, Sliskovic et al. 2004).

Furthermore, different physiological stimuli have been shown to regulate PDI activity. Mikami and colleagues demonstrated that a fasting period promoted a decrease in the expression and activity of this enzyme, but these recovered on refeeding (Mikami, Genma et al. 1998). Given that the transition from fasting to feeding modifies both PDI content and activity, PDI activation might contribute to changes in insulin levels and action after a meal.

The involvement of insulin *per se* in the regulation of PDI activity is unclear. On one hand, Varandani suggested that the low levels of insulin in the fasted state contribute to a decrease in PDI activity (Varandani 1974). On the other hand, Nieto and colleagues have shown that high insulin levels inhibited PDI gene expression (Nieto, Mira et al. 1990). Kulkarni and co-workers showed that PDI associates with the insulin 5'UTR, both *in vitro* and *in vivo* (Kulkarni, Muralidharan et al. 2011). They also found that in the presence of anti-PDI antibody, glucose-stimulated translation of insulin was abrogated and PDI overexpression in beta cells resulted in activation of insulin secretion (Kulkarni, Muralidharan et al. 2011). These confounding conclusions make it difficult to state with confidence the mechanisms and factors involved in the regulation of PDI activity.

PDI activity alteration has also been implicated in different pathologies. Zhou showed that downregulation of PDI by sepsis is associated with an increase in proinflamatory cytokine production. On this basis, it is suggested that PDI may have anti-inflammatory actions (Zhou, Jacob et al. 2008).

1.2.4.3. Insulin Clearance Evaluation

Hyperinsulinemia, a key feature of insulin resistance and early stages of T2D, can result from hypersecretion of insulin, reduction in insulin clearance or a combination of both. As already mentioned, insulin is secreted concomitantly with C-peptide in a 1:1 molar ratio.

Moreover, is known that insulin is secreted directly into the portal vein and during first pass through the liver, 50 to 70% is removed. Meanwhile, C-peptide is not extracted to a significant degree by the liver hence its peripheral circulating levels reflect the quantity of insulin that was secreted by the beta cells. Thus, hepatic insulin clearance can be related to the levels of circulating C-peptide (reflecting the initial amount of insulin secreted) and insulin (reflecting the portion that survived hepatic clearance) (Polonsky, Given et al. 1988). In humans and mice, mathematical modeling allows the calculation of insulin secretion after a challenge of glucose based on the longitudinal profile of C-peptide levels (Polonsky, Given et al. 1988). However, c-peptide kinetics has not been measured in rats and consequently the calculation of insulin secretion in these animals based on this method is not possible. In this setting, insulin clearance is evaluated from the ratio of circulating c-peptide to insulin following a glucose challenge.

1.3. Nitric Oxide (NO)

NO is a heterodiatomic free radical with a large range of physiological functions (Figure 13). Its functions include neurotransmission, regulation of platelet function, blood pressure control, and the killing of pathogens and tumor cells as part of the immune system. NO induces vasorelaxation via soluble guanylyl cyclase activation (Zai, Rudd et al. 1999).

NO is transported in the circulation in the form of S-nitrosothiols involving albumin or low-molecular weight thiols. NO diffuses freely from its source of synthesis across effector cell membranes nonspecifically due to its small size and rapid diffusibility (Zai, Rudd et al. 1999). Also, compared with other biological free radicals it has less reactivity due to its neutral charge.

NO is postulated to play an important role in the regulation of insulin secretion since the NOS-NO enzymatic pathway is present in the islets of Langerhans and in the beta cells (Eckersten and Henningsson 2012). However, the precise role of NO in modulating insulin secretion is controversial. On the one hand, there are a large number of studies demonstrating an inhibition of insulin secretion when NO production is decreased. On the other hand, there are numerous studies demonstrating a stimulatory effect of NO on insulin secretion, while others suggest that changes in NO levels do not alter insulin secretion rates.

There are also *in vitro* evidence suggesting that NO impairs insulin clearance by inhibiting IDE (Cordes, Bennett et al. 2009). However, even with all the evidence suggesting an important involvement of NO on insulin metabolism, the exact mechanisms by which this occur is unknown and *in vivo* evidences of this are slow to appear.

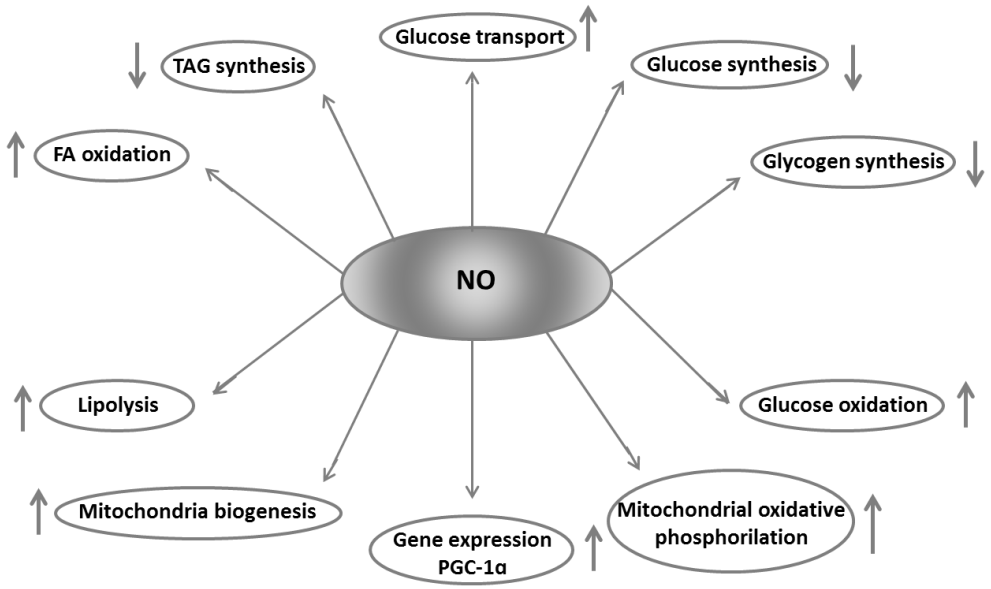

Figure 13: Effects of physiologic levels of NO on energy substrate metabolism: NO is involved on both glucose and lipid metabolism regulation as well as in mitochondria function.

1.3.1. NO Synthesis Pathway

The biosynthesis of NO requires L-arginine and oxygen as substrates. It requires also the following cofactors: tetrahydrobiopterin (BH4), nicotinamide adenine dinucleotide phosphate (NADPH), Ca^{2+}, calmodulin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). NO is produced by NOS which can be found in plasma membrane, cytoplasm, nuclear rough endoplasmic reticulum and mitochondria. This enzyme transforms L-arginine into L-citruline and NO (figure 14).

There are 3 isoforms of NOS: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). nNOS and eNOS are constitutive isoforms (cNOS) because they are expressed constitutively at low levels in a variety of cell types and tissues, whereas iNOS is normally not expressed at a significant level in basal state being only induced by a certain immunological stimuli. nNOS was first identified in neurons and is described to be

involved in synaptic signaling and memory at the same time that is involved in control of insulin secretion. eNOS mediates smooth muscle vasorelaxation and it is suggested to promote increase of substrate availability in insulin target tissues (Zhao, Xu et al. 2009). iNOS is highly expressed in many cell types including macrophages and hepatocytes and produces a larger amount of NO than cNOS and for longer periods. Contrary to cNOS, iNOS is fully active in the absence of exogenous Ca^{2+} or calmodulin (Jobgen, Fried et al. 2006).

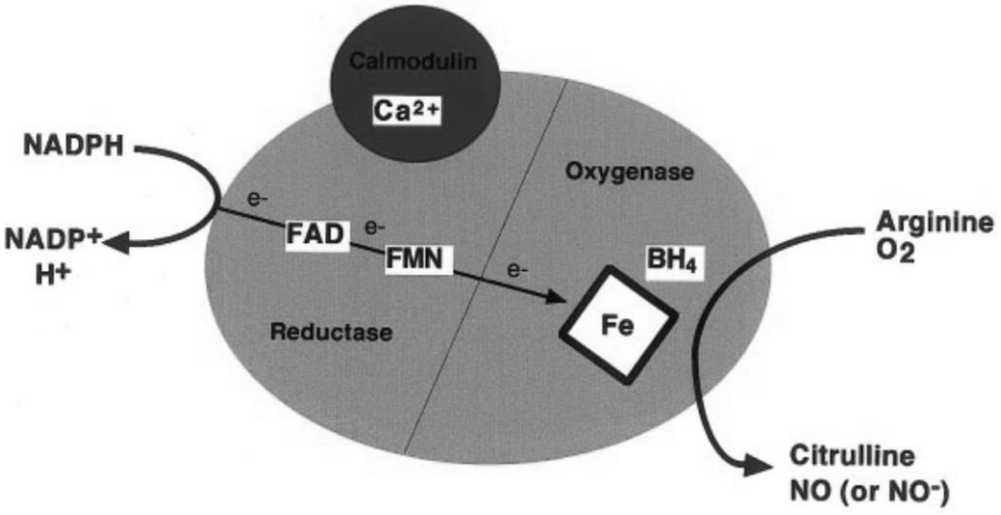

Figure 14: Electrons (e–) are donated by NADPH to the reductase domain of the enzyme and proceed via FAD and FMN redox carriers to the oxygenase domain. There they interact with the haem iron and BH_4 at the active site to catalyze the reaction of oxygen with L-arginine, generating citruline and NO as products. In some circumstances NO^- may be a product instead of NO. Electron flow through the reductase domain requires the presence of bound Ca^{2+}/CaM (Alderton, Cooper et al. 2001).

For the activation of NOS isoforms each one has to associate into active dimers to achieve the correct structure with all the active domains in association: flavin, heme and pterin domains. After the assembly of the enzymes they can be regulated by different transcriptional mechanisms involving mRNA splicing, protein-protein interactions and covalent modifications (Alderton, Cooper et al. 2001).

1.3.2. NO in Liver and Brain

NO production has an important role in many insulin-sensitive tissues that are involved in nutrient storage and energy metabolism, namely skeletal muscle, adipose tissue, heart and

liver. Since liver is directly involved in the regulation of insulin clearance and there are also clues demonstrating the involvement of hepatic NO in this process, this section focuses on NO metabolism in liver. Moreover, since it is already known that hepatic NO production is sensitive to central nervous signals, via the parasympathetic system, the possible role of the brain in modulating the hepatic actions of NO will also be discussed.

In liver, cNOS is uniformly distributed in hepatocytes and in the endothelium of hepatic arteries, terminal hepatic venules and sinusoids, whereas iNOS is expressed primarily in the cytoplasm of periportal hepatocytes (Jobgen, Fried et al. 2006). In hepatocytes, the NO pathway has a beneficial effect on mitochondrial biogenesis, regulates gluconeogenesis by inhibiting it in a dose-dependent manner, inhibits glycogen synthesis by decreasing the activity of glycogen synthase, and reduces glycolysis. Besides hepatic glucose metabolism, NO is also involved in the regulation of lipid metabolism at the level of lipogenesis and lipolysis. Whether NO stimulates or inhibits lipid oxidation or synthesis appears to be dependent on the isoforms on NOS stimulated, tissue site and intracellular redox state (Jobgen, Fried et al. 2006).

In brain NOS has been localized within many areas, consistent with acting as a neurotransmitter in the central nervous system (Zheng, Mayhan et al. 2006). NO is the common link between insulin, leptin and glucose signaling in glucose-sensing neurons (Canabal, Song et al. 2007). In the work of Shankar, infusion of a NOS inhibitor [N^G-Monomethyl-L-Arginine (L-NMMA)] had different effects depending if was administrated i.c.v. or systemically. L-NMMA was only able to promote glucose intolerance when given i.c.v., demonstrating that central nitrergic neuronal pathways may be involved in glucose

homeostasis. Since only plasma insulin levels were assessed, they were not able to determine if the decrease in central NO availability impaired the insulin secretory response or whether it increased the insulin clearance rate or both (Shankar, Zhu et al. 1998). Hence, there is 1) a need to resolve central and peripheral NO effects on overall plasma insulin levels and 2) determine if these are mediated through changes in insulin secretion, insulin clearance or both.

Hypothalamic NO that is generated in central nervous system can then diffuse to other tissues and act as an endocrine factor or can activate the autonomic nervous system, which will modulate the glucose utilization and insulin metabolism by peripheral tissues.

It has been shown that vagus nerve activity is regulated by the NO-dependent signal (Cabou, 2008). NO will activate the parasympathetic nervous system which, by enervating

peripheral tissues, will lead to release of acetylcholine (Ach) in those tissues thereby potentiating NO production. In muscle tissue this NO promotes an increase in glucose utilization, in pancreas it stimulates insulin and inhibits glucagon secretion and in liver it increases insulin sensitivity and alters insulin degradation (Cabou, Cani et al. 2007). Moreover, insulin infusion in the hypothalamus also caused a progressive increase in blood flow via sympathetic nerve signaling. Microinjection of a NO donor in the PVN produced a significant decrease in sympathetic nerve activity (Zhang, Mayhan et al. 1997). So, both sympathetic and parasympathetic fibers are regulated by NO derived from hypothalamic signals.

In animal models of insulin resistance and diabetes, alterations in ANS activity due to an impaired hypothalamic NO production was suggested to contribute to impaired glucose tolerance and insulin actions. In the work of Shankar and colleagues, the central inhibition of NOS promoted hyperglycemia, insulin resistance and low insulin levels. The low insulin levels were attributed to a dysfunction in insulin secretion, and the possibility that altered insulin clearance could also play a role was not considered. Thus, a central hypothesis of this work is that the alterations in central NO production can modify hepatic insulin clearance as well as pancreatic insulin secretion (Shankar, Zhu et al. 1998).

The interaction between NO and IDE activity has also been described in brain tissue extracts. Brain hemispheres of aged mice showed increased IDE activity in NOS2 $^{(-/-)}$ animals, where endogenous NO production by NOS2 is absent, compared to wild-type controls (Kummer, Hulsmann et al. 2012). So, the involvement of NO in insulin clearance could be an important component in the regulation of insulin bioavailability via liver and/or brain NO production.

1.3.3. NO and Insulin Resistance

It is well known that NO influences insulin signaling and skeletal muscle glucose uptake in opposite ways for physiological and pathophysiological conditions such as T2D and obesity Under physiological conditions, NO increases glucose tolerance by increasing insulin-dependent glucose uptake in skeletal muscle (Balon, Jasman et al. 1999) while also stimulating the expression of fatty acid oxidation enzymes (Jobgen, Fried et al. 2006). The work of Natali and coworkers, showed that in normal subjects the inhibition of NO production caused a significant deterioration of glucose tolerance, supporting the hypothesis that NO is essential for the correct response to a challenge of glucose. In this work they also showed that the

impairment of glucose tolerance was associated with increased insulin clearance and inhibition of insulin secretion (Natali, Ribeiro et al. 2013).

However, in pathophysiological states such as insulin resistance, there are contradictory results regarding the role of NO in insulin signaling and glucose and lipid metabolism. There are reports demonstrating that NO levels are increased, decreased or normal in humans or animals with insulin resistance. These contradictory data may result from differences in the etiology of insulin resistance (i.e. hepatic versus peripheral) and the presence of associated pathologies in the subjects, which can also by themselves influence NO synthesis. These findings also may suggest two different relationships between NO levels and insulin sensitivity in healthy and pathophysiological states: insulin resistance can lead to a compensatory increase in the NO concentration while, in healthy subjects, higher insulin sensitivity is defined in part by higher rates of NO synthesis (Zavaroni, Platti et al. 2000).

For example, for healthy subjects given a glucose tolerance test, an increase in NO levels induce vasodilation that enhances the effects of insulin in skeletal muscle and appears to compensate individuals that are in the lower end of the normal insulin secretion range (Zavaroni, Platti et al. 2000). On the other hand, an increase in NO levels secondary to increased inflammatory state was implicated in the impairment of skeletal muscle glucose uptake and insulin signaling (Cha, Song et al. 2011). Furthermore, the work of Pilon and colleagues demonstrated that iNOS-derived tyrosine nitrosation of IRS1 may be responsible for the establishment of insulin resistance in skeletal muscle and the consequent alterations in metabolism (Pilon, Charbonneau et al. 2012). In addition, studies showed that iNOS-derived NO was also responsible for a decrease in IRS2 expression (Tanioka, Tamura et al. 2011). Taken together, the studies of Pilon et al. and Tanioka et al. support the idea that exacerbated production of NO due to inflammation may be responsible for both alterations in IRS1 and IRS2-mediated signaling that in turn result in defective glucose and lipid metabolism.

The pro-inflammatory effect of FFAs has also been attributed to iNOS activation and increased NO levels leading to a decrease in insulin-mediated glucose transport (Bedard, Marcotte et al. 1997).The link between FFA and NO-mediated impairment of insulin signaling implicates NO as a mediator of insulin resistance in obesity and dyslipidemia.

1.4. Insulin Resistance Models and Insulin Clearance

1.4.1. Metabolic Alterations in Insulin Resistance

Insulin resistance is the common feature of a variety of diseases such as obesity, hypertension and T2D and is described as a condition in which normal plasma insulin levels does not adequately produce normal insulin response (Figure 15). It is characterized by hyperinsulinemia, hypertriglyceridemia and is widely considered to be an initial feature of T2D (Thresher, Podolin et al. 2000).

Both genetic and environmental factors seem to contribute to the development of insulin resistance. The pathogenesis of insulin resistance is not completely known but it is suggested that high plasma levels of FFAs and glucose, chronic inflammation and mitochondrial dysfunction may contribute to it (Gallagher, Leroith et al. 2010). In addition, there is increased secretion of adipocytokines from adipose tissue that also contribute to the disturbance of lipid metabolism (Sumiyoshi, Sakanaka et al. 2006).

The metabolic consequences of these dysfunctions are increased gluconeogenesis in the fasted state, reduced insulin suppression of hepatic glucose production in the post-prandial state, reduced insulin stimulation of glucose uptake, increased production of lipids in liver (DNL), increased release of FFAs from adipose tissue and decreased rates of FFA clearance via oxidation and esterification beta cell dysfunction, impaired eNOS activity due to impaired insulin signaling via PI3K pathway, etc. (Commerford, Bizeau et al. 2001; Gallagher, Leroith et al. 2010).Together, these metabolic alterations promote systemic complications that include hypertension, dyslipidemia and hyperglycemia (Gallagher, Leroith et al. 2010).

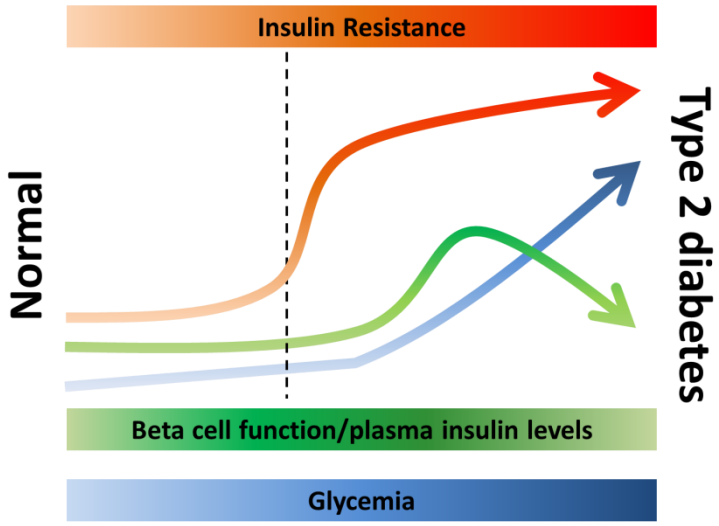

Figure 15: Progression of T2D: insulin resistance is described to appear when normal plasma insulin levels are not sufficient to control plasma glucose levels; at this time point beta cell secretes more insulin leading to hyperinsulinemia in order to compensate for the hyperglycemia; over time, beta cell develops inability to produce sufficient insulin to correct glycemia and beta cell dysfunction is developed; therefore, maintained hyperglycemia is settled and insulin resistance evolves to over T2D.

In its initial stages, insulin resistance is characterized by hyperinsulinemia. Several studies have implicated reduced insulin clearance as a contributing factor to the development of hyperinsulinemia. For example, Erdmann and colleagues demonstrated that in early stages of hyperinsulinemia linked to obesity there is hypersecretion of insulin as well as impairment of insulin clearance, with the latter factor being more strongly associated with hyperinsulinemia (Erdmann, Kallabis et al. 2008).

ANS activity dysfunction has also been found to associate with severe insulin resistance, demonstrating the important role of central signals in the regulation of peripheral metabolism (Ribeiro, Lautt et al. 2005). Some studies showed that insulin resistance per se is intimately associated with disturbances in the sympathetic nerve activity that may also be linked with the onset of hypertension – a common feature of the insulin resistant state (Anderson, Hoffman et al. 1991). Moreover, hypothalamic inflammation has been associated with diet-induced insulin resistance, promoting disturbances in leptin and insulin signaling, and consequent alterations on food intake, energy expenditure and glucose homeostasis (Milanski, Arruda et al. 2012).

1.4.2. High-Sucrose Diet (HSD) Animal Model

Translational research involves the transformation of knowledge from basic science to impacting public health. It identifies novel molecular mechanisms underlying a disease and this knowledge leads to discovery of diagnostic biomarkers, development of new therapies, and lifestyle strategies for disease prevention. With a multifactorial disease such as T2D, it is crucial to have models to study and understand the molecular mechanisms. Since T2D development has both genetic and environmental factors, with animal models we can precisely manipulate genes or alter the type and/or quantity of food ingested, exercise, etc. So, a particular disease mechanism can be studied under highly controlled conditions where other confounding factors are minimized. At same time animal models are a key tool to study T2D in a more integrative manner since tissue signaling and metabolic interconnections can be observed (McMurray and Cox 2011). Mice and rat models have been widely used for mammalian T2D study. They have the great advantage of a short reproductive cycle, are easy to maintain and cost effective, and having a short life span that allows the interaction between aging and T2D development to be studied over a practical interval. Mice have a specific advantage over rats in that they can be more easily manipulated genetically, whereas rats have the advantage of providing larger tissue or fluid samples for analysis.

Although there is no perfect animal model to mimic the T2D disturbances found in humans, several rodent models have features that closely resemble some aspects of human T2D. Some models are based on healthy animals treated with streptozotocin (STZ) to mimic pancreatic beta cell failure, however the STZ dose has to be controlled and low to avoid the complete destruction of pancreas, which would be a feature of type 1 diabetes (T1D). Other models use genetically altered mice that mimic T2D polymorphisms. Due to the predominance of life style factors in the pathogenesis of T2D animal models with healthy background genotype and phenotype that are subject to different diets and/or exercise patterns are also important tools in translational T2D research (McMurray and Cox 2011).

The urbanization of Western societies has been associated with the increase in consumption of high caloric foods containing high quantities of saturated fats and refined sugars. This, in combination with a more sedentary life style, is considered to be the main factor that explains the growing incidence of T2D in these populations (Shafrir, Ziv et al. 2006).

To mimic the disturbances of T2D related to food intake the high sucrose diet (HSD) model has been widely used (Wright, Hansen et al. 1983; Chicco, D'Alessandro et al. 2003; Sumiyoshi,

Sakanaka et al. 2006). HSD leads to development of hyperinsulinemia, hypertriglyceridemia, increases in pancreatic islet size and insulin content, ketosis as well as defective insulin-stimulated glucose utilization (Wright, Hansen et al. 1983; Shafrir, Ziv et al. 2006). Since basal levels of plasma glucose do not typically increase in this model it is useful for the study of insulin resistance without confounding effects arising from the loss of glycemic control. Moreover, the HSD animal model also reproduces many of the metabolic abnormalities that are seen in insulin-resistant or T2D patients (Wright, Hansen et al. 1983). Additionally, it has been shown that parasympathetic nervous activation is impaired in HSD animals and, therefore, central mechanisms of metabolism regulation must also be altered in this model (Ribeiro, Lautt et al. 2005).

1.4.3. HSD and Insulin Clearance

It is well established that HSD-fed animals show hyperinsulinemia when tested for glucose tolerance. Whether this is due to insulin secretion stimulation, insulin clearance reduction, or a combination of both, is not known. The work of Wright et al. already suggested the modification of insulin clearance as a contributor to hyperinsulinemia in this model. They showed that in the insulin suppression test, in which endogenous insulin secretion was abolished, the sucrose fed animals still showed increased plasma insulin levels. They hypothesized that sucrose feeding leads to a decrease in the rate of insulin clearance from plasma (Wright, Hansen et al. 1983). In another study where the effects of sucrose vs. fructose diet supplements on insulin action were compared, showed that fasting and post-prandial plasma insulin levels in the sucrose fed group were elevated and it was suggested that impaired insulin clearance was a contributing factor (Thresher, Podolin et al. 2000). Apart from these studies, the contribution of impaired insulin clearance to hyperinsulinemia in HSD animal models has not been specifically addressed.

Nevertheless, suggestions that hyperinsulinemia is linked with impaired insulin clearance can be indirectly inferred from other HSD study data. For example, it is known that HSD promotes an increase in plasma FFAs levels. At the same time it is also known that insulin clearance is downregulated by FFAs (Hennes, Dua et al. 1997; Hamel 2009). Therefore, increased FFAs in HSD animals may be responsible for decreasing in insulin clearance and thereby contributing to hyperinsulinemia.

Moreover, the insulin resistance state promoted by HSD is characterized by increased NO levels. It was already described that *in vitro*, NO is able to inhibit the main enzyme involved in insulin degradation. Therefore, it can be postulated, that insulin resistance provoked by HSD is linked to impairment of insulin clearance.

1.5. Anti-diabetic Drug and Insulin clearance

1.5.1. Thiazolidinediones (TZDs) and Insulin Clearance

Thiazolidinediones (TZDs) are widely used insulin-sensitizing pharmacological agents that have adipogenic effects on adipocytes and have marked antidiabetic effects in animal models of T2D. It is generally accepted that the gamma isoform of the peroxisome proliferator-activated receptor (PPARγ) is the molecular target for the adipogenic effects of TZDs. PPARγ is a nuclear transcription factor mainly expressed in adipose tissue and at much lower concentrations in skeletal muscle, liver, pancreas, heart and spleen (Spiegelman 1998). It binds to DNA sequence elements present in the regulatory regions of genes encoding enzymes that modulate lipid metabolism (Lehmann, Moore et al. 1995).

TZDs reverse the abnormalities in glucose and lipid metabolism by ameliorating insulin resistance in adipose tissues, liver and muscle. TZDs increase plasma adiponectin levels, activate AMPK, stimulate FA oxidation, and inhibit hepatic FA synthesis (Belfort, Harrison et al. 2006). They also have anti-inflammatory effects since they are able to rescue from the inflammatory state found in steatohepatitis patients (Browning and Horton 2004). There are different types of TZDs (e.g. pioglitazone, troglitazone, rosiglitazone) and the exact mechanisms of action that differentiate the effects of each drug are not well known.

The hepatic actions of TZD may be explained by alterations in adipose tissue secretion of endocrine signaling molecules, namely adiponectin (Belfort, Harrison et al. 2006). It was already shown that with TZD treatment there is a strong correlation between the increase in plasma adiponectin levels and the reduction in endogenous glucose production (Bajaj, Suraamornkul et al. 2004). Moreover, in perfused rat livers, treatment with a TZD drug inhibits expression of key hepatic enzymes of the gluconeogenic pathway, such as phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-Pase) and pyruvate carboxylase (PC), demonstrating also a direct hepatic action. Moreover, it also reduces free

fatty acids release from adipose tissue and decrease visceral fat content, effects that can contribute to a reduction of hepatic gluconeogenesis (Gastaldelli, Miyazaki et al. 2006).

TZD-based therapy is often associated with an increase in fat mass and weight gain. The increase in fat mass can be largely explained by improvement of insulin sensitivity in adipose tissue, which will result in decreased release of FFAs from fat depots, increased FFAs uptake and accumulation and consequent increase in body fat mass (Bays, Mandarino et al. 2004). The increase in fat mass is commonly associated with disturbances present in obesity and T2D. While promoting the increase in fat mass, TZDs also promote the redistribution of that fat, with reduced visceral and increased subcutaneous fat content. This redistribution is an important mechanism by which TZDs improve glucose metabolism (Gastaldelli, Miyazaki et al. 2006).

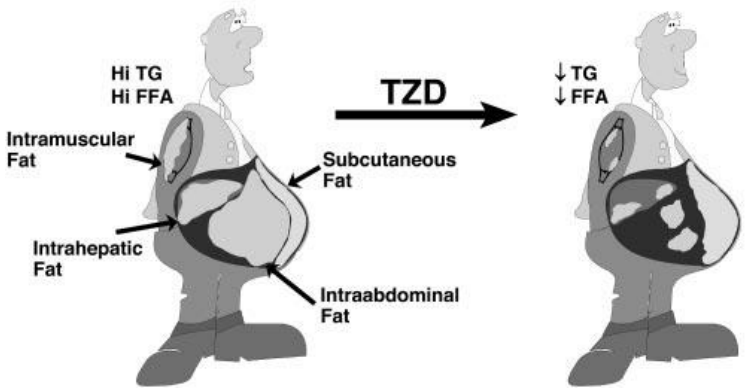

Figure 16: Effect of TZDs treatment on fat distribution [adapted from (Bays, Mandarino et al. 2004)].

It has been described that the improvement in insulin sensitivity and glucose control promoted by TZDs does not have direct effects on beta cell secretion, despite promoting the normalization of plasma insulin levels (Osei, Gaillard et al. 2004). However, this relationship is controversial since some TZDs have been shown to directly normalize insulin secretion and consequently decrease plasma insulin levels. Thus, we may conclude that some TZDs may directly alter beta cell activity while others do not (Kim, Abbasi et al. 2005). Studies in both humans and rats, found that besides alterations in insulin secretion, the normalization of plasma insulin levels were also ascribed to an increase in insulin clearance (Kim, Abbasi et al. 2005; Osei, Gaillard et al. 2007; Lamontagne, Pepin et al. 2009). The lowering of plasma FFAs may explain the improvement in insulin clearance since it is known that excessive FFAs inhibit insulin clearance. Therefore, it is important to evaluate the involvement of insulin clearance on

the regulation of plasma insulin levels after TZDs treatment, since this mechanism can explain alterations in plasma insulin levels that cannot be attributed to changes in beta cell secretion.

CHAPTER 2- HYPOTHESES AND OBJECTIVES

2. Hypotheses and Objectives

2.1. Hypotheses

The first hypothesis for this work is that the process of insulin clearance is important in the post-prandial state, being responsible for the correct plasma insulin bioavailability after meal ingestion.

Second, we tested the hypothesis that *in vivo*, hepatic NO decreases insulin clearance by inhibiting IDE activity. This alteration will be responsible for an increase in insulin bioavailability.

The third hypothesis for this work is that hypothalamic nitric oxide regulates hepatic insulin clearance via parasympathetic nervous signal as well as hepatic lipid metabolism.

Finally, the fourth hypothesis is that troglitazone, a TZD drug, protects against hepatic fatty liver in HS-fed rodents by decreasing hepatic DNL and improving hepatic insulin clearance by increase in hepatic IDE activity.

2.2. Objectives

In order to test the previous detailed hypothesis we established some major objectives. The main objective of this work is to understand and integrate all the knowledge about insulin bioavailability and its importance to glucose homeostasis and lipid metabolism.

It is also our objective to understand the mechanisms involved in insulin clearance regulation, one important and neglected process. There is growing evidence that insulin clearance is responsible for modulation of insulin bioavailability both in physiology and pathophysiology.

In physiologic conditions, levels of circulating insulin in the post-prandial state are extreme important, in order to organisms manage nutrients intake. However, little is known about the specific role of insulin clearance in this state. Therefore, understanding this function is other objective of this work.

Since living organisms are complex and formed by interconnections between all tissues and organs, we have also the purpose to understand the connections between brain, liver and gut signals to the regulation of insulin clearance and, consequently, insulin bioavailability.

Finally, it is our objective to look for the mechanism involved in the action of TZDs drugs, widely used to treat insulin resistance or T2D, and understand the importance of insulin clearance in the drugs action.

CHAPTER 3 – METHODOLOGICAL CONSIDERATIONS

3. Methodological Considerations

3.1. Peripheral pharmacologic modulation of NOS activity and NO levels

In this work, it was performed all body NOS enzymatic modulation by the use of pharmacologic compounds to manipulate NO levels.

Namely, NOS activity inhibition was achieved by injecting animals with a non-specific inhibitor, NG-nitro-L-arginine methyl ester (L-NAME). This inhibitor is described to act over the three isoforms of NOS, nNOS, iNOS and eNOS, although it seems to have higher activity over the cNOS, meaning nNOS and eNOS. However, the selectivity and specificity of this and other NOS inhibitors has been studied at isolated enzymes levels, which do not exactly reflect potency at the cellular level or in isolated organ systems (Boer, Ulrich et al. 2000).

Therefore, L-NAME an analog of the NOS substrate, L-arginine, has been used in animal and human studies as non-specific NOS inhibitors and it has been described to inhibit all the NOS isoforms in a cell or in vivo context (Sprangers, Sauerwein et al. 1998; de Castro Barbosa, Jiang et al. 2013; Natali, Ribeiro et al. 2013).

Moreover, different routes of administration and doses have been used to L-NAME infusion. For instance, in the work of Hajrasouliha both acute and chronic L-NAME treatment was performed in rats. This was one of the studies by which we based our doses and routes of administration (Hajrasouliha, Tavakoli et al. 2004). For acute treatment we used intravenous (i.v.) infusion and the dose of 35ug/kg/min and for the chronic treatment we performed subcutaneous (s.c.) injection every day in a dose of 10mg/kg/day. In these all body treatment we saw as expected an increase in the heart rate and in mean arterial pressure. However, it was already described, with a similar NOS inhibitor (L-NMMA) that these alterations in the blood flow do not alter blood delivery to different organs (Meneilly, Elliott et al. 2001).

In addition, NO levels were increased with the infusion of a NO donor, 3-morpholinosydnonimine (SIN-1) in the fast state. The mechanism of action of this drug as a NO donor is well described in the literature and has many implications like cardiovascular effects, stimulation of muscarinic acetylcholine receptors, repair functions in neurogenesis, etc. (Rojas Wahl 2004). Previously, our lab showed that the i.v. infusion of this drug could be a mechanism to evaluate the effect of NO levels increase in different conditions (Fernandes, Guarino et al. 2012). The work of An and Cherrington supported our choice in order to achieve the objective

to increase NO levels in the fast state and evaluate in which extent this approach mimics the post-prandial state concerning insulin metabolism (An, DiCostanzo et al. 2008). They already have described that the decomposition of SIN-1 yields NO and superoxide. Both can then nitrosilate glutathione to generate S-nitrosoglutathione (GSNO), that, according to Lautt's studies, can act as an intermediate in the increase of insulin sensitivity occurring in the post-prandial state (Lautt 2005). Finally, another important fact supporting the choice of SIN-1 use is that this drug has little effect on total hepatic blood flow, avoiding parallel and confounding factors.

3.2. Hypothalamic NO production manipulation

In order to test one of the hypotheses, the central NO-signal involvement on the regulation of insulin clearance were evaluated in a group of animals. In these animals, NOS inhibition was performed by the infusion of L-NAME directly in the brain. To manipulate NO levels in all brain or in specific regions of the hypothalamus a stereotaxic apparatus had to be used, which allow the implantation on specific cannulas. The implantation of these cannulas is followed by a week of animal recover, after which L-NAME was injected both, acute in the final day of the experiment, and chronically, during 2 weeks. To evaluate the possible role of NO produced in all brain over insulin clearance and IDE activity, i.c.v. cannulas were implanted following the specific coordinates for the release of the drug in the lateral ventricle. In this way the L-NAME will then diffuse spreading all over the brain.

Moreover, it is known that hypothalamus has an important role concerning glucose and lipid metabolism, food intake, etc.. Therefore, we focused our attention for specific regions of the hypothalamus, with the implantation of double cannulas with specific measures to reach those regions. The VMH nucleus was tested only for the acute NOS inhibition due to the lack of insulin clearance alterations in those animals. This region was chosen since it is rich in leptin receptors and therefore is important for mediating the glucoregulatory actions of leptin (Fujikawa, Berglund et al. 2013). Moreover, it is enervated by sympathetic and parasympathetic nervous that reaches peripheral tissues like liver and muscle. Regarding PVN region, it acts as an integrative station of signals from other regions of the hypothalamus and transmits for autonomic nerves. It is also insulin, leptin and glucose sensitive. Moreover, is rich in NO producing neurons. Therefore, the PVN was a target for the evaluation of central regulation of insulin clearance both acute and chronically. After one week of recover of

animals implanted with VMH or PVN directed cannulas, they were acutely infused with L-NAME (VMH and PVN) or chronically for 2 weeks, every-day (PVN).

3.3. Metabolic evaluation by NMR - ^2H_2O method

NMR became a useful technique to study conformational and configurational questions concerning structures of biological molecules (e.g., drugs, proteins and DNA), and for obtaining anatomical, functional and metabolic information from biological systems.

One of the biggest advantages of this technique, due to its non-invasive nature, is that it can be used both *in vivo* and *in vitro*. By using stable isotopes it also has, from a safety perspective, an enormous advantage over radioactive tracer methodologies.

One important isotope in metabolism studies is deuterium (^2H). Since the natural abundance of ^2H is really low (~0.015%) it can frequently be neglected. Following the administration of a deuterated tracer different positional ^2H enrichment in a molecule of interest can be associated with different metabolic pathway fluxes. ^2H-NMR spectroscopy allows the quantification of the different metabolic pathways by evaluation of the ^2H positional enrichments of given intermediary metabolites.

The use of ^2H_2O does not suffer from some of the disadvantages associated with other labeled precursors. The rapid equilibrium with body water removes frequently encountered problems of tracer zonation. ^2H_2O is optimal for evaluating metabolic fluxes since its distribution is independent of any effects of nutritional or physiological effects. The ingestion of a small loading dose, maintained indefinitely by enrichment of the drinking water, for example, allow the use of this tracer in *in vivo* studies with different animals species.

^2H_2O has been widely used to measure, for example, hepatic glycogen synthesis and inform the role of hepatic glycogenesis in glucose homeostasis in physiology and pathophysiology conditions like diabetes and obesity (Delgado, Silva et al. 2009; Soares, Viega et al. 2009; Barosa, Silva et al. 2012; Delgado, Martins et al. 2013) or to evaluate rates of lipogenesis and lipid metabolism regulation (Silva, Martins et al. 2011).

As already mentioned above, hepatic glycogen can be synthesized from glucose via the so called “direct pathway” or from gluconeogenic precursors by the indirect pathway (Newgard, Hirsch et al. 1983).

The estimates of direct *vs* indirect pathway contributions to hepatic glycogenesis rely on some key assumptions concerning exchange of metabolite and water hydrogens. These assumptions have been extensively examined by our group in previous works (Delgado, Silva et al. 2009; Soares, Carvalho et al. 2012). The overestimation of indirect pathway contribution and the correspondent under evaluation of direct pathway results from G6P position 5 enrichment by processes other than indirect pathway flux, such as transaldolase exchange. Therefore, the purpose of the final study herein is to correctly evaluate the impact of transaldolase exchange over hepatic glycogenesis in different species.

The rat is widely used as a small animal model of diabetes and liver carbohydrate metabolism hence there is widespread interest in studying hepatic glycogen fluxes in this animal. Nowadays interest by commercial and academic entities has also appeared in the context of the analysis of fish metabolism in order to increase its production and decrease the costs of that process. In the case of fish metabolism analysis the $^{2}H_2O$ also appears as very reliable technique, due to the easy use of it in fish tank water (Viegas, Mendes et al. 2011).

CHAPTER 4 – RESULTS

SUB-CHAPTER 4.1. – REGULATION OF INSULIN BIOAVAILABILITY BY HEPATIC CLEARANCE: THE ROLE OF NITRIC OXIDE

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[*]**These senior authors contributed equally to this work**

Submitted to: Diabetes

Abstract

Objective: The mechanisms that regulate insulin clearance, which will regulate insulin bioavailability in combination with insulin secretion, have been overlooked. We tested the hypothesis that, *in vivo*, NO decreases hepatic IDE activity and consequently insulin clearance, leading to increase in insulin bioavailability in the post-prandial state.

Research Design and Methods: Insulin clearance, NO levels, hepatic nitric oxide synthase (NOS), IDE and protein disulfide isomerase (PDI) were analyzed in male Wistar rats, after L-nitroarginine methyl ester (L-NAME) acute or chronic administration. They were also assessed for hepatic insulin signaling and glucose transporters expression.

Results: In physiologic conditions, with fasting and after a bolus of glucose, insulin clearance decreased with the increase in NO levels. Moreover, hepatic IDE activity decreased in the post-prandial state, and hepatic PDI activity increased. Acute and chronic depletion of NO levels promoted similar increase in hepatic IDE activity and insulin clearance. Therefore we showed an inverse relationship between NO levels and insulin clearance. Even though, acute NO depletion led to a decrease in insulin bioavailability with no alterations in insulin secretion, chronic NO depletion promoted a stimulation on insulin secretion resulting in a normalization of peripheral insulin levels. The acute and chronic NOS inhibition promoted both an increase in PDI activity. Acute animals showed an increase in hepatic insulin signaling and glucose transporters expression, on the analysis of the chronic animals only GLUT4 hepatic glucose transporter expression was decreased.

Conclusion: We showed that NO is a physiological regulator of insulin clearance, by inhibition of hepatic IDE activity, and resulting in insulin bioavailability increase in the post-prandial state.

Keywords: Insulin bioavailability, Insulin clearance, Nitric oxide, Insulin degrading enzyme, Protein disulfide isomerase.

Introduction

The transition between fasting and feeding presents a challenge to the body´s glucose homeostatic mechanisms. For effective maintenance of euglycemia under these conditions, the concentrations of circulating insulin need to be finely tuned so that the appearance of absorbed glucose in the circulation is matched by insulin-mediated disposal into peripheral tissues. The concentration of circulating insulin is a function of both β-cell secretion and first pass clearance by the liver of the newly secreted insulin.

Following a meal, the β-cells sense the increase in portal vein glucose levels and in response these cells secrete insulin (DeFronzo 2004). During its first pass through the liver, 50-70% of this newly-secreted insulin is cleared from the blood (Duckworth, Bennett et al. 1998). The remaining circulating insulin is also subsequently cleared by the kidneys and muscle (Ferrannini, Wahren et al. 1983; Duckworth, Bennett et al. 1998). Thus, there is a 2-3 fold gradient of insulin levels between the portal vein and that of the general circulation which is dynamically controlled by hepatic insulin clearance (Ferrannini, Wahren et al. 1983; Shapiro, Tillil et al. 1987). This process is predominantly mediated by the actions of two enzymes: insulin degrading enzyme (IDE) and protein disulfide isomerase (PDI). These enzymes have different actions regarding insulin removal and degradation. IDE is responsible for the detachment of insulin from its receptor in combination with alterations of pH in the degradation vesicles, and the proteolytic cleavage of both insulin chains (Duckworth, Bennett et al. 1998). PDI converts insulin into its two component chains by cleavage of the disulfide bonds, leading to the release of chain A and chain B to the circulation (Lambert and Freedman 1983; Tang and Tsou 1990).

Impairment of hepatic insulin clearance has been observed in first-degree relatives of type 2 diabetics (Rudovich, Rochlitz et al. 2004) and in obese insulin resistant subjects (Valera Mora, Scarfone et al. 2003). Hepatic insulin clearance has been demonstrated to have a tighter association with insulin resistance and hyperinsulinemia than other well known factors such as beta cell function and insulin secretion, lipid metabolism dysfunctions, lifestyle factors, among others (Ferrannini and Balkau 2002; Erdmann, Kallabis et al. 2008; Lee, Haffner et al. 2013). Previous studies also demonstrated a decrease in insulin clearance associated with aging (Basu, Breda et al. 2003) and with increased liver fat accumulation (Kotronen, Vehkavaara et al. 2007). Despite the apparently significant role of hepatic insulin clearance in adjusting peripheral insulin levels during feeding and the strong possibility that its impairment

contributes to hyperinsulinemia in the setting of insulin resistance, the mechanisms that govern hepatic insulin clearance remain poorly understood.

Nitric oxide (NO) has been reported to regulate insulin secretion and peripheral insulin sensitivity. Moreover, *in vitro* studies by Cordes and colleagues (Cordes, Bennett et al. 2009) have shown that NO also alters the activity of IDE, hence identifying a possible role of NO in regulating insulin clearance. In healthy humans, where NO levels were pharmacologically manipulated, there was a correlation between plasma NO levels and the fraction of insulin undergoing hepatic clearance (Natali, Ribeiro et al. 2013). However the physiological role of NO on insulin clearance upon fast and fed state and the activities of hepatic IDE and PDI were not assayed in these studies. Therefore it was not possible to determine if NO was modulating insulin clearance by directly regulating these enzymes. The aim of the current study was to investigate the relationship between NO levels and insulin clearance and whether NO affects IDE and PDI activities in an *in vivo* animal model. The goal was to test the hypothesis that *in vivo*, hepatic NO decreases insulin clearance and increases peripheral insulin bioavailability by inhibiting IDE and PDI.

Materials and Methods

Reagents

D-glucose, insulin, GSH, L-NAME and SIN-1 were purchased from Sigma-Aldrich (Portugal). Sodium pentobarbital (Eutasil) was obtained from Ceva (Portugal). Heparin and NaCl 0.9% were purchased from BBraun (Portugal). FRET substrate Mca-GGFLRKHGQ-EDDnp was purchased from CRB Discovery (Cambridge, UK). Antibodies were obtained from Santa Cruz, Cell Signaling and Invitrogen.

Animals

Male Wistar rats at 12 weeks of age were used for the *in vivo* experiments. All animals were housed under standard conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard commercial diet and water (ad libitum) till the night before the experiment. All experimental procedures were conducted according to the EU guidelines for the use of experimental animals (86/609/EEC).

Surgical procedures

After a 24h fasting period, animals were anesthetized using sodium pentobarbital (65 mg/kg, intraperitoneally). During the experiment, anesthesia was maintained by continuous intravenous infusion of sodium pentobarbital (10mg/kg/h), using a B. Braun Perfusor automatic pump (Lisboa, Portugal). Body temperature was maintained at 37.0 ± 0.5 °C by means of a Homeothermic Blanket Control Unit (Harvard Apparatus Inc., USA). Tracheotomy was performed to allow spontaneous respiration throughout the experiment. The femoral artery and the femoral vein were cannulated and an arterial-venous external shunt was inserted (Lautt, Wang et al. 1998). This shunt allows blood collection from the arterial side and infusion of substances directly on the vein. Blood pressure was also monitored during all the experimental procedure. Animals were randomly distributed by each treatment group.

Experimental protocols for alteration of peripheral NO levels

To deplete peripheral NO, animals were administered with L-NAME, a NOS inhibitor, either acute or chronically. The acute infusion was performed intravenously in the external loop in the dose of 35ug/kg/min and a rate of 1.5ml/min, in the final day. The chronic administration was performed during 4 weeks by daily subcutaneously injection of L-NAME in the dose of 10mg/kg. Another group of animals were injected intravenously with SIN-1, a NO donor, in the fasted state at a dose of 10 mg/kg. Fifteen minutes after the SIN-1 infusion, livers were harvested, frozen in liquid nitrogen and kept at -80 ºC till further analysis. For each experiment control animals were infused with saline by the same route used for the treated animals.

Assessment of glucose tolerance

Glucose tolerance was assessed by an intra-enteric glucose tolerance test (IEGTT). Briefly, a laparotomy was performed to allow the implantation of a catheter [Abbocath 24G Optiva IV (Johnson & Johnson, Italy)] in the initial portion of the intestine for the administration of glucose at 2g/kg (glucose solution at 0.2% in water). Blood samples were obtained at different time points from the arterial side of the external shunt, before and after the glucose bolus (-60, -30, 0, 15, 30, 60, 90, 120 min), and plasma samples were kept at -20° after centrifugation. Glycemia was monitored during the IEGTT through the enzymatic glucose oxidase method, using a glucose analyzer (YSI Model 1500 Sport). Animals were euthanized with a lethal bolus of anesthetic at different time points after the glucose bolus (0, 30, 60, 120 min) and livers were harvested, immediately frozen in liquid nitrogen and kept at -80° prior to further analysis.

Assessment of insulin clearance

Plasma insulin and c-peptide levels were quantified in the fasted state and during the IEGTT by ELISA-based assay kits (Rat Insulin Ultrasensitive kit and Rat C-peptide kit, Mercodia, Sweden). Insulin clearance was calculated by the ratio between area under the curve of c-peptide, a surrogate of insulin secretion, and the area under the curve of plasma insulin levels.

Assessment of hepatic NO levels

Liver was degraded by mechanical disruption using a piston in a buffer containing 25mM Tris-HCl (pH7.4), 1mM EDTA and 1mM EGTA. Extracts were centrifuged and supernatant deproteinized with absolut ethanol. Hepatic nitric oxide levels were measured by a chemiluminescent-based technique using a Sievers 280 NO Analyzer (Sievers Instruments, USA) as previously described (Afonso, Patarrao et al. 2006).

Evaluation of hepatic NOS, IDE and PDI activities

For NOS activity quantification a buffer composed of 25mM Tris-HCl, pH 7.4, 1mM EDTA, and 1mM EGTA was used to homogenize tissue in combination with mechanical homogenization. The resulting extracts were centrifuged for 15 min at 10000 g and 4 ºC and supernatants were measured for NOS activity using the Ultra-Sensitive Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research, U.S.A.).

For quantification of IDE activity, liver tissue was also homogenized by mechanical homogenization in a buffer containing 170 mM NaCl and 2mM EDTA. To the homogenate, the FRET substrate Mca-GGFLRKHGQ-EDDnp was added and a fluorometric assay performed as previously described (Miners, Kehoe et al. 2008).

PDI activity was measured after incubation of homogenized liver tissue for 1h at 37° with KHR buffer supplemented with 0.26 mM $CaCl_2$ and 0.2 g/L collagenase. Samples were then centrifuged at 4° and 13000rpm for 5min 2 times, with recovery of the pellet containing the hepatocytes between centrifugations and resuspended in KHR buffer. The final pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 1:200 dilution of tablet protease cocktail inhibitor and 1% Triton X-100. The mixture was submitted to 5 bursts of low-level sonication and then centrifuged at 13000 rpm for 30 min at 4°. The supernatant was kept and PDI activity was measured at 630 nm by an optical density assay over a 60 minute period.

The reaction mixture was composed of distilled water, 10 mM potassium phosphate buffer (pH 7.4), 8 mM GSH and 1.16mg/mL insulin with a final volume of 186 uL. To this, 10 ul of the supernatant was added to each well in a multi well plate. In this assay the PDI promotes the cleavage of insulin present in the reaction mixture and the insulin degradation products cause an increase in optical density that is proportional to the units of PDI present.

Assessment of protein expressions

Protein extracts for Western blot analysis were obtained using a lysis buffer (1M Tris-HCl, pH 7.5, 0.2M EGTA, 0.2M EDTA, 1% Triton-X 100, 0.1M sodium orthovanadate, 2g/L sodium fluoride, 2.2 g/L sodium pyrophosphate, 0.27M sucrose) to homogenize liver tissue. Samples were centrifuged and total protein lysates were denaturated with sample buffer and heated for 5 min at 95 ºC, after which they were kept at -20°. Total protein lysates from liver were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrotransferred on a polyvinylidene difluoride membrane, and probed with the respective antibodies: NOS2 (sc-650), NOS3 (sc-654), IDE (sc-27265), PDI (sc-20132), IRS1 (sc-7200), Glut1 (sc-7903), Glut2 (sc-9117), Glut4 (sc-7938) (Santa Cruz Biotechnology, Santa Cruz, CA), Akt (92725), pAkt (5473) (Cell Signaling), pIRS1 (44816G) (Invitrogen),. Then, membranes were exposed to radiograph film (Super RX, Fuji Medical X-Ray Film; Fujifilm, Japan) and developed with developer and fixing liquids (AGFA, Mortsel, Belgium) under appropriate dark room conditions. Protein levels were normalized to β-actin (A5316, Sigma) for each sample.

Statistical analysis

Data was expressed as means ± standard error. Statistical significance was calculated using Student's t test or ANOVA (Bonferroni post hoc test). At $p < 0.05$ differences were accepted as statistically significant.

Results

Prandial hepatic nitric oxide correlates with regulation of insulin clearance

In normal chow fed animals, tested for glucose tolerance, plasma glucose levels increased immediately after the bolus of glucose with a maximum level at 30 min after that bolus (figure 17-A). The quantification of plasma insulin and c-peptide levels during the test made possible to calculate insulin clearance as described in the methods section. Insulin clearance decreased in comparison to the basal fasting state concomitantly with the increase in plasma glucose levels allowing more insulin bioavailable for peripheral action. By the final 60 min of the test, insulin clearance had rebounded such that reached the baseline fasting values (figure 17-B).

During this test, hepatic NO levels were also assessed by the quantification of hepatic nitrate and nitrite levels. Hepatic NO levels increased in the first 30 min of the test, after which there was a drop until baseline fasting values were reached by the end of the test (figure 17-C).

These alterations in hepatic NO levels were simultaneous with an initial increase in hepatic NOS activity followed by a drop to fasting levels by the end of the test (figure 17-D).

When assessing enzymatic activities of the main enzymes involved in insulin degradation, we observed contrasting effects on IDE and PDI during the transition from fasted to fed state. For IDE, there was a significant decrease in activity during the initial 30 min after the glucose bolus, but its activity was subsequently restored to basal fasting levels over the last hour of the test (figure 17-E). For PDI, its activity rapidly increased to a maximum at 30 min after glucose administration, followed by a return to basal fasting levels (figure 17-F).

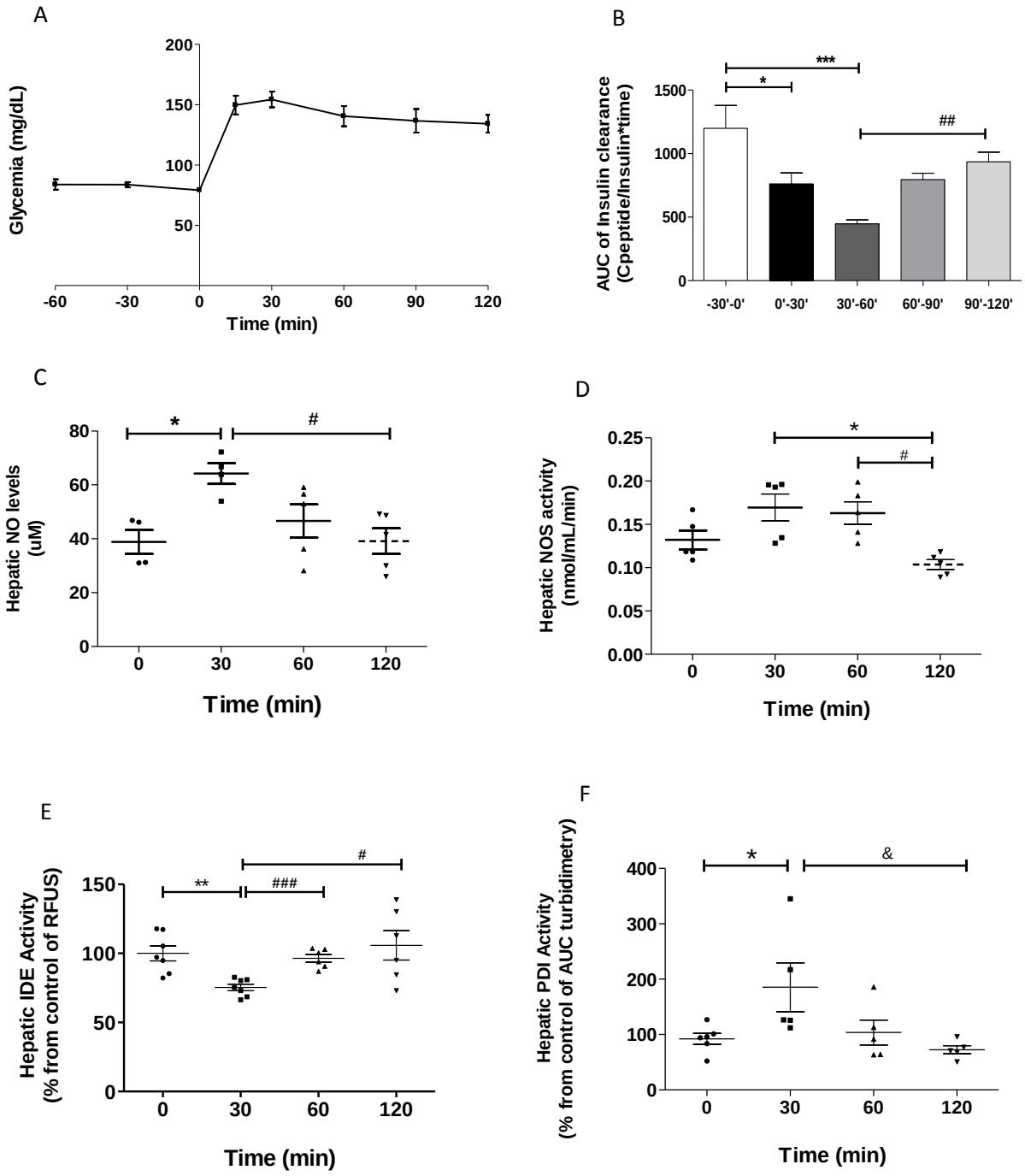


Figure 17: Assessment of insulin clearance and activities of related-enzymes during a glucose tolerance test in physiologic conditions: in a dynamic situation after a bolus of glucose, given intra-enterically, glucose profile was assessed (A) at the same time that insulin clearance was evaluated before and after the bolus of glucose (B); hepatic NO levels and NOS activity were quantified during the test (C and D); hepatic IDE and PDI activities were evaluated at different time points (E and F) (statistical analysis performed by One-Way ANOVA with statistical significance considered at *, # and &, $p<0.05$; ** and ##, $p<0.01$; *** and ###, $p<0.001$).

Correlation of NO levels with hepatic IDE activity in the fasted state

In the fasted state, IDE is active and endogenous NO production and levels are at a basal state. To determine the effects of an acute increase in hepatic NO levels on hepatic IDE activity, we artificially increased hepatic NO levels in fasted animals by infusion of the NO donor, SIN-1, and measured IDE activity. As shown in Figure 18-A, SIN-1 administration resulted in a 4-fold increase of hepatic NO levels. This was associated with a significant decrease in hepatic IDE activity, shown in Figure 18-C. Conversely, when basal hepatic NO levels were reduced by one-half using L-NAME acutely - an inhibitor of NOS - as shown in figure 18-B, hepatic IDE activity showed a significant increase (figure 18-D). Therefore, *in vivo* hepatic NO levels are inversely correlated with hepatic IDE activity.

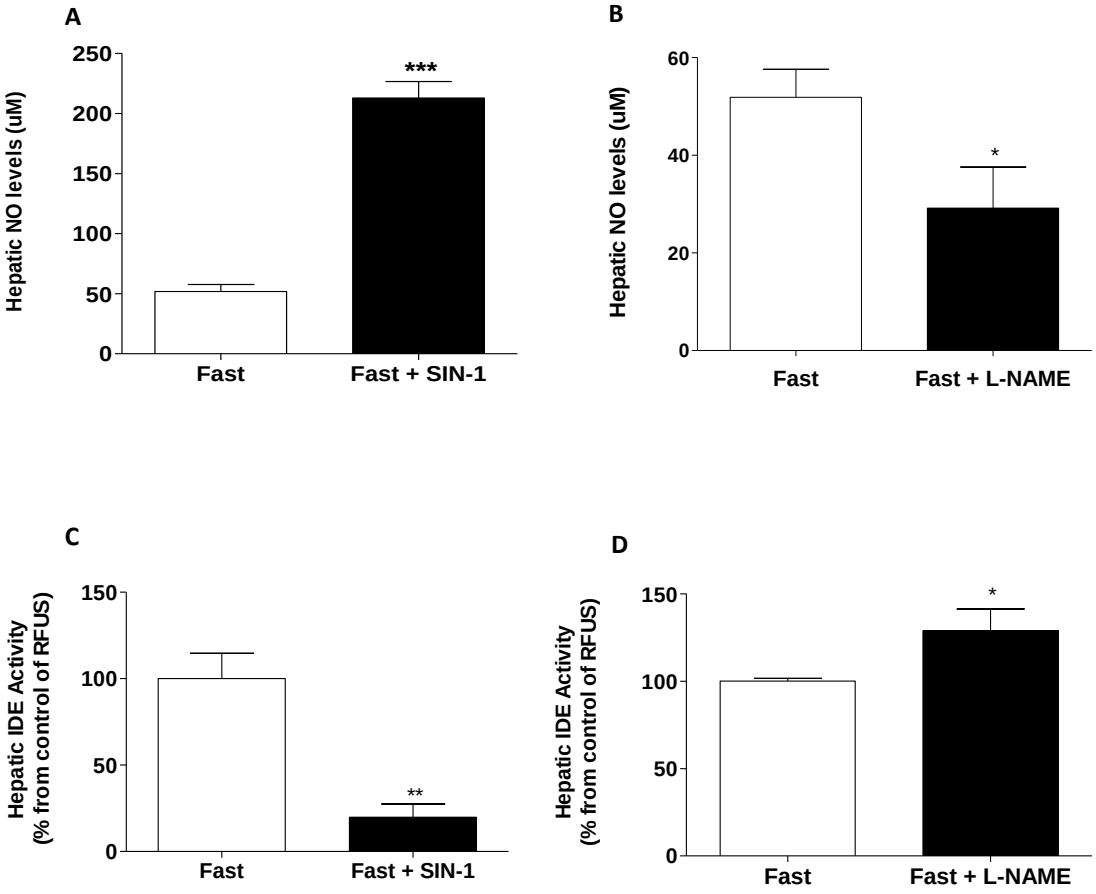


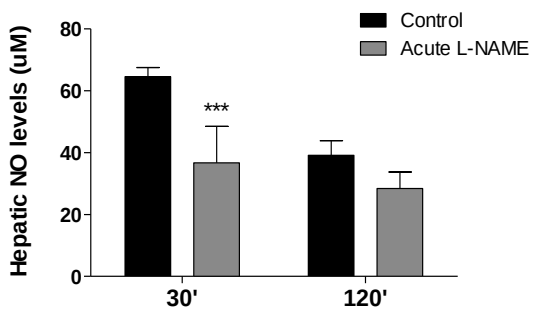
Figure 18: Correlation between hepatic NO levels and hepatic IDE activity in the fast state: modulation of hepatic NO levels by infusion of a NO donor, SIN1, or inhibition of NOS activity with L-NAME in the fast state (A and B); evaluation of hepatic IDE activity with NO levels manipulation in the fast state (C and D) (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Impact of NO on insulin bioavailability via modulation of insulin clearance *in vivo* from the fast to the fed state

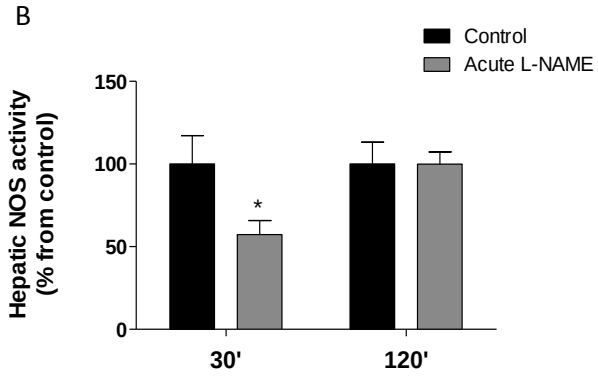
To test the involvement of NO in the dynamic regulation of insulin clearance during the transition from fasting to the post-prandial state, animals treated with both acute and chronic L-NAME intravenous infusion were submitted to an IEGTT. For these animals, glucose excursions, insulin secretion, insulin levels and clearance were measured. After sacrifice, NO levels, NOS, IDE and PDI activities were evaluated. In addition, the status of hepatic insulin signaling was also assessed.

As expected, L-NAME treatment reduced significantly hepatic NO levels when this molecule was assessed in livers excised at 30 min after the bolus of glucose (figure 19-A). At the end of the glucose tolerance test these were already similar between the two groups. The decrease in NO levels at 30 min after the bolus of glucose is consequence of a decrease in hepatic NOS activity at that time point, which also normalized at the end of the test (figure 19-B). At the end of the intra-enteric glucose tolerance test there were no changes in hepatic NOS isoforms protein expression (figure 19-C).

A



B



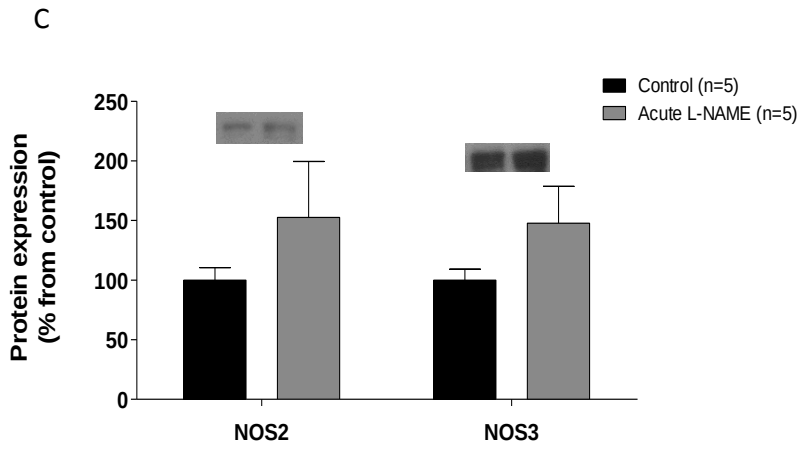


Figure 19: Evaluation of NO metabolism at 30 min and 120 min of an intra-enteric glucose tolerance test after acute NOS inhibition: NO levels (A), NOS activity (B) were assessed in livers after 30 and 120 min of a glucose, and NOS isoforms expression (C) were assessed only after 120 min of a glucose bolus given directly in the intestine (statistical analysis performed by Student's t test between control and L-NAME group with statistical significance considered at *, $p<0.05$; ***, $p<0.001$).

Acute NOS inhibition by L-NAME resulted in a decrease in glucose excursion during the IEGTT (figure 20-A). In the same animals, plasma insulin levels were decreased in the first hour of the test (figure 20-B). Insulin secretion over the first 30 min was not significantly different from control animals but showed a significant increase over the ensuing 90 min (figure 20-C). Acute L-NAME treatment promoted an increase in insulin clearance in the period immediately after the glucose bolus (o to 60 min) and during the entire 120 min of the test (figure 20-D).

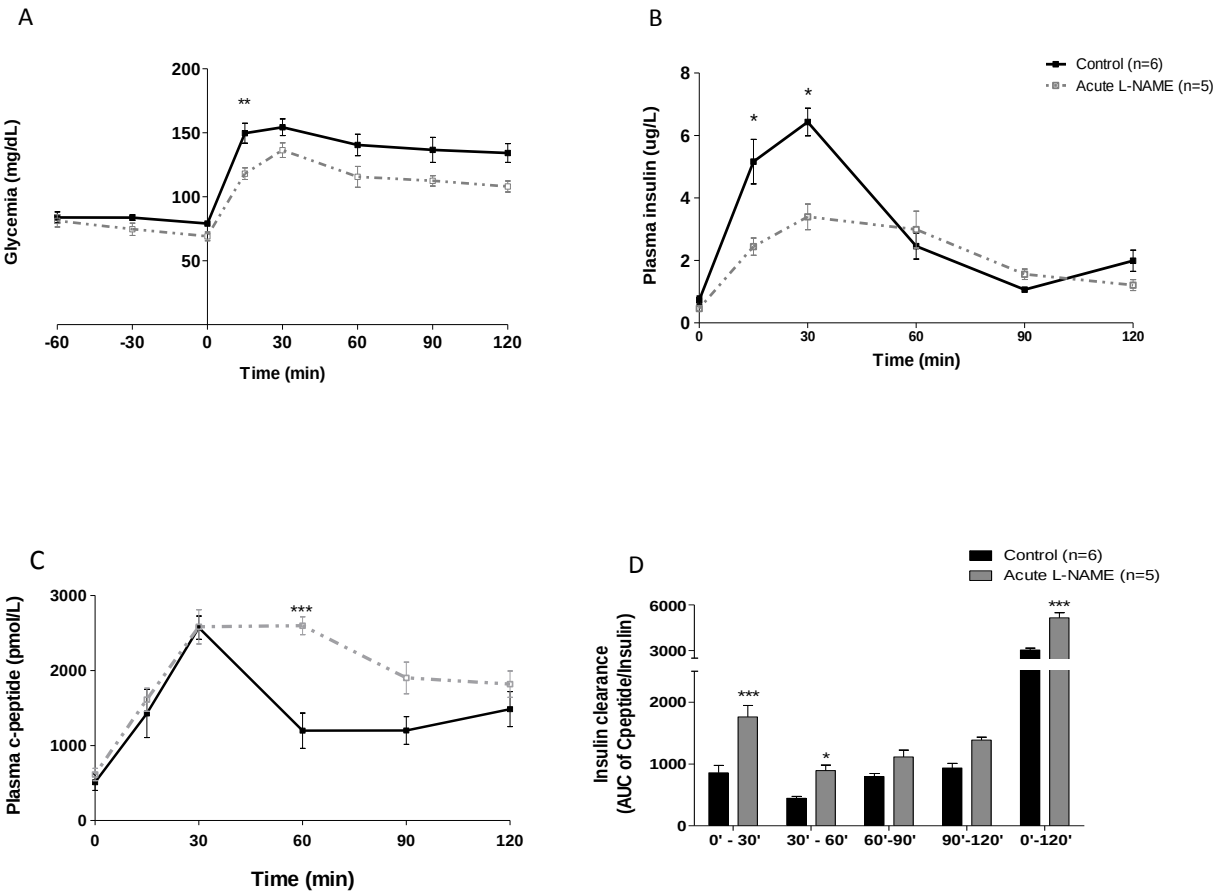


Figure 20: Evaluation of insulin clearance in animals with acute L-NAME treatment in a dynamic situation: glycemic profile was assessed during an intra-enteric glucose tolerance test after NOS inhibition (A); quantification of plasma insulin (B) and c-peptide (C) levels and calculation of correspondent insulin clearance rates (D) during the glucose tolerance test in animals acutely treated with L-NAME [statistical analysis performed by Two-Way ANOVA (A, B and C) and Student's t test (D) with statistical significance considered at *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$].

Hepatic IDE activity, in L-NAME treated animals, was increased both at 30 and 120 min after the bolus of glucose (figure 21-A) while IDE expression was also enhanced at the end of the test (figure 21-C). Hepatic PDI activity showed an increase at 30 min and 120 min (figure 21-B) with no alterations on its expression at the end of the test (figure 21-C). At the same time hepatic insulin signaling was enhanced as seen a significant increase in IRS1/Akt expression (figure 22-A). In addition, there were increases in the expression of GLUT2 and GLUT4 (figure 22-B), suggesting a stimulation of hepatic glucose transport.

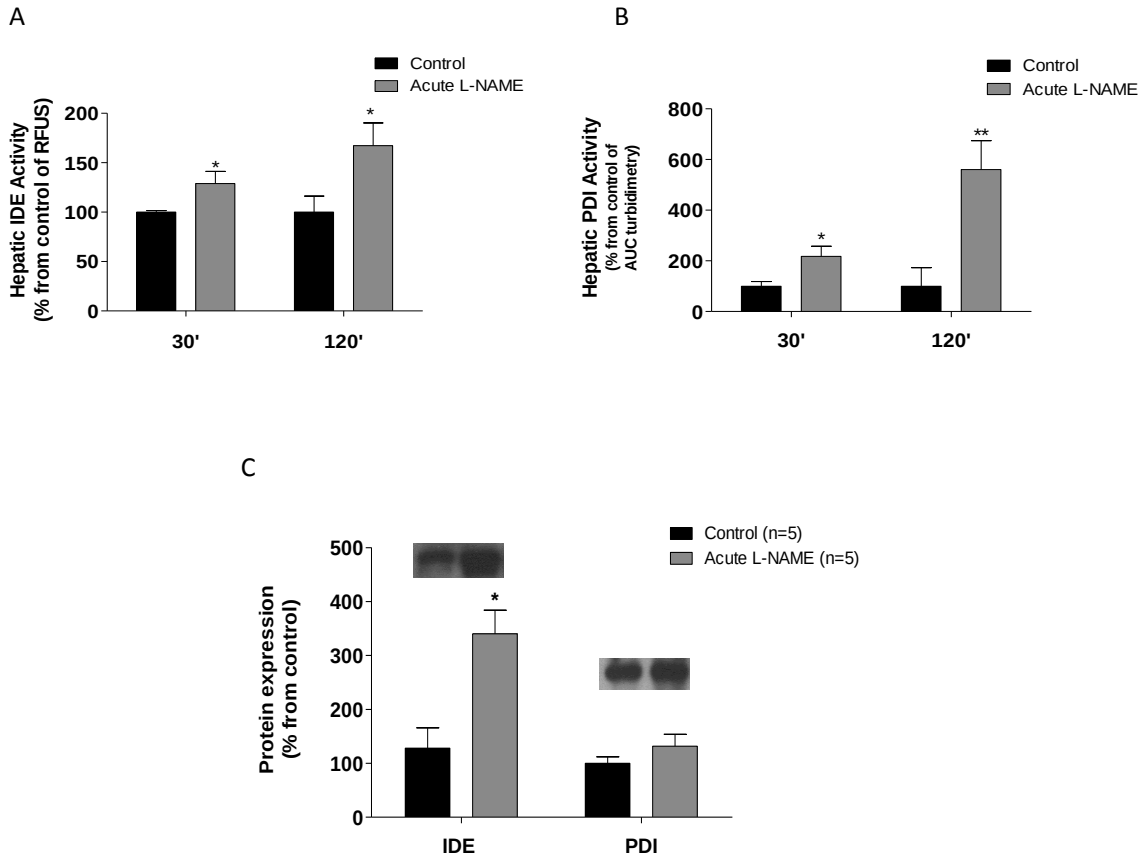


Figure 21: Analysis of hepatic IDE and PDI activity (A and B) and hepatic IDE and PDI protein expression (C) in animals treated with acute L-NAME infusion; enzymatic activities were assessed after 30 min and 120 min of the intra-enteric glucose bolus and protein expression was assessed only at the end of the 120 min of the glucose tolerance test (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$; **, $p<0.01$).

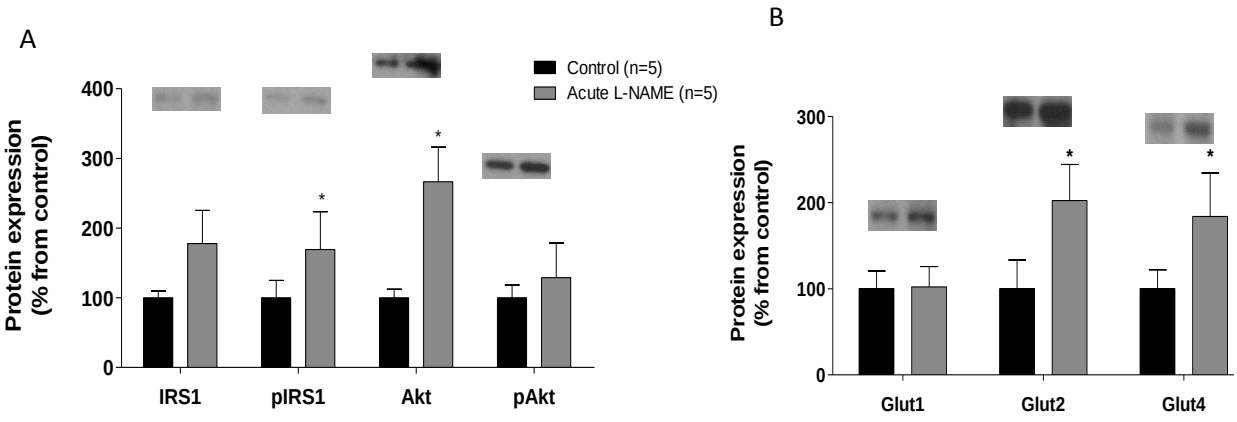


Figure 22: Assessment of hepatic insulin signaling via IRS1/Akt pathway (A) and glucose transporters expression (B) in livers at 120 min after a bolus of glucose in animals with acute NOS inhibition (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$).

Chronic NOS inhibition by subcutaneous injection of L-NAME during 4 weeks promoted depletion of NO levels in liver (figure 23-A), consequence of a decrease in NOS activity (figure 23-B) with no alterations in hepatic NOS2 and 3 isoforms expression (figure 23-C). Chronic NOS inhibition promoted no alterations in glucose excursion (figure 24-A) and plasma insulin levels (figure 24-B), in comparison to controls. Insulin secretion was systematically increased during IEGTT (figure 24-C). Insulin clearance during the first 30 min of the IEGTT was significantly increased compared to control animals (figure 24-D), like the overall insulin clearance during the entire 120 min of the glucose tolerance test.

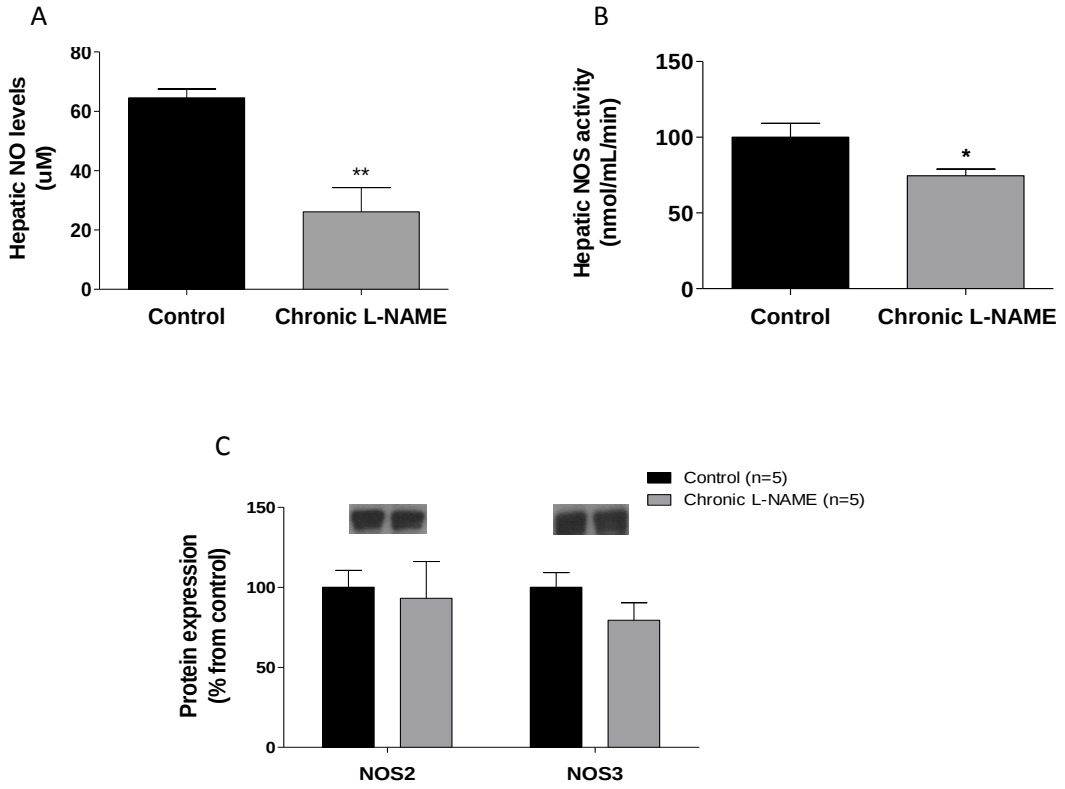


Figure 23: Evaluation of NO metabolism after chronic NOS inhibition: NO levels (A), NOS activity (B) and NOS isoforms expression (C) were assessed in livers after 120 min of a glucose bolus given directly in the intestine (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$; **, $p<0.01$).

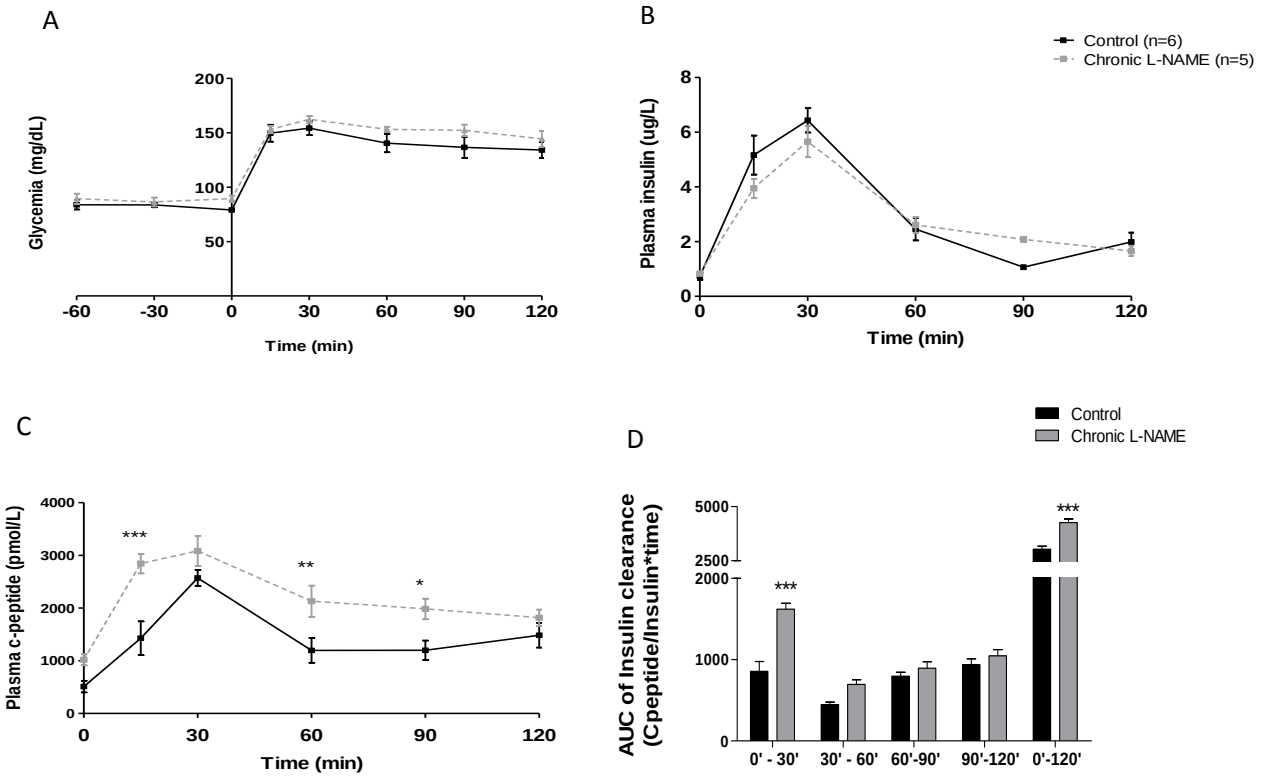


Figure 24: Evaluation of insulin clearance in animals with chronic L-NAME treatment during an intra-enteric glucose tolerance test: glycemic profile was assessed during the intra-enteric glucose tolerance test in animals with chronic NOS inhibition (A); quantification of plasma insulin (B) and c-peptide (C) levels and calculation of correspondent insulin clearance rates (D) during the glucose tolerance test in animals chronically treated with L-NAME [statistical analysis performed by Two-Way ANOVA (A, B and C) and Student's t test (D) with statistical significance considered at *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$].

In L-NAME animals both hepatic IDE and PDI activities were increased (figure 25-A and B) with no alterations on IDE and PDI expression (figure 25-C) at the end of the test. Concerning hepatic insulin signaling no alterations in the expression of the enzymes involved on it were observed (figure 26-A) 120 min after the bolus of glucose. On the analysis of hepatic glucose transporters only GLUT4 was decreased in L-NAME treated animals (figure 26-B).

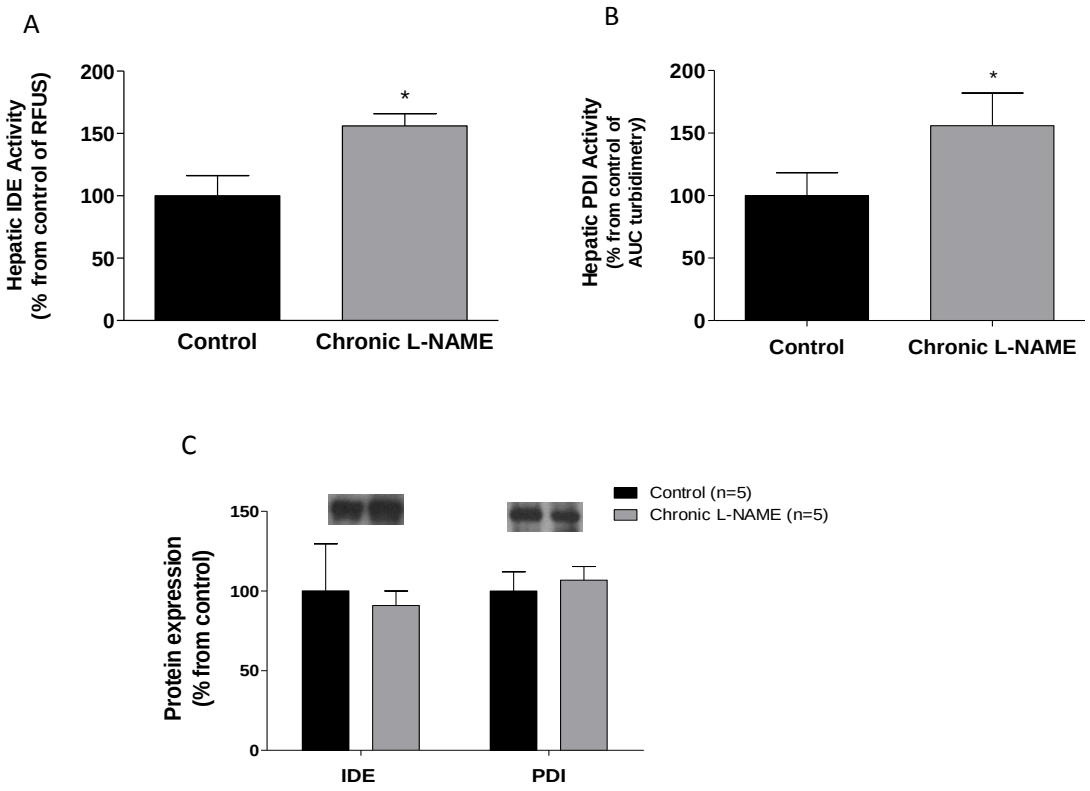


Figure 25: Analysis of IDE and PDI activity (A and B) and IDE and PDI protein expression (C) in livers at 120 min after the intra-enteric glucose bolus and treated with chronic L-NAME injection (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$).

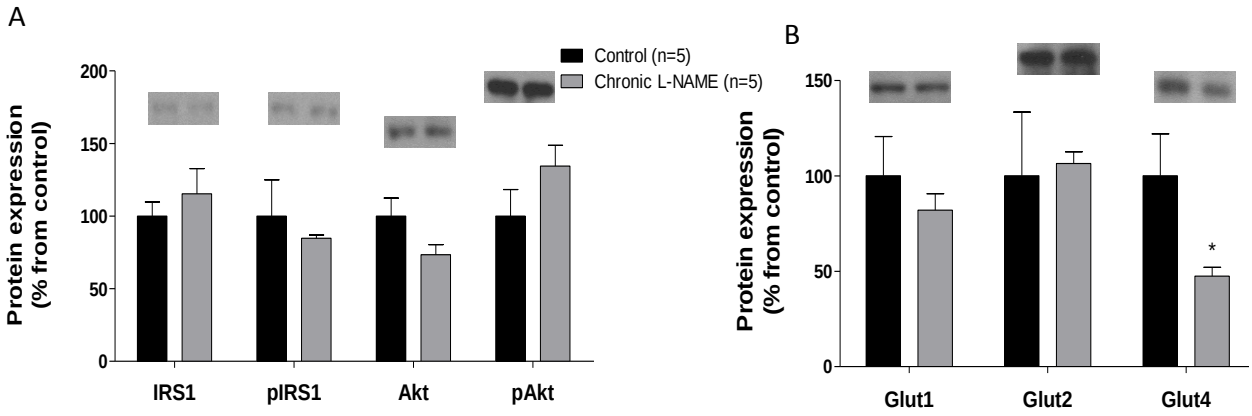


Figure 26: Assessment of hepatic insulin signaling via IRS1/Akt pathway (A) and glucose transporters expression (B) in livers at 120 min after a bolus of glucose in animals with chronic NOS inhibition (statistical analysis performed by Student's t test with statistical significance considered at *, $p<0.05$).

Discussion

The results of this study indicate that, following an enteric glucose load, changes in hepatic insulin clearance are highly involved in the fine-tuning of circulating insulin levels, as a requirement to maintain euglycemia. This process was found to be tightly linked to changes in hepatic NO levels, through changes in NOS activity. Under physiologic conditions, the role of NO is related to a biphasic pattern of insulin clearance, following the same glucose load. We observed that in the initial 60 min post-glucose where plasma glucose reached the highest levels, simultaneously an increase in hepatic NO levels as a consequence of increase in hepatic NOS activity was attained. Additionally, for the same period, we also observed a decrease in hepatic IDE activity, followed by a recovery to basal levels in the final hour of the test, in phase with the changes in insulin clearance. Moreover, the acute and chronic systemic inhibition of NO production confirmed the link between hepatic NO levels, hepatic IDE activity, and insulin clearance. In these situations we observed that hepatic NO depletion promoted an increase in insulin clearance that was associated with stimulation of hepatic IDE activity.

The rapid decrease in insulin clearance that occurs parallel to beta cell insulin secretion provides a mean for rapidly adjusting the levels of circulating insulin to counterbalance plasma glucose excursions. Previous human studies reported a decrease in hepatic insulin clearance after an oral glucose bolus (Shapiro, Tillil et al. 1987). This was explained in terms of hepatic insulin uptake being temporarily saturated following a burst of insulin secretion that acutely raised portal vein insulin concentrations. This simple model did not take into account that the hepatic capacity for insulin clearance might be rapidly evolving in this interval. A loss of this flexibility in postprandial hepatic insulin clearance may contribute to persistent hyperinsulinemia that among other things can decrease peripheral insulin sensitivity (Ferrannini and Balkau 2002; Erdmann, Kallabis et al. 2008).

Insulin clearance correlates with changes in the activity of the two main enzymes involved in this process. Hepatic IDE activity was decreased after the intestinal bolus of glucose thereby at least partly explaining the observed decrease in insulin clearance at this time. Cordes *et al* demonstrated *in vitro* that NO is able to inhibit IDE activity (Cordes, Bennett et al. 2009). We observed that *in vivo*, the decreased insulin clearance and reduced hepatic IDE activity were associated with increased hepatic NO levels suggesting that NO was responsible for attenuating insulin clearance through inhibition of IDE. On the other hand, inhibition of the NOS activity resulted in increased IDE activity. Regarding PDI activity, the other main component enzyme of insulin degradation, we observed a decrease in the fasting state with a

maximal activity during the initial 60 min of the glucose tolerance test. This is in agreement with Mikami *et al*. who found that after 3 days of fasting in rats, there was a decrease in PDI activity, which recovered with refeeding (Mikami, Genma et al. 1998). PDI has been reported to have different functions in different cells, namely in platelet activation/aggregation, proteins adhesion, infection and pathology of many diseases including human immunodeficiency virus (HIV), cancer and pathogenic infections (Raturi and Mutus 2007).

Indeed, it has also been suggested that PDI acts as an anti-inflammatory agent, and therefore its activation after the intestinal bolus of glucose could be a response to increases in pro-inflammatory species (Zhou, Jacob et al. 2008). Moreover, PDI activity was also found to be dependent on the ratio of reduced to oxidized glutathione (GSH/GSSG) being attenuated in a more oxidizing environment where GSH/GSSG is low (Raturi and Mutus 2007). It is well described that glutathione levels are decreased in the fast state(Tateishi, Higashi et al. 1977; Guarino, Afonso et al. 2003; Lautt 2007), which may explain in part our finding of lower PDI activity in the fast state compared to after the glucose load. Furthermore, PDI functions as a glutathione-insulin transhydrogenase, promoting the reductive cleavage of disulfide bonds between the two chains of insulin with concomitant formation of glutathione disulfide (Varandani 1974). Whether the insulin levels *per se* are involved in the regulation of PDI activity is unclear. Varandani suggested that the low levels of insulin in the fasted state contributed to a decrease in PDI activity (Varandani 1974). Meanwhile, we observed that with higher levels of insulin attained following a glucose load, PDI activity was increased, suggesting it may be induced by insulin. The immediate products of PDI are the separated insulin A and B chains. There is evidence of the presence of insulin chains in the circulation and that they have biological activity (Meek and Bolinger 1966; Varandani and Shroyer 1987; Yang, Huang et al. 2000; Duckworth, Fawcett et al. 2004). From our results NO does not appear to be a regulator of PDI activity as it increases physiologically with a bolus of glucose independently of NO manipulation. Thus, the role of PDI in regulating post-prandial hepatic insulin clearance remains unclear and further experiments addressing this point should be established.

To understand the involvement of NO in the regulation of insulin clearance we acutely manipulated NO levels by infusion of a NO donor and by inhibiting endogenous NO synthesis. The results of these experiments indicate that hepatic NO levels are inversely correlated with insulin clearance during fasting as well as in the postprandial state. Natali *et al.* demonstrated that NO depletion via acute L-NAME infusion in humans promoted an increase in insulin clearance (Natali, Ribeiro et al. 2013). However, they also observed that the depletion of NO also inhibited insulin secretion, which is in contradiction to our results. These contradictory

effects of NO on insulin secretion observed in rats *versus* humans may be due to species differences in beta cell response to changes in NO levels.

In addition, acute NOS inhibition led to a stimulation of hepatic insulin signaling cascade via IRS1/Akt pathway with a concomitant increase in hepatic GLUT4 and GLUT2 expression. This can at least partly explain the improved glucose tolerance found in these animals. In previous studies performed in dogs it has been described that NO depletion promotes a decrease in insulin signaling and increase in hepatic glucose output (Moore, Dicostanzo et al. 2008). However, they describe that this effect is observed only when the compounds were directly given to the liver by portal vein infusion. In our study, we infused L-NAME intravenously hence some of the hepatic effects may have been indirectly caused by the effects of NO depletion in other tissues. For example, central nervous system activity involving the sympathetic-parasympathetic switch is sensitive to NO levels and alterations in its functions could results in altered hepatic insulin sensitivity via parasympathetic nerve signals (Patel, Li et al. 2001; Zheng, Mayhan et al. 2006). In addition, it has been demonstrated that when non-hepatic glucose metabolism is modulated post-prandially by systemic NO manipulation it is hard to detect the possible effects of NO on hepatic glucose metabolism (An, DiCostanzo et al. 2008; Macedo, Lima et al. 2013).

When NO synthesis was chronically inhibited over 4 weeks, resulting in constantly high IDE activities and insulin clearance, plasma insulin levels were preserved by a compensatory increase in insulin secretion. Moreover, we also showed that acute and chronic NO depletion promoted different alterations in insulin signaling activation and glucose transporters expression. In contrast to acute NOS inhibition, chronic NO depletion did not alter the insulin signaling cascade and transporter expression with the exception of GLUT4. Since acute and chronic NO inhibition led to similar increases in insulin clearance but had different consequences on plasma insulin levels and hepatic insulin signaling, it seems that other factors related to chronic NOS inhibition might have affected insulin actions. Therefore, the acute treatment with NOS inhibitor evidences the lack of the immediate and physiologic role of the NO while a more prolonged alteration combines the physiologic role with adaptive mechanism of the aforementioned hepatic direct and indirect actions of NO (Cherrington 2005; An, DiCostanzo et al. 2008).

In conclusion, this work demonstrated that, *in vivo*, NO inhibits IDE activity and this is associated with a reduction in hepatic insulin clearance. Thus, the transient increase in NO levels following a glucose load serves to temporarily boost peripheral insulin levels by reducing

insulin clearance. In contrast, PDI activity was increased following the glucose load and this change was not influenced by NO levels. Whether chronic NO maintained elevated levels leads to hyperinsulinemia, by sustained inhibition IDE, further resulting in a decrease in the number of insulin receptors of other tissues/organs and being in the genesis of insulin resistance needs further experimental assessments. Nevertheless it is clear that hepatic NO is an important signaling molecule for the *in vivo* regulation of IDE and consequently of peripheral insulin bioavailability.

Acknowledgements

The authors acknowledge financial support from the Portuguese Foundation for Science and Technology (research grant PTDC/DTP-EPI/0207/2012). FOM held a fellowship from the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/51194/2010).

SUB-CHAPTER 4.2. - HYPOTHALAMIC NITRIC OXIDE LEVELS CONTRIBUTE TO THE REGULATION OF HEPATIC INSULIN CLEARANCE AND LIPID METABOLISM

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Abstract

The bioavailability of insulin for peripheral tissues is defined by the rates of insulin secretion and hepatic insulin clearance. Whether the control of insulin clearance is solely dependent on hepatic mechanisms or also involves hypothalamic regulation is currently uncertain. We hypothesized that nitric oxide (NO) production by central/hypothalamic axis modulates insulin clearance and therefore peripheral insulin bioavailability.

Male Wistar rats were infused with a single dose of NG-nitro-L-arginine methyl ester (L-NAME) into intracerebroventricular (i.c.v.), ventromedial hypothalamus (VMH) or paraventricular nucleus of the hypothalamus (PVN) (acute treatment). Other groups were infused every day for 2 weeks into the i.c.v. or PVN (chronic treatment).

Acute i.c.v., acute PVN and chronic PVN treatments all resulted in increased insulin clearance with consequent decrease in insulin bioavailability. Acute VMH treatment did not alter insulin clearance therefore it is not involved in insulin clearance regulation via NO. Beta cell sensitivity was diminished only with the chronic PVN treatment. Acute PVN treatment resulted in suppressed lipogenic pathway and diminished hepatic free fatty acid content. Chronic PVN treatment resulted in increased lipogenesis and hepatic triglyceride levels. The hepatic insulin signaling cascade via the IRS1/Akt pathway and hepatic glucose transporters expression were decreased by acute PVN treatment but were overexpressed with chronic PVN treatment.

In conclusion, NO levels in the PVN region of the hypothalamus have a significant influence on hepatic insulin clearance and peripheral insulin bioavailability. These effects were also associated with changes in hepatic triglyceride levels as well as expression of hepatic lipogenic pathway enzymes, glucose transport proteins and insulin signaling components.

Keywords: Insulin clearance, hypothalamus, liver, nitric oxide, metabolism, triglycerides.

Introduction

Mammalian metabolism is characterized by the maintenance of blood glucose levels within a relatively narrow physiological range during transitions between feeding and fasting. The control of systemic glucose levels is mediated by insulin, whose circulating levels in turn are finely tuned by a combination of nutrient-induced β-cell secretion and first-pass hepatic clearance. Hepatic insulin clearance is mediated by insulin degrading enzyme (IDE) and protein disulfide isomerase (PDI) (Osei, Gaillard et al. 2007; Lamontagne, Pepin et al. 2009). Recently has been suggested that nitric oxide (NO) attenuates hepatic insulin clearance (Natali, Ribeiro et al. 2013), possibly by inhibition of hepatic IDE activity (Cordes, Bennett et al. 2009) thereby playing a role in regulating circulating insulin levels. In addition to such direct hepatic mechanisms, NO levels in extrahepatic tissues may also contribute to the regulation of hepatic insulin clearance. This conclusion follows from the finding that liver-specific alterations of NO levels had less effect on insulin clearance than systemic NO changes (Sadri and Lautt 1999; Guarino, Afonso et al. 2003; Moore, Dicostanzo et al. 2008).

Of the possible extrahepatic sites where NO could influence hepatic insulin clearance, the hypothalamus is highly implicated. It is known to be a key regulator of hepatic energy metabolism and endocrine functions. It is also implicated as an important component of central glucometabolic regulation since it contains specialized neurons whose activities are regulated by changes in circulating glucose levels (Uyama, Geerts et al. 2004). Moreover, NO generation within the hypothalamus has been shown to influence energy and fuel homeostasis, in particular glucose metabolism (Canabal, Song et al. 2007). These actions appear to be mediated via changes in sympathetic neuronal signaling to the pancreas, liver and muscle (Cabou, Campistron et al. 2008). Thus, artificial manipulation of NO levels in the PVN region demonstrated that elevated NO levels have inhibitory effects on sympathetic neuronal activities (Zhang, Mayhan et al. 1997; Zhou, Sun et al. 2014). Among other things, this may have important implications for the pathophysiology of glucose intolerance and diabetes. For example, elevated PVN NO levels may contribute to the decreased sympathetic nerve activity and consequent glucose-induced insulin secretion impairment during diabetes (Takeda, Tsuura et al. 2001; Burattini, Di Nardo et al. 2009; Lee, Lorenzo et al. 2013).

Hence, we hypothesized that hypothalamic NO contributes to the regulation of hepatic insulin clearance. To test this hypothesis, we manipulated hypothalamic NO levels and

evaluated these effects on systemic insulin bioavailability, as defined by pancreatic insulin secretion and first-pass hepatic insulin clearance. The hypothalamus consists of different regions that are characterized by specialized neurons whose activity is regulated by changes in glucose levels. Moreover, hypothalamus is crucial on the modulation of autonomic nervous system (ANS). Therefore, hypothalamus is a key player of central glucometabolic regulation (Uyama, Geerts et al. 2004). We focused our attention on two specific areas: the ventromedial (VMH) and paraventricular nuclei (PVN), due to their known effects on systemic glucose and lipid metabolism (Dampney 2011). The VMH sends sympathetic signals to peripheral tissues promoting catabolic activities such as gluconeogenesis and lipolysis (Dampney 2011). Through its sensitivity to insulin, leptin and glucose levels, the PVN is responsive to the status of glycemia as well as that of whole-body energy metabolism. In consequence, PVN exerts its effects on glucose and energy metabolism in peripheral tissues via vagus activity (Zhang and Fogel 2002) and by triggering the release of various hormones from the pituitary gland (Uyama, Geerts et al. 2004; Zheng, Mayhan et al. 2006). Thus, we further hypothesized that changes in NO levels within PVN and VMH regions mediate different effects on hepatic insulin clearance and insulin bioavailability. To test this hypothesis, we specifically inhibited NO generation in each of these regions and compared the effects of these interventions on hepatic insulin clearance and systemic insulin bioavailability.

Methods

Reagents

All reagents were of the highest degree of purity availably. D-glucose, insulin and L-NAME were purchased from Sigma- Aldrich (Portugal). Ketamine and xylazine were obtained from a local veterinary company (Portugal). Heparin and 0.9% NaCl were purchased from BBraun (Portugal).

Animals

12 weeks old male Wistar rats, from in-house animal facilities in Santiago de Compostela, were used. All animals were housed under conventional conditions with controlled temperature, humidity and light (12 h light–dark cycle), and were provided with a standard

commercial diet and water (ad libitum) till the night before the experiment. All experimental procedures were conducted according to the EU guidelines for the use of experimental animals (86/609/EEC).

Surgical procedures

Male Wistar rats were submitted to brain surgery using a stereotaxic apparatus to the implantation of a single cannula for i.c.v., or double cannulae, for PVN and VMH-specific infusions. After the bregma localization the following coordinates were used: Lat: 1.2mm, AP: 1.0mm for lateral ventricle (i.c.v.), AP: -1.8mm, Lat: +/- 0.4mm, DV: -8.0mm for PVN and AP: -2.52mm, Lat: +/- 0.6mm, DV: -9.2mm for VMH.

Experimental protocols for hypothalamic NO depletion via L-NAME

To deplete hypothalamic NO levels, animals were administered with L-NAME, a NOS inhibitor, either acute or chronically. For acute administration, bolus infusions of 250ug of L-NAME in 2μL of saline (or 2μL of saline only in the control animals) were performed in each side of the brain on the day of the experiment. This procedure was applied to i.c.v, as well as PVN- and VMH-specific studies. Chronic L-NAME administration was performed in i.c.v. and PVN-specific animals only. Here, the infusion procedures described were repeated daily for 14 days prior to the analyses of glycemic status and insulin clearance. For each experiment, control animals were infused daily with saline by the same route used for the L-NAME treated animals.

Assessment of glucose tolerance

After a 24 h fasting period, all animals were submitted to an oral glucose tolerance test (OGTT) (2g glucose/kg) performed 45 min after the final L-NAME infusion. Blood samples were obtained at different time points from a cut in the tail, before and after the glucose bolus, and plasma samples were kept at -20°after centrifugation. Plasma glucose levels were monitored during the 120 min of the OGTT using a standard glucometer. Animals were then euthanized by cervical dislocation and liver was harvested, immediately frozen in liquid nitrogen and kept at -80° to further analysis.

Assessment of insulin clearance

Plasma insulin and c-peptide levels were quantified in the basal fasted state and during the OGTT by ELISA-based assay kits (Rat Insulin Ultrasensitive kit and Rat C-peptide kit, Mercodia, Sweden). Insulin clearance was calculated by the ratio between area under the curve of c-peptide, a surrogate of insulin secretion, and area under the curve of plasma insulin levels at fast state and at each time point during the OGTT.

Assessment of protein expression

Protein extracts for Western blot analysis were obtained using a lysis buffer (1M Tris-HCl, pH 7.5, 0.2M EGTA, 0.2M EDTA, 1% Triton-X 100, 0.1M sodium orthovanadate, 2g/L sodium fluoride, 2.2 g/L sodium pyrophosphate, 0.27M sucrose) to homogenize liver tissue. Samples were centrifuged and total protein lysates were kept at -20°. Total protein lysates from liver were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrotransferred on a polyvinylidene difluoride membrane, and probed with the respective antibodies: insulin receptor substrate 1 [IRS1 (sc-7200)], Glut1 (sc-7903), Glut2 (sc-9117), Glut4 (sc-7938), sterol regulatory element binding protein 1 [SREBP1 (sc-8984)], fatty acid synthase [FAS (sc-20140)], diacyl glycerol transferase [DGAT (sc-66859)] (Santa Cruz Biotechnology, Santa Cruz, CA, USA); acetyl CoA carboxylase [ACC (04-322)], pACC (07-303) (Merck Millipore, Darmstadt, Germany); ATP citrate lyase (ACL 4332), pATP citrate lyase (pACL4331), Akt (92725), pAkt (5473);pIRS1 (44816G) (Invitrogen, Life Technologies, Scotland, UK). Then, membranes were exposed to radiograph film (Super RX, Fuji Medical X-Ray Film;Fujifilm, Japan) and developed with developer and fixing liquids (AGFA, Mortsel, Belgium) under appropriate dark room conditions. Protein levels were normalized to β-actin (A5316, Sigma) for each sample.

Assessment of hepatic FFAs and TGs and plasma FFAs

For hepatic TG extraction 50 mg of powdered frozen liver was added to 500 μL of a 2:1 chloroform:methanol mixture. The mix was homogenized in a tissue lyzer and placed for 1.5-3 h in a cold room with moderate shaking. Afterwards, 300 μL of miliQ water was added and the

mixture was centrifuged in Eppendorff tubes for 20 min at 13000 rpm at room temperature. The bottom organic phase was separated and allowed to evaporate in air.

For hepatic TG and FFA quantification, samples were dissolved in chloroform and submitted to vigorous vortex and analysis performed by the use of commercial kits: for TGs Spinreact kit (1001314) and for FFA Wako kit (434-91795). For FFA quantification in plasma, a sample of plasma was directly applied to the Wako FFA kit.

Statistical Analysis

Data was expressed as means ± standard error. The significance of differences in parameters between different groups was calculated through two-tailed Student's t tests. At $p < 0.05$, differences were accepted as statistically significant.

Results

Central nervous system NO depletion and insulin clearance

Male Wistar rats were tested for glucose tolerance after both acute and chronic central L-NAME infusion. The acute treatment was performed with an L-NAME injection 45 min before the oral bolus of glucose in three different regions: i.c.v, PVN and VMH. The chronic treatment was performed every day during 2 weeks by both i.c.v. and PVN infusion.

The OGTT glycemic profile was similar between control and L-NAME animals in all the tested sites (data not shown). Insulin secretion, assessed by the quantification of c-peptide levels in the plasma during the OGTT, was also similar for all treated and control groups (figure 27-A and D). In acute VMH animals plasma insulin levels and insulin clearance was not altered, therefore this animals were not evaluated for further parameters neither were evaluated with a chronic treatment (figure 27-B and C). In i.c.v. and PVN animals under acute treatment plasma insulin levels were significantly increased (figure 27-B), but with chronic L-NAME treatment only the PVN group showed increased plasma insulin levels (figure 27-E). Therefore, insulin clearance was decreased in both i.c.v. and PVN animals with acute treatment (figure 27-C), while with chronic treatment insulin clearance was only decreased in PVN-infused animals

(figure 27-F). As seen in figure 27-C, the decrease on insulin clearance in PVN animals was around half of the decrement seen in the i.c.v. animals.

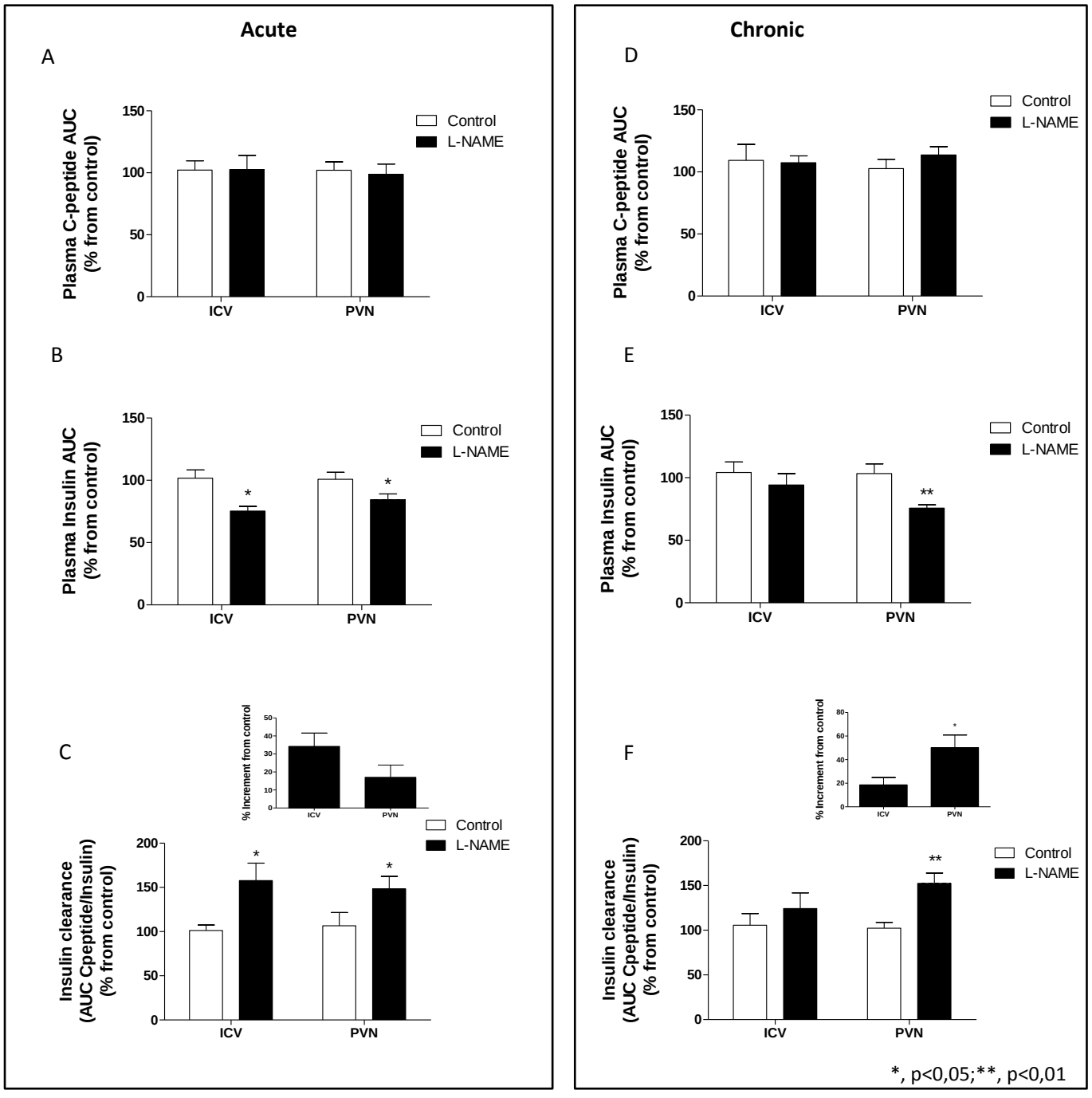

Figure 27: Insulin metabolism regulation after acute and chronic L-NAME infusion in the lateral ventricle and in the hypothalamus: animals were infused with the NOS inhibitor L-NAME in all brain (i.c.v.) and in two regions of the hypothalamus (VMH and PVN) 45 min before an OGTT (acute treatment – A to C) or only in i.c.v. and in the PVN during 2 weeks (in the chronic treatment – D to F); insulin secretion was assessed by the quantification of plasma c-peptide levels (A and D); plasma insulin levels were assessed in all the different groups of animals (B and E); and insulin clearance was quantified by the ratio between c-peptide and plasma insulin levels (C and F).

Chronic hypothalamic NO depletion and beta cell sensitivity

In order to determine if beta cell function was altered by acute and chronic administration of L-NAME into the PVN region, plasma glucose concentrations were plotted against c-peptide levels, representative of insulin secretion. Slopes of the lines were compared and it was observed that β-cell function in the acute treatment did not differ from saline-infused controls (figure 28-A) while the chronic infused group had a significant reduction in β-cell function (figure 28-B).

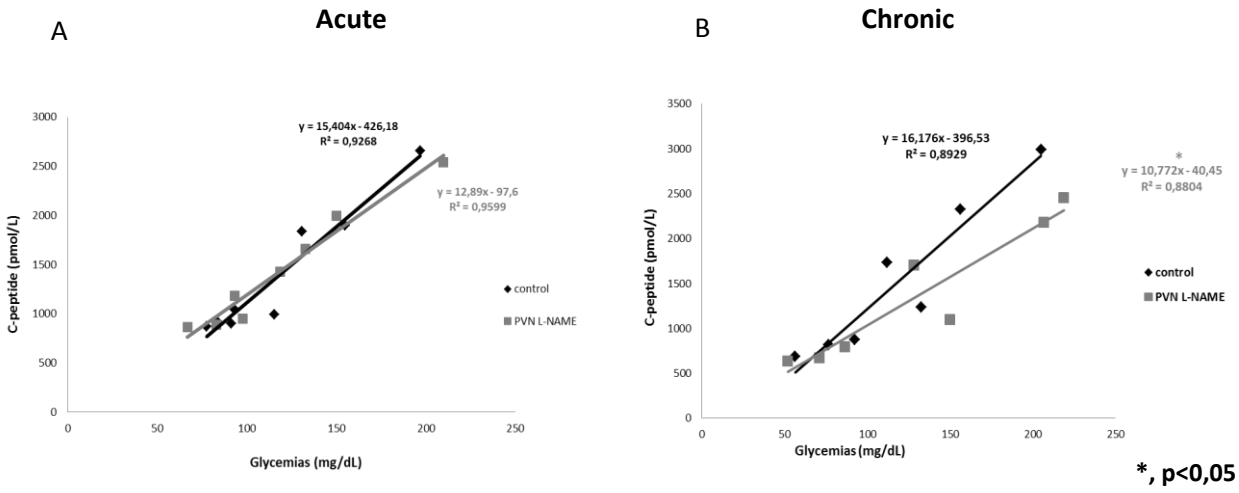

Figure 28: Impact of PVN-NOS inhibition on beta cell function: after acute (A) or chronic (B) L-NAME infusion in the hypothalamic region of the PVN the glycemias during OGTT were plotted against corresponding c-peptide levels; the slopes of the lines allow the comparison of beta-cell sensitivity.

Hypothalamic NO alters hepatic insulin signaling cascade activation and glucose transporters expression

The assessment of insulin clearance regulation by NO produced in the PVN region of the hypothalamus was complemented with the analysis of the impact on hepatic insulin signaling and glucose transporters expression.

The acute PVN animals showed an inhibition of the IRS1/Akt pathway with the reduction in the hepatic expression of IRS1, pIRS1 and pAkt (figure 29-A). Hepatic Glut1, Glut2 and Glut4 expression in these animals was also decreased (figure 29-B).

In the chronic PVN animals, contrary to the acute animals, there was an increase in the expression of total hepatic IRS1 and Akt (figure 29-C) and a stimulation of the hepatic expression of Glut1, with no alterations on Glut2 and Glut4 expression (figure 29-D).

Hypothalamic NOS inhibition and hepatic lipid metabolism

We evaluated the impact of NOS inhibition on the PVN region of the hypothalamus over FFA, TG and expression of de-novo lipogenesis pathway enzymes.

With the acute treatment plasma FFA were not changed (figure 30-A) and hepatic FFA were decreased with the NOS inhibition (figure 30-C). Hepatic TGs in these animals were similar between control and L-NAME group (figure 30-C). When proteins expression was evaluated there was an increase on the ratio pACC/ACC and a decrease on the ratio pACL/ACL (figure 31-A), suggesting a decrease in lipogenesis. The protein involved in the assembling of TGs, DGAT, was increased in the liver of this acute L-NAME group (figure 31-A).

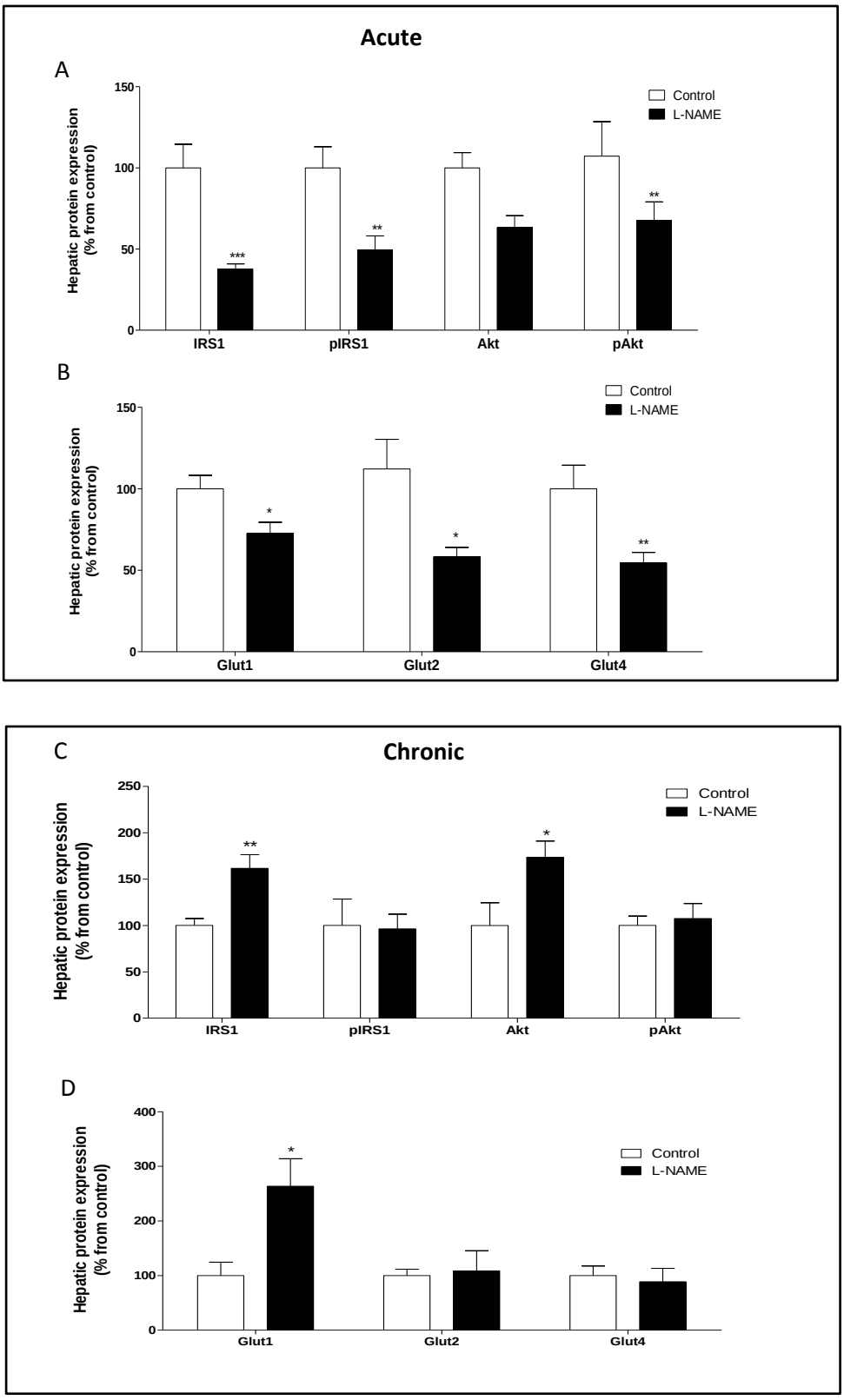

Figure 29: Impact of acute and chronic PVN-NOS inhibition on hepatic insulin signaling and glucose transport: after acute (A and B) or chronic (C and D) L-NAME infusion in the hypothalamic region of the PVN hepatic expression of proteins involved in insulin signaling cascade (A and C) and hepatic expression of glucose transporters was evaluated (B and D).

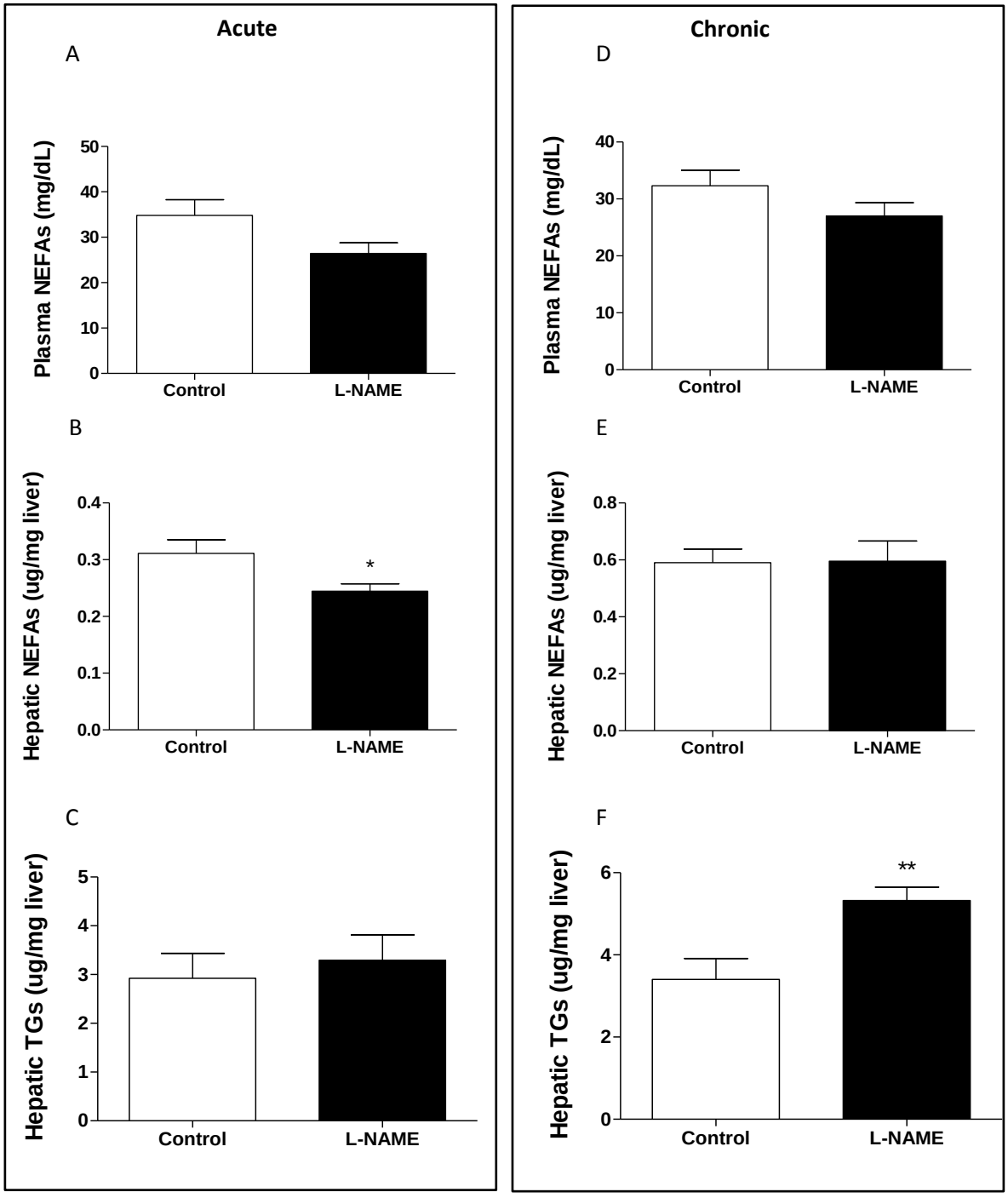

Figure 30: Impact of PVN-NOS inhibition on the levels on FFAs and TGs in plasma and liver: animals that were acutely (A-C) or chronically (D-F) infused with L-NAME in the hypothalamic region of the PVN were assessed for plasma (A and D) and hepatic (B and E) FFAs and hepatic levels of TGs (C and F).

Concerning chronic PVN infused animals, they showed similar levels of plasma (figure 30-D) and hepatic FFAs (figure 30-E) comparing to control group and a significant increase of hepatic

TGs (figure 30-F). These animals also showed a decrease in the ratio pACC/ACC and an increase in the ratio pACL/ACL demonstrating a stimulation of lipogenesis (figure 31-B) albeit FAS hepatic expression was diminished (figure 31-B).

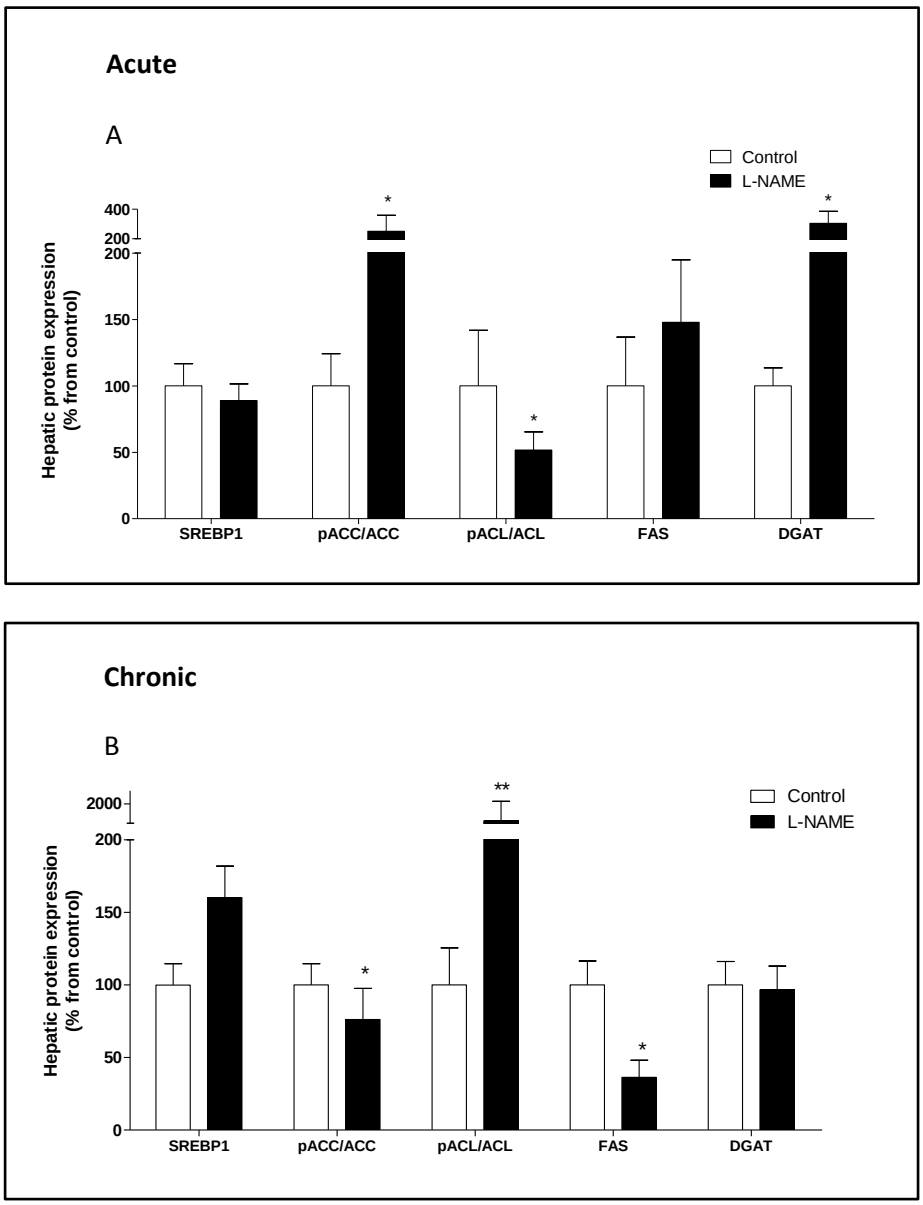

Figure 31: Regulation of lipid metabolism-related proteins expression by the hypothalamic region of the PVN: animals were acutely (A) or chronically (B) infused with L-NAME in the hypothalamic region of the PVN; the impact of that treatment in the regulation of lipid metabolism was assessed by the evaluation of proteins expression.

Discussion

In this study we investigated the involvement of hypothalamic NO levels in the regulation of hepatic insulin clearance and peripheral insulin bioavailability. Our major finding was that alterations in PVN NO levels have an effect on hepatic insulin clearance. This was accompanied by changes in hepatic lipid levels and expression of lipogenic enzymes as well as expression of insulin signaling pathway proteins.

It is already known that NO can act both directly on hepatic metabolism as well as indirectly via the brain and central nervous system. Sprangers *et al.* have shown in isolated hepatocytes that the direct effects of NO in liver results in modulation of glucose metabolism (Sprangers, Sauerwein et al. 1998). Lautt and others showed that beside these direct effects, there is also a NO-neurohumoral mechanism involving hepatic parasympathetic nervous system that also contributes to the regulation of glucose disposal (Lautt, Macedo et al. 2001). They found that hepatic parasympathetic nerve firing induces the release of NO that in turn activates a cascade that culminates in the stimulation of insulin action and glucose uptake (Lautt, Macedo et al. 2001; Guarino, Afonso et al. 2003; Lautt 2005). In this study, we attempted to resolve the actions of hepatic and hypothalamic NO levels in the regulation of hepatic insulin clearance. Rezende *et al.* showed that administration of ciliary neurotrophic factor (CNTF) in a type 2 diabetes rat model decreased their insulin levels and improved their glycemic control (Rezende, Santos et al. 2012). This reversal of hyperinsulinemia was shown to be associated with increases in hepatic IDE activity and first-pass insulin clearance. They suggested that this concerted improvement in both circulating glucose and insulin levels change requires a high level of coordination between central and peripheral glucoregulatory mechanisms (Rezende, Santos et al. 2012).

In our work the inhibition of NO synthesis in PVN both acutely and chronically promoted an increase in insulin clearance leading to a decrease in plasma insulin bioavailability. This supports our hypothesis that central NO levels are involved in the regulation of insulin clearance. PVN already has been described to be a prominent target for many brain regions and it acts in the coordination of the autonomic nervous system because it connects with both parasympathetic and sympathetic centers (Saper, Loewy et al. 1976; Kalsbeek, La Fleur et al. 2004; Dergacheva, Dyavanapalli et al. 2014). However, PVN NO levels alone do not fully account for the NO-dependent central effects of hepatic insulin disposal since i.c.v. infusion of the NO-inhibitor had a greater effect on insulin clearance compared to PVN infusion. Since

VMH infusion of the NO-inhibitor had no effect on insulin clearance, the actions of NO in this brain region do not appear to have a role in the regulation of hepatic insulin clearance. However, other brain region(s) and/or mechanisms may compensate for a deficiency in central NO levels thereby decoupling central NO levels from hepatic insulin clearance. This may explain the insensitivity of hepatic insulin clearance to chronic whole-brain NO depletion. Shankar et al. reported that acute i.c.v. infusion of another NOS inhibitor, L-NMMA, promoted a decrease in plasma insulin levels and improved insulin sensitivity (Shankar, Zhu et al. 1998). They suggested that the reduction in insulin levels was due to reduced β-cell secretion, but they did not assess insulin clearance. Our studies suggest that hepatic insulin clearance is in fact a dominant factor in explaining the changes in circulating insulin levels secondary to acute alterations in cerebral NO levels.

When we looked to the alterations promoted by the chronic L-NAME infusion in the PVN region of the hypothalamus, we conclude that the role of NO over insulin clearance is maintained supporting the importance of PVN NO levels in the regulation of insulin clearance. Moreover, it seems that the 2-week depletion of NO in the PVN starts to alter β-cell function, suggesting its possible role in regulating β-cell insulin secretion as well as hepatic insulin clearance. In concert with the maintained increase in insulin clearance, we observed a stimulation of insulin signaling via IRS1/Akt. It is known that high levels of insulin in circulation activate a negative feedback effect (Duckworth, Bennett et al. 1998; Ye 2007) that decreases the availability of insulin receptors (Tarsitano, Paffaro et al. 2007). Therefore, an increase in the clearance of insulin via NOS inhibition in the PVN can be viewed as a possible mechanism for increasing insulin sensitivity (Rudovich, Pivovarova et al. 2009). Additional research is needed, for example with the quantification of insulin receptors, to specifically address this question about the compensatory mechanisms that preserve insulin signaling in the face of reduced insulin bioavailability.

The inhibition of NO production in the PVN region by chronic L-NAME treatment was also shown to increase hepatic lipid levels. An increase in hepatic TG levels was previously reported in chronic NO-depleted animals by Zheng and colleagues (Zheng, Wang et al. 2002). It is important to emphasize that the changes in hepatic TG levels observed in our study were relatively modest compared to those seen with 14 days of high fat or high sugar feeding (Ferramosca, Conte et al. 2014). Nevertheless, alterations in hypothalamic NO may contribute to diet-induced changes in hepatic TG levels, since systemic NO levels are also modified by different diets (Perreault and Marette 2001). The absence of significant changes in hepatic TG

levels after acute NO inhibition may in part reflect the relatively slow turnover of the hepatic TG pool coupled with relatively low fractional synthetic rates for de novo lipogenesis.

To determine the possible mechanisms accounting for the observed hepatic TG levels the decrease in hepatic insulin signaling following acute hypothalamic L-NAME administration was accompanied by a decrease in lipogenesis pathway enzyme expression. However, animals that had chronic L-NAME treatment showed increase in lipogenesis pathway enzyme expression. Despite these contrary effects of acute and chronic NO production inhibition in the PVN region of the hypothalamus both groups maintain or even increase the accumulation of hepatic TGs. In the acute group the maintenance of hepatic TGs is achieved by the stimulation of DGAT expression which is converting hepatic FFAs into TGs. The chronic group evidences an accumulation of hepatic TGs due to the observed stimulation of lipogenic pathway. Moreover, we cannot forget that lipogenesis is one of many metabolic origins for hepatic TGs. We have to take in account that we are not measuring uptake of lipids from the diet, rates of export of VLDL-TGs to the circulation, etc, which will alter the TGs pool in the liver. In another work, the inhibition of melanocortin system in the brain promoted also an increase in lipogenesis, due to increase of the ratio sympathetic/parasympathetic system activity (Wiedmer, Chaudhary et al. 2012). Nogueiras and colleagues also showed that KO of melanocortin receptors in mice promoted fat storage by tissue-specific increase of insulin sensitivity (Nogueiras, Wiedmer et al. 2007). Additionally, NO availability decrease, for example in hypertension states, has been described to inhibit the melanocortin system activity and the alteration of both parameters regulates insulin and glucose metabolism (do Carmo, Bassi et al. 2011). This can explain our increase in lipogenesis in the chronic animals infused in the PVN with L-NAME and rises evidences for the modulation of insulin clearance by hypothalamic signals in the treatment of diabetes or obesity dysfunctions. Additional work is needed with our experimental study in order to evaluate the importance of ANS in the observed modulation of insulin clearance by the PVN-derived NO signal.

In conclusion, we can state that hypothalamic NO has a role on insulin clearance, with high relevance to the PVN region. A maintained defect in hypothalamic NO production increases hepatic insulin signaling and glucose transporters expression. Finally, the major metabolic impact of chronic PVN-NO depletion occurs at the levels of lipogenic pathway modulation and is associated with changes in hepatic TG levels, raising the idea for a possible intervention on hepatic steatosis, obesity and/or T2D patients by hypothalamic NO modulation.

Acknowledgements

The authors acknowledge financial support from the Portuguese Foundation for Science and Technology (research grants: PIC/IC/82956/2007 and PTDC/DTP-EPI/0207/2012).FOM held a fellowship from the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/51194/2010).

SUB-CHAPTER 4.3. - MECHANISMS BY WHICH THIAZOLIDINEDIONES PROTECT AGAINST SUCROSE-INDUCED FATTY LIVER AND HYPERINSULINEMIA

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Submitted to: PLoS One

Abstract

Objective: Thiazolidinediones (TZD) are known to ameliorate fatty liver in type 2 diabetes. To date, the underlying mechanisms of their hepatic actions remain unclear.

Research design and methods: Hepatic triglyceride content and export rates were assessed in 2-week high-sucrose-fed Wistar rats treated with troglitazone (HS-T) and compared with untreated HS rodent controls (HS-C). Fractional *de novo* lipogenesis contributions to hepatic triglyceride were quantified by analysis of triglyceride enrichment from deuterated water. Hepatic insulin clearance and nitric oxide (NO) status during a meal tolerance test were also evaluated.

Results: TZD significantly reduced hepatic triglyceride (4.2±0.3 HS-T versus 8.6±1.2 mg/g liver HS-C, $p<0.01$), decreased *de novo* lipogenesis contribution to hepatic triglyceride (16 ± 1 HS-T versus 22 ± 1% HS-C, $p<0.01$) and increased postprandial NEFA clearance rates (-0.057 ± 0.0007 HS-T versus -0.0013 ± 0.0004 %/min HS-C, $p<0.01$). During a meal tolerance test, plasma insulin area-under-the curve was significantly lower (42 ± 6 HS-T versus 206 ± 44 A.U.HS-C, $p<0.01$) while blood glucose and plasma C-peptide levels were not different. Insulin clearance was significantly increased (12598 ± 3053HS-T versus 3042 ± 720 A.U. HS-C, $p<0.001$) and was associated with augmented hepatic insulin-degrading enzyme (IDE) activity (118.8 ± 4.3 HS-T versus 96.7 ± 4.5 % HS-C, $p<0.05$). Finally, hepatic NO was significantly decreased (43.89 ± 6.9 HS-T versus 182.2 ± 44.8 uM HS-C, $p<0.05$).

Conclusions: Overall, TZD show direct actions on liver by reducing hepatic *de novo* lipogenesis and increasing hepatic insulin clearance. Alterations on hepatic insulin clearance are associated with changes in IDE activity, with possible modulation of NO levels.

Introduction

Thiazolidinediones (TZD) are widely used agents for improving glycemic control in Type 2 diabetes (T2D) patients. Thiazolidinediones have been also shown to decrease hepatic triglyceride levels in those patients that present non-alcoholic fatty liver disease - a frequent complication of T2D (Belfort, Harrison et al. 2006; Ratziu and Poynard 2006). The TZD activate the gamma isoform of the peroxisome proliferator-activated receptor (PPARγ) (Lehmann, Moore et al. 1995), a nuclear transcription factor that is highly expressed in adipose tissue but is poorly expressed in other insulin sensitive tissues such as skeletal muscle, liver, pancreas, heart and spleen (Ferre 2004). Thus, adipose tissue is considered to be the main site of action for TZD and much of their systemic beneficial effects have hitherto been explained via their effects on adipocyte physiology and metabolism. These include accelerated adipocyte uptake, oxidation and esterification of circulating non-esterified fatty acids (NEFA), thereby reducing the ectopic lipid burden and its interference of insulin signaling in other tissues such as the skeletal muscle and liver. This is accompanied by alterations in adipokine secretion profile that can further improve control of glucose and lipid metabolism in these tissues (Guan, Li et al. 2002; Boden, Homko et al. 2005). However, adipose tissue may not be the exclusive site of action for TZD. Lipoatrophic patients have negligible adipose tissue mass, and the same is true for a mouse model of late onset lipoatrophy. Yet in both cases, TZD therapy improved diabetes and hyperlipidemia (Burant, Sreenan et al. 1997; Arioglu, Duncan-Morin et al. 2000).

In the liver, TZD have been shown to reduce the expression of gluconeogenic enzymes in animal models (Afonso, Patarrao et al. 2006) and attenuate gluconeogenic fluxes in T2D patients (Miners, Kehoe et al. 2008). In addition to being a principal control site for carbohydrate metabolism, the liver is also highly involved in regulating systemic lipid fluxes via re-esterification, lipogenesis and very low density lipoproteins (VLDL)-export. Furthermore, it plays an active role in controlling the levels of circulating insulin via first-pass clearance of secreted insulin mediated by insulin degrading enzyme (IDE) and protein disulfide isomerase (PDI) (Osei, Gaillard et al. 2007; Lamontagne, Jalbert-Arsenault et al. 2013). Recently has been suggested in humans that nitric oxide (NO) regulates insulin clearance (Natali, Ribeiro et al. 2013) moreover its mechanism might be linked to the NO inhibitory capacity of the IDE enzyme (Cordes, Bennett et al. 2009). Its relevance might be tied with lately convincing data that putts forward the observation that increases in insulin clearance is associated with

improvement of insulin sensitivity, however the mechanism behind this observation is still unclear (Ader, Stefanovski et al. 2014; Bril, Lomonaco et al. 2014).

Thus the development of steatosis and hyperinsulinemia, that in addition to glucose intolerance are defining features of the insulin resistant state, may reflect dysfunctional hepatic lipid metabolism as well as impaired hepatic insulin clearance. Thus, we hypothesized that TZD ameliorate steatosis and hyperinsulinemia through changes in hepatic lipid fluxes and insulin clearance. We tested this hypothesis by measuring the effects of a TZD (troglitazone) on hepatic lipid fluxes and insulin degradation in rodent models of short-term (14-day) high sucrose feeding. This model is useful for specifically probing splanchnic complications of diet-induced insulin resistance since steatosis and hyperinsulinemia are established before significant increases in whole-body adiposity. It is also well known that high sucrose feeding induces a substantial increase in *de novo* lipogenesis (DNL) such that this pathway becomes a significant contributor to hepatic triglyceride synthesis (Chong, Fielding et al. 2007; Richelsen 2013). Therefore we also hypothesized that the reversal of steatosis in this setting by TZD involves the attenuation of hepatic DNL.

Methods

Materials: Troglitazone was procured from Sangyo, Japan. $^{2}H_2O$ (99% enriched) was acquired from CortecNet (Voisins-Le-Bretonneux, France), sucrose from Panreac (Castellar del Vallès, Barcelona, Spain) and other reagents from Sigma Aldrich (Steinheim, Germany).

Animals: All animal protocols conformed to the NIH guide and the Institutional guidelines for the Care and Use of Laboratory Animals. The experiments were approved by the Ethics Committee of the Faculty of Medical Sciences at the New University of Lisbon. Thirty-six adult male Wistar rats were maintained in a 12 h light/12 h dark cycle (lights on from 7 am to 7 pm) with ad libitum access to food and water. Animals were randomly separated in standard chow (SC), high sucrose (HS)-fed rodents (HS-C) and troglitazone-treated HS-fed rodents, (0.2%, included in the diet) (HS-T). The animals were maintained on these diets for 14 days. Two parallel studies were conducted with 18 animals per study, 6 per diet regime.

In Study 1, hepatic DNL and VLDL export were measured. At 7 pm of day 13, all animals of study 1 received a loading dose of 99% $^{2}H_2O$ (3g/100 g body weight) and the drinking water

was also supplemented with ^2H_2O to a 3% final enrichment. Following overnight ad libitum feeding, animals were sacrificed the next morning by ketamine intraperitoneal injection (100 mg/kg body weight). Upon sacrifice, the liver and epididymal adipose tissue was excised, weighted and immediately freeze-clamped in liquid nitrogen until further analysis.

In Study 2, food was withdrawn on the last evening (day 13) and animals were fasted overnight. On the morning of day 14, rats were allowed ad libitum access to their respective diets for 120 minutes. At pre-determined intervals, plasma NEFA, glucose, insulin and C-peptide levels were quantified. Rate constants for the decrease in plasma NEFA concentrations were derived from the logarithm-transformed curves of the relative reduction in plasma NEFA concentrations from 0 to 120 min . Livers were excised and immediately freeze-clamped in liquid nitrogen until further analysis for enzyme activities, NO levels and protein expression.

Quantification of hepatic DNL: Hepatic triglycerides can be derived from plasma NEFA, which are taken up via lipoprotein transport and esterified to triglycerides after hepatic uptake. However, hepatic triglycerides can also be formed *in situ* by DNL of fatty acids from acetyl-CoA. Hepatic DNL was quantified using ^2H_2O as a tracer, as previously reported (Delgado, Silva et al. 2009; Soares, Carvalho et al. 2012). From the ^1H and ^2H NMR data, triglyceride methyl ^2H-enrichment levels were estimated and by relating these enrichments values to that of plasma water, the contribution of DNL to total hepatic triglycerides was calculated.

About half of each liver was powdered and a Folch extraction, with 20 ml chloroform:methanol (2:1) per gram of dried tissue, performed. The mixture was continuously agitated for 20 min at room temperature and then centrifuged for 5 min at 4^{o}C and 3000 rpm. The supernatant was vigorously mixed with 100 ml 0.9% (w/v) NaCl and then centrifuged for 5 min at 4^{o}C and 3000 rpm. The upper phase was discarded and the lipid-containing lower phase was recovered and evaporated to dryness. Afterwards, dried lipids were dissolved in 2 ml hexane/methyl tertiary butyl ether (MTBE) (200:3) solution for purification by solid phase extraction (SPE).

For triglyceride purification, DSC-18 SPE columns (2 g) were initially washed with 12 ml of hexane/MTBE (96:4) followed by 12 ml hexane. The lipid fraction was added to the column and further washed with 10 ml hexane/MTBE (200:3). To recover triglycerides, 12 ml hexane/MTBE (96:4) were eluted in the column and 1 ml fractions were collected. For

identification of the fractions containing triglycerides, thin layer chromatography (TLC) was performed using a mixture of petroleum ether, diethyl ether and acetic acid in the proportions of 8.0:2.0:0.1 as elutant and visualization by iodine. Finally, triglyceride fractions were combined and evaporated to dryness for NMR analysis.

For the acquisition of ^{1}H and ^{2}H NMR spectra, the purified triglyceride extract was dissolved in 300 µl of chloroform and deuterated pyrazine was used as an internal ^{2}H-enrichment standard. ^{1}H and ^{2}H NMR spectra were acquired at 25^{o}C with a 14.1 T Varian Spectrometer (Varian, Palo Alto, CA, USA) equipped with a 3-mm broadband probe. Proton-decoupled ^{2}H NMR spectra were acquired without lock. Acquisition parameters included an FID acquisition time of 5 s, a delay of 2 s and a 90^{o}pulse width. Between 500 and 1000 transients were acquired to achieve adequate signal to noise for signal area analysis. Spectra were referenced for TMS using the peaks of chloroform and pyrazine resonances, at 7.27 and 8.60 ppm, respectively. Before Fourier transformations, ^{1}H and ^{2}H NMR spectra were multiplied by 0.5 and 1.0 Hz Lorentzian functions, respectively, and signal areas determined using the signal deconvolution routine of the PC based NMR processing software NUTS proTM (Acorn, Freemont, CA). Plasma water ^{2}H-enrichments were determined from plasma by ^{2}H NMR spectroscopy analysis, as described previously . Briefly, a 10 µl volume of plasma was mixed with a known amount of acetone, and the ^{2}H-enrichments were determined using a standard curve constructed previously using $^{2}H_2O$ enrichment standards against the constant natural abundance ^{2}H signal of the acetone.

Quantification of hepatic very-low density lipoproteins (VLDL)-TG export rates: Hepatic VLDL-triglycerides export rate were determined according to Millar *et al. (Millar, Cromley et al. 2005)* . On the morning of day 14 following overnight *ad libitum* feeding, rats were given an intraperitoneal injection of poloxomer 407 (P-407) (1,000 mg/kg body weight).

Plasma triglycerides were evaluated immediately before and at pre-established time intervals after P-407 injection. Hepatic VLDL-triglycerides export rates were derived from the slope of the curves of plasma triglycerides concentrations at 0-90 min.

Biochemical assays: Plasma glucose was assessed using a standard glucometer whereas the quantitative determination of plasma insulin and C-peptide levels was achieved by means of enzyme-linked immunosorbent assay (ELISA) (Mercodia). Plasma NEFA levels were assessed

using an in vitro enzymatic colorimetric method assay (Wako Chemicals GmbH). Plasma triglycerides and hepatic and epididymal adipose tissue triglycerides were determined, following a Folch extraction of the tissue samples, by an automated clinical chemistry analyzer (Olympus AU400 Chemistry Analyzer).

Assessment of insulin clearance: After quantification of plasma insulin and C-peptide levels, insulin clearance was calculated by the ratio between area under the curve of C-peptide, a surrogate of insulin secretion, and area under the curve of plasma insulin levels for each analyzed point.

Assessment of hepatic NO levels: Liver was degraded by mechanical disruption using a piston in a buffer containing 25mM Tris-HCl (pH7.4), 1mM EDTA and 1mM EGTA. Extracts were centrifuged and supernatant denatured with ethanol. Hepatic nitric oxide levels were measured by a chemiluminescent-based technique using a Sievers 280 NO Analyzer (Sievers Instruments, USA) as previously described (Afonso, Patarrao et al. 2006).

Evaluation of hepatic nitric NO synthase (NOS), IDE and PDI activities: For NOS activity quantification a buffer composed of 25mM Tris-HCl, pH 7.4, 1mM EDTA, and 1mM EGTA was used to degrade the tissue in combination with mechanical homogenization. The resulting extracts were centrifuged for 15 min at 10000 g and 4 ºC and supernatants were measured for NOS activity using the Ultra-Sensitive Assay for Nitric Oxide Synthase kit (Oxford Biomedical Research, U.S.A.). For quantification of IDE activity, liver tissue was also degraded by mechanical homogenization in a buffer containing 170 mM NaCl and 2mM EDTA. To the homogenate, the FRET substrate Mca-GGFLRKHGQ-EDDnp was added and a fluorometric assay performed as previously described (Miners, Kehoe et al. 2008). PDI activity was measured after incubation of homogenized liver tissue for 1h at 37° with KHR buffer supplemented with 0.26 mM $CaCl_2$ and 0.2 g/L collagenase. Samples were then centrifuged at 4° and 13000rpm for 5min 2 times, with recovery of the pellet containing the hepatocytes between centrifugations and resuspension in KHR buffer. The final pellet was resuspended in lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 1:200 dilution of tablet protease cocktail inhibitor and 1% Triton X-100. The mixture was submitted to 5 bursts of low-level sonication and then centrifuged at 13000 rpm for 30 min at 4°. The supernatant was kept and PDI activity was measured at 630 nm by an optical density assay over a 60 minute period. The reaction mixture was composed of

distilled water, 10 mM potassium phosphate buffer (pH 7.4), 8 mM GSH and 1,16mg/mL insulin with a final volume of 186 uL. To this, 10 ul of the supernatant was added to each well in a multi well plate. In this assay the PDI promotes the cleavage of insulin present in the reaction mixture and the insulin degradation products cause an increase in optical density that is proportional to the units of PDI present.

Assessment of protein expression: Protein extracts for Western blot analysis were obtained using a lysis buffer (1M Tris-HCl, pH 7.5, 0.2M EGTA, 0.2M EDTA, 1% Triton-X 100, 0.1M sodium orthovanadate, 2g/L sodium fluoride, 2.2 g/L sodium pyrophosphate, 0.27M sucrose) to homogenize liver tissue. Samples were centrifuged and total protein lysates were kept at -20°. Total protein lysates from liver were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, electrotransferred on a polyvinylidene difluoride membrane, and probed with the respective antibodies: IDE (sc-27265) and PDI (sc-20132), (Santa Cruz Biotechnology, Santa Cruz, CA). Then, membranes were revealed in a ChemiDoc apparatus. Protein levels were normalized to β-actin (A5316, Sigma) for each sample.

Statistical analysis: Data are expressed as mean ± standard error mean of at least five animals per group. Statistical significance was calculated using one-way ANOVA (Bonferroni post hoc test).

Results

Baseline glycemic and lipidemic parameters for the group fed with standard-chow (SC) and the group fed with high sucrose (HS-C): Plasma NEFA and triglycerides following an overnight fast or after normal overnight feeding were similar for SC and HS-T (Table 2). Weight gain over the 2-week feeding period was not different between SC and HS-C, although daily caloric intake was significantly increased for HS-C. Total epididymal adipose tissue triglyceride content was not different between SC and HS-C (Table 2).

Table 2: Animal characteristics.

	Standard chow	High sucrose	High sucrose + troglitazone
Initial weight (g)	293 ± 18	300 ± 16	285 ± 8
2-week weight increase (%)	29 ± 3	23 ± 3	23 ± 2
Caloric intake (kcal/day)	129 ± 5	174 ± 15*	156 ± 8
Plasma NEFA (mg/dl)[1]	47 ± 7	51 ± 6	7 ± 1**,##
Plasma NEFA (mg/dl)[2]	62 ± 9	49 ± 12	57 ± 11
Plasma triglycerides (mg/dl)[1]	98 ± 5	113 ± 16	100 ± 18
Plasma insulin (μg/l)[2]	0.4 ± 0.1#	0.9 ± 0.1*	0.2 ± 0.1##
Plasma glucose (mg/dl)[1]	119 ± 5	114 ± 5	124 ± 6
Plasma glucose (mg/dl)[2]	80 ± 4	82 ± 4	85 ± 4
HOMA-IR	1.84 ± 0.62#	4.40 ± 0.71*	1.10 ± 0.22##
Total epididymal adipose tissue triglycerides (mg/g body wt) [2]	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.2

*p<0.05 and ** p<0.01 relative to the SC group; # p<0.05 and ## p<0.01 relative to the HS group. [1] Following overnight *ad libitum* feeding. [2] Following overnight fast.

However, in agreement with earlier studies (Huang, Lin et al. 2010), hepatic triglyceride levels were 3-fold higher in HS-C compared to SC (Figure 32A). Moreover, fractional DNL rates (Figure 32B) were increased approximately two-fold in HS-C-fed compared to SC-fed rats. As shown in Figure 33, postprandial export of hepatic triglyceride via VLDL showed a tendency to be increased in HS-C compared to SC (p=0.16). Also, the clearance of fasting plasma NEFA levels following a 120 minute feeding period was significantly slower for HS-C compared to SC, as shown in Figure 34.

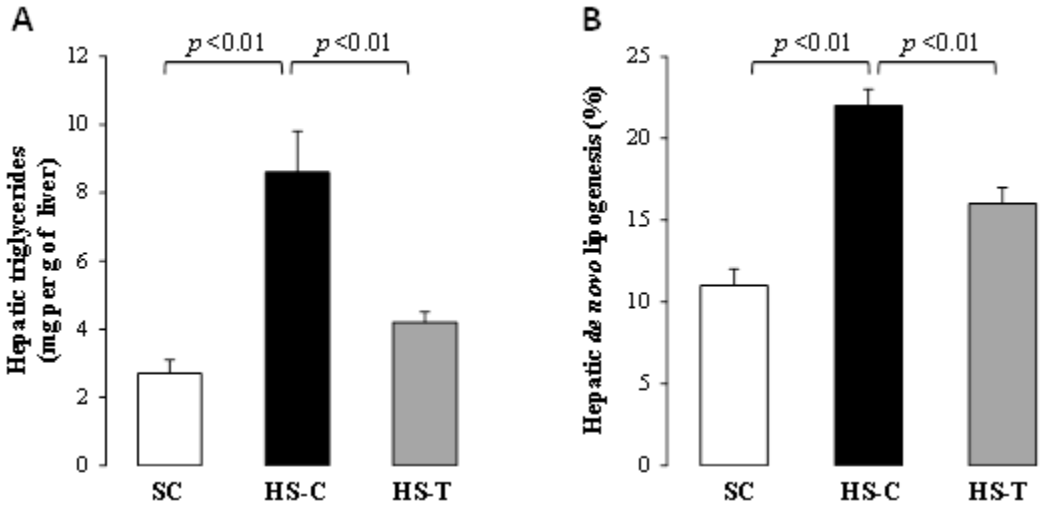

Figure 32: A. Hepatic triglycerides levels following overnight *ad libitum* feeding and; B. Contribution of hepatic *de novo* lipogenesis, quantified by using deuterated water as a tracer followed by analysis using ^{2}H Nuclear Magnetic Resonance spectroscopy, in standard-chow (SC), high-sucrose (HS-C) and in 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents.

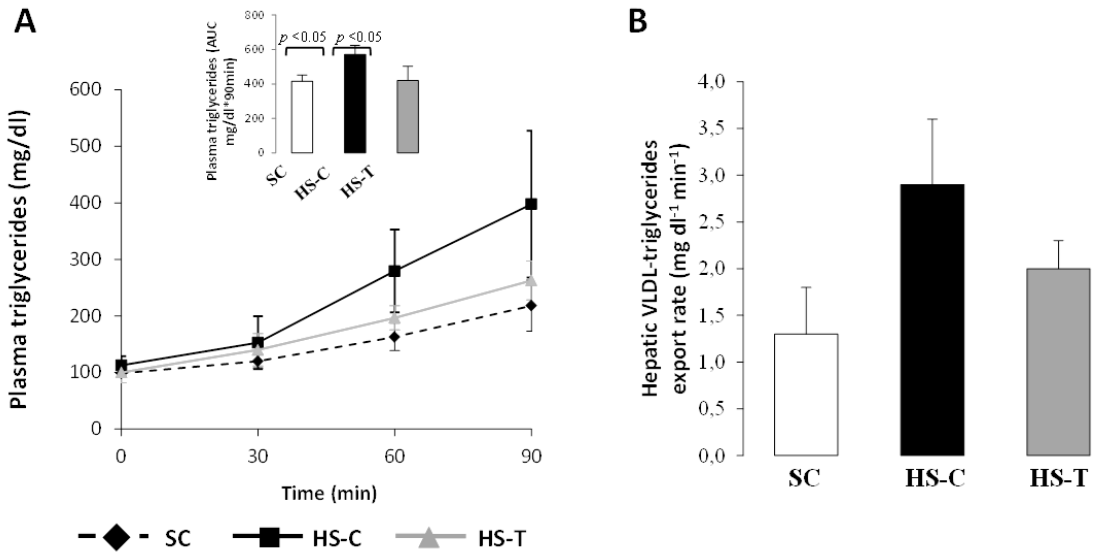

Figure 33: Quantification of very low density lipoproteins (VLDL)-triglycerides export rates following inhibition of adipose tissue lipolysis, by means of poloxomer 407 injection, after overnight *ad libitum* feeding. A. Graphical representation of the accumulation of plasma triglycerides during 90-min; and B. Average of the slopes of the curves of plasma triglycerides concentrations at 0-90 min for standard-chow (SC), high-sucrose (HS-C) and 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents.

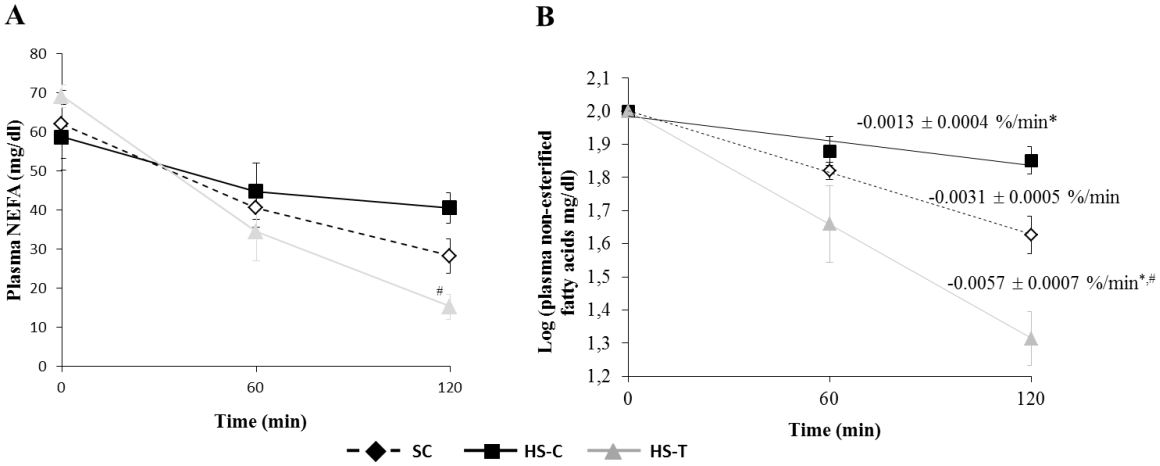

Figure 34: A. Absolute values and; B. Logarithm-transformed curves of the relative reduction in plasma NEFA concentrations from 0 to 120 min refeeding, following overnight fast, in standard-chow (SC), high-sucrose (HS-C) and in 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents. *p<0.05 relative to SC and $^{\#}p$<0.05 relative to HS-C are indicated.

Plasma glucose levels either after overnight fasting or after normal overnight feeding were not significantly different between SC and HS-C groups indicating that glycemic control was maintained with HS feeding. However, fasting plasma insulin levels were significantly higher for HS-C *versus* SC. This translates to a significantly higher HOMA-IR index (see Table 2) and indicates a compensated insulin resistant state for HS-C relative to SC. Meal-induced blood glucose, plasma insulin, C-peptide excursions and insulin clearance were not different between HS-C and SC (Figure 35).

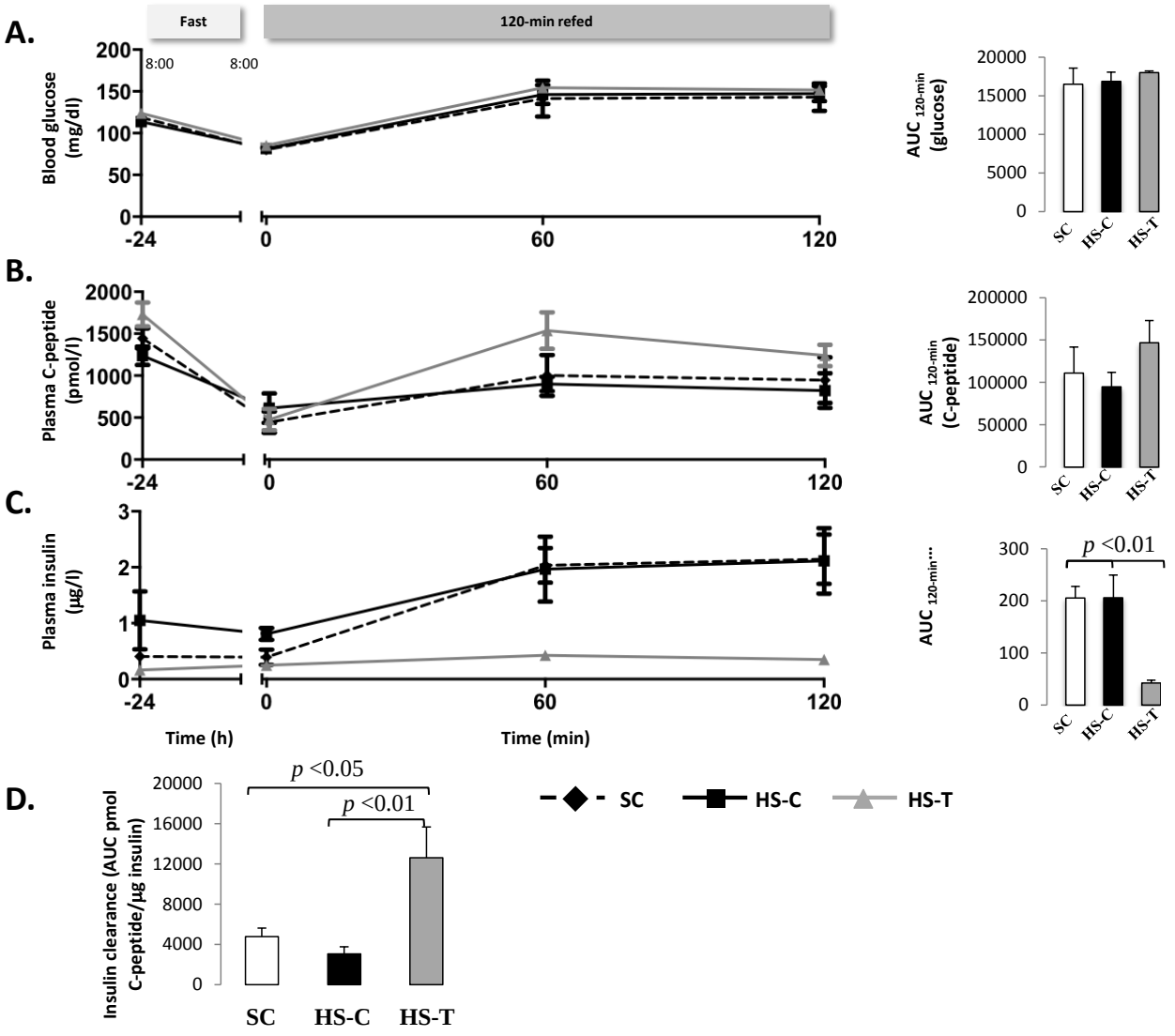

Figure 35: Following overnight fast, animals were submitted to 120-min refeeding. A. Blood glucose; B. Plasma C-peptide; and C. plasma insulin profiles and integration of the area under the curve (AUC); D. insulin clearance integration of the AUC during 120-min refeeding in standard-chow (SC), high-sucrose (HS-C) and in 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents.

Hepatic IDE activity was not different between HS-C and SC animals, but PDI activity was significantly decreased in HS-C when compared to SC animals (Figure 36A). Regarding IDE and PDI expression there were no alterations between SC and HS-C fed animals (Figure 36B).

Finally, there were no alterations in NO levels and NOS activity in HS-C fed animals at 2 weeks of sucrose feeding (Figure 37).

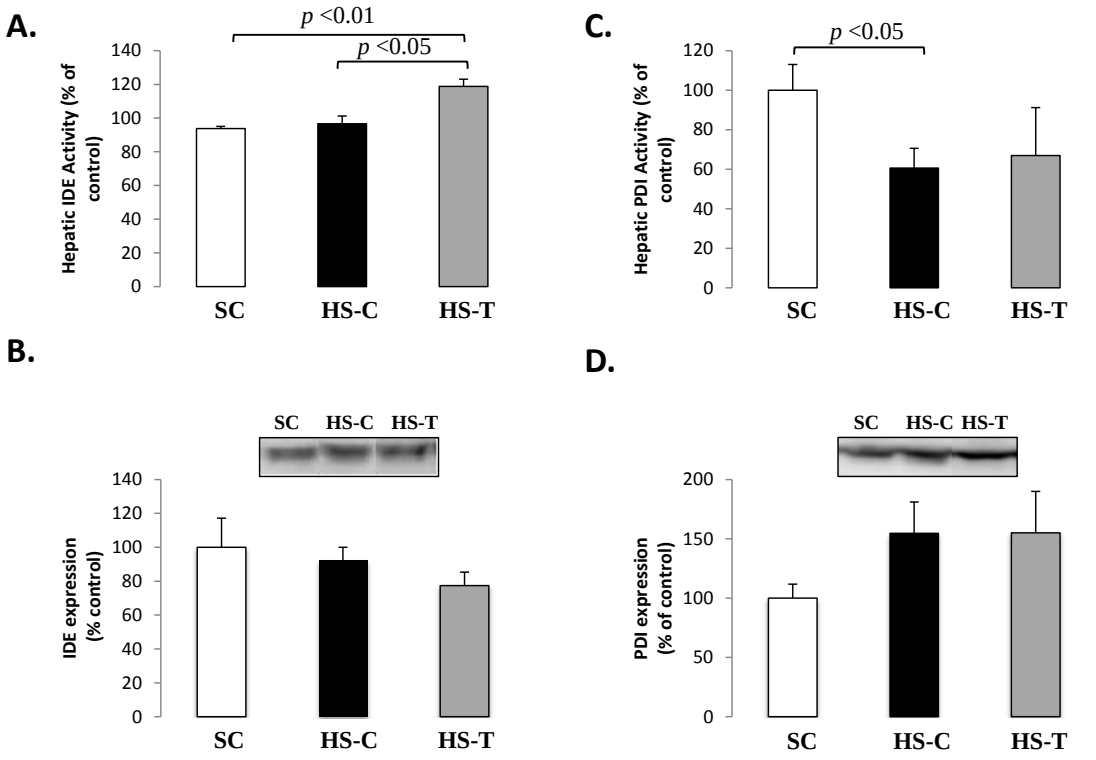

Figure 36: Quantification of hepatic enzymes activity and expression A. Hepatic insulin degrading enzyme (IDE) activity; B. Hepatic IDE expression; C. Hepatic protein disulfide isomerase (PDI) activity; and D. Hepatic PDI expression after 120-min refeeding in standard-chow (SC), high-sucrose (HS-C) and in 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents.

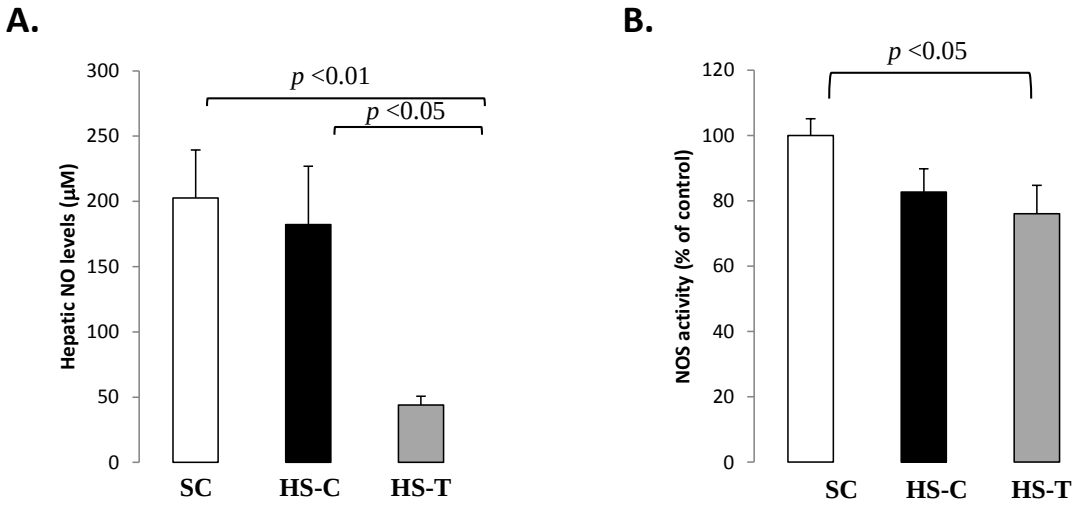

Figure 37: Evaluation of hepatic nitric oxide (NO) production; A. Hepatic NO levels; B. Hepatic NO synthase (NOS) activity after 120-min refeeding in standard-chow (SC), high-sucrose (HS-C) and in 2-weeks troglitazone-treated high-sucrose fed (HS-T) rodents.

Effects of troglitazone administration in rats fed with a high sucrose diet: For animals that were placed on a high sucrose diet and also administered with troglitazone (HS-T), neither weight gain nor caloric intake over the 2-week feeding period were different compared to HS-C or SC. Epididymal adipose tissue triglyceride content was not different compared to either SC or HS-C. However, hepatic lipid levels were significantly reduced compared to HS-C and were indistinguishable from SC. These reductions in hepatic triglyceride were associated with significant reductions in fractional DNL rates to levels that were similar to SC (Figure 32B). The rate of hepatic VLDL-triglyceride export tended to be reduced in HS-T compared to HS-C (Figure 33). HS-T had similar fasting plasma NEFA and triglycerides levels to both HS-C and SC (Table 2) suggesting that there was no significant effect of troglitazone supplementation on fasting whole-body triglyceride dynamics. However under fed conditions, HS-T had significantly lower plasma NEFA levels compared to both HS-C and SC (Table 2). Moreover, troglitazone potentiated the drop in plasma NEFA levels following the transition from fasting to feeding, with the fractional decrease in NEFA concentration exceeding both HS-C and SC groups, as shown in Figure 34.

Plasma glucose levels either after overnight fasting or after normal overnight feeding were not significantly different between HS-T and either HS-C or SC groups. However, HS-T had significantly reduced fasting plasma insulin and HOMA-IR values compared to HS-C and were similar to SC regarding insulin sensitivity (Table 2). The HS-T group had a pattern of plasma glucose excursion following a meal that was identical to HS-C and SC groups. While meal-induced C-peptide profiles were not significantly different between the three groups, plasma insulin levels were significantly lower in HS-T compared to either HS-C or SC (Figure 35). These data indicate that the reduced insulin levels were due to increased hepatic insulin clearance rather than reduced pancreatic insulin secretion (Figure 35D).

The increase in insulin clearance correlates with an increase in hepatic IDE activity for HS-T (Figure 36). Regarding PDI activity, HS-T animals showed a tendency to restore the decrease seen in HS-C animals (Figure 36). IDE and PDI expression were not altered in HS-T animals (Figure 36). The NO, an inhibitor of IDE activity, was significantly diminished in HS-T group and this was associated to a decrease in NOS activity. Therefore the observed increase in insulin clearance can be linked to decreased hepatic NO levels, that in turn were associated with increased IDE activity (Cordes, Bennett et al. 2009) (Figure 37).

Discussion

TZD are widely used pharmacological agents that improve several aspects of insulin resistance including fatty liver and hyperinsulinemia (Belfort, Harrison et al. 2006; Ratziu and Poynard 2006). The purpose of the current study was to better understand the underlying hepatic mechanisms of these actions in the setting of sucrose-induced hepatic insulin resistance. Our data show that troglitazone prevented the development of fatty liver following 2 weeks of high sucrose feeding in rat models. Kawaguchi *et al.* previously showed that pioglitazone protected against steatosis in a 2-week choline-deficient animal model (Kawaguchi, Sakaida et al. 2004).

Our results demonstrate that: 1) troglitazone prevents the development of fatty liver in 2-week HS-fed rodents not only by reducing plasma NEFA, (presumably by improved adipocyte NEFA uptake) but also by decreasing hepatic DNL; 2) these anti-lipogenic actions are accompanied by decreased plasma insulin levels due to increased hepatic insulin clearance with no change in insulin secretion; 3) increased insulin clearance is associated with elevated hepatic IDE activity; and 4), elevated IDE activity is explained by a fall in levels for two of its endogenous inhibitors: NO and NEFA.

If the observed decrease in plasma NEFA levels is due to a more efficient uptake and storage in adipocytes, this is expected to result in an increased adipose tissue mass. While for our study, the 2-week TZD-therapy did not significantly augment epididymal adipose tissue mass, a 4-week rosiglitazone-based therapy in Zucker lean and fatty rats, significantly increased the total weight of epididymal fat pads (Sotiropoulos, Clermont et al. 2006). Therefore, it is possible that for over a longer period of HS feeding with troglitazone administration, epididymal fat mass would have also shown an increase over untreated HS-fed animals.

The fatty acyl sources of hepatic triglycerides include plasma NEFA released from adipocytes during fasting, dietary fat intake during feeding, and hepatic DNL. Although TZD are well known to stimulate DNL in adipocytes, their effects on hepatic lipogenesis are more controversial. Here, we showed that troglitazone therapy was associated with a decrease in fractional DNL for HS-fed rodents. Likewise, pioglitazone reduced steatosis in a diet-induced steatohepatitis animal model through suppression of liver lipogenic gene expression, including sterol regulatory element-binding protein-1c and fatty acid synthase (Ota, Takamura et al.

2007). On the other hand, prolonged rosiglitazone- and troglitazone-based therapy in obese KKA^{γ} mice, expressing elevated hepatic PPARγ was associated with a worsening of steatosis and activation of lipogenic genes and DNL (Bedoucha, Atzpodien et al. 2001). This suggests that administration of TZD when hepatic expression of PPARγ is constitutively high may promote rather than curtail hepatic steatosis.

Recently, Beysen *et al.* demonstrated that rosiglitazone and pioglitazone had different effects on hepatic DNL rates in T2D subjects (Beysen, Murphy et al. 2008). A possible explanation is that the actions of a given TZD may be further potentiated by cross-reactivity with PPARα, whose agonists are known to increase hepatic fatty acid oxidation activity and reduce hepatic lipogenic fluxes (Tenenbaum and Fisman 2012). Moreover, in addition to functioning as PPAR agonists, TZD may also differentially modify PPAR α and γ expression. Thus troglitazone, an agonist for both PPARα and PPARγ, was also found to increase PPARα expression while reducing PPARγ expression in mononuclear cells of obese subjects (Aljada, Ghanim et al. 2001). In the liver, where constitutive PPARα levels are much higher than those of PPARγ, the anti-lipogenic effects of troglitazone are best explained through its promotion and stimulation of PPARα.

TZD therapy is also associated with a reduction in peripheral serum insulin levels that accompanies an improvement in insulin sensitivity (Osei, Gaillard et al. 2004; Kim, Abbasi et al. 2005). However, the mechanism that accounts for the reduced circulating insulin levels remains uncertain. After insulin is secreted into the portal vein, a significant and variable fraction (40-70%) is immediately cleared by the liver, this fraction being sensitive to physiological and nutritional parameters that also inform insulin sensitivity (Radziuk and Morishima 1985). Hence, plasma insulin levels reflect both β-cell secretion as well as hepatic insulin clearance. Insulin clearance is initiated by the binding of insulin to its hepatic receptor. Internalization of the bound insulin complex is critical not only for insulin clearance but also for hepatic insulin actions. It has been suggested that 80% of secreted insulin binds to liver receptors. After binding, insulin can be either fully degraded or be returned to the circulation either intact or partially degraded. Insulin clearance is primarily mediated by IDE and PDI (Duckworth, Bennett et al. 1998). In troglitazone-treated HS-fed rodents, β-cell insulin secretion, quantified using plasma C-peptide levels as a surrogate (Polonsky, Pugh et al. 1984; Polonsky and Rubenstein 1984), was not altered. Hence, the observed decrease in plasma insulin levels in troglitazone-treated rodents is explained by increased hepatic insulin

clearance. In agreement with our study, rosiglitazone therapy increased hepatic insulin clearance in nondiabetic insulin-resistant, impaired glucose tolerant and T2D subjects (Kim, Abbasi et al. 2005; Osei, Gaillard et al. 2007).

Both glucose and NEFA are regulators of IDE activity (Hamel 2009; Pivovarova, Gogebakan et al. 2009). Since postprandial glucose excursions were very similar between the three groups the differential effects of glucose on insulin clearance between the groups were likely to be insignificant. NEFA, besides inhibiting insulin binding and actions, may also inhibit its degradation via IDE (Hamel 2009). Accordingly, an acute twofold increase of NEFA in lean subjects promoted a pronounced reduction in insulin clearance independently of glucose levels (Hennes, Dua et al. 1997; Xiao, Giacca et al. 2006) Thus, the decrease in plasma NEFA levels mediated by TZD relieves the inhibition of insulin degradation thereby boosting hepatic insulin clearance.

Previously, Wei and coworkers have shown that another TZD drug, pioglitazone, increased insulin clearance in a model of diet induced obesity (Wei, Ke et al. 2014). However, in this work their animal model had an increment in insulin clearance dependent on the diet treatment and independent on the TZD treatment, which it is contrary to the expected and to all the previous works that they refer on their discussion. Moreover, they assumed in the *in vitro* studies that the effects of pioglitazone, FFAs, glucagon and TNF-α on IDE mRNA levels could represent effects on this enzyme activity, which is known to be incorrect (Wei, Ke et al. 2014).

NO has also been shown to be a regulator of IDE activity. Cordes and colleagues showed that NO directly binds to IDE leading to it inhibition (Cordes, Bennett et al. 2009). We showed that TZD treatment resulted in a significant decrease of hepatic NO levels and NOS activity. Previous studies demonstrated that pioglitazone administration led to a decrease in inducible NOS activation in obese T2D subjects (Esterson, Zhang et al. 2013). These specific effects were only observed in T2D patients, where iNOS activity is elevated compared to healthy subjects.

While IDE is considered to be the primary enzyme for hepatic insulin clearance, PDI, which is also expressed by the liver, can also degrade internalized insulin. Our data indicate that hepatic PDI activity and expression were not correlated with either IDE activity or insulin clearance. Nevertheless, it is possible that PDI may have a role in insulin degradation that is downstream or complementary to that of IDE.

In conclusion our data demonstrate that troglitazone protects against fatty liver induced by a short-term period of high sucrose feeding via several concerted mechanisms involving both adipose tissue and liver. In addition to the canonical mechanism of increased adipocyte NEFA uptake and esterification thereby reducing plasma NEFA, we also demonstrated an inhibition of hepatic DNL and also reduced hepatic NO levels and NOS expression. The reduction in hepatic lipid and NO, both of which are inhibitors of IDE, were correlated with an increase in IDE activity and hepatic insulin clearance. This increased insulin clearance explained the maintenance of normal plasma insulin levels concomitant with the preservation of insulin sensitivity. These novel hepatic actions of TZD offer more precise therapeutic strategies for reversing the onset of non-alcoholic fatty liver disease and countering hyperinsulinemia in T2D subjects.

Acknowledgements

The authors acknowledge financial support from the Portuguese Foundation for Science and Technology (research grants PTDC/EEB-BIO/99810/2008 and PTDC-SAU-MET-111398-2009). The NMR spectrometers are part of the National NMR Network and were purchased in the framework of the National Programme for Scientific re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and the Portuguese Foundation for Science and Technology. TCD and FOM held a fellowship from the Fundação para a Ciência e Tecnologia, Portugal (SFRH/BPD/46197/2008 and SFRH/BD/51194/2010, respectively).

SUB-CHAPTER 4.4. - DISPOSITION OF [U-$^{2}H_{7}$] GLUCOSE INTO HEPATIC GLYCOGEN IN RAT AND IN SEABASS

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Published on: Comparative Biochemistry and Physiology-Part A, 166, 316-322, 2013

Abstract

The stimulation of hepatic glycogenesis is an ubiquitous response to a glucose challenge and quantifying its contribution to glucose uptake informs its role in restoring euglycemia. Glycogenesis can be quantified with labeled water provided that exchange of glucose-6-phosphate hydrogen 2 (G6P-H2) and body water via glucose-6-phosphate isomerase, and exchange of positions 4, 5 and 6 hydrogens (G6P-H456) via transaldolase, are known. These exchanges were quantified in 24-hour fasted rats (n=6) and 21-day fasted seabass (n=8) by administration of a glucose load (2000 mg.kg^{-1}) enriched with [U-$^{2}H_{7}$]glucose and by quantifying hepatic glycogen ^{2}H-enrichments after 2 hours (rats) and 48 hours (seabass). Direct pathway contributions of the glucose load to glycogenesis were also estimated. G6P-H2 and body water exchange was 61±1% for rat and 47±3% for seabass.

Transaldolase-mediated exchange of G6P-H456 was 5±1% for rat and 10±1% for seabass. Conversion of the glucose load to hepatic glycogen was significant in seabass (249 ± 54 mg.kg^{-1}) but negligible in rats (12 ± 1 mg.kg^{-1}). Preload plasma glucose levels were similar for seabass and rats (3.3 ± 0.7 and 4.4 ± 0.1 mMol.L^{-1}, respectively) but post-load plasma glucose was significantly higher in Seabass compared to rats (14.6 ± 1.8 *versus* 5.8 ± 0.3 mMol.L^{-1}, p<0.01). In conclusion, G6P-H2 and body water exchange is incomplete for both species and has to be accounted for in estimating hepatic glycogen synthesis and direct pathway activities with labeled water tracers.

Transaldolase-mediated exchange is insignificant. Hepatic direct pathway glycogenesis plays a prominent role in seabass glucose load disposal but a negligible role in the rat.

Keywords: Direct pathway, transaldolase, glycogenesis, gluconeogenesis.

Introduction

The clearance of a glucose load is used to assess the capacity of an organism to control plasma glucose levels and measurements of hepatic glycogen synthesis under these conditions inform the role of hepatic glycogenesis in glucose homeostasis. Alterations in net hepatic glycogen synthesis rates can reveal hormonal or cell signaling defects in glycemic control, for example defective insulin secretion in Type 1 Diabetes (Bischof, Krssak et al. 2001) or impaired insulin signaling in Type 2 Diabetes (Torres, Fujimoto et al. 2011). These measurements also provide insight into the importance of hepatic glycogenesis relative to other mechanisms of glucose disposal for dissipating postprandial hyperglycemia. Compared to mammals, carnivorous fish such as seabass (DicentrarchuslabraxL.) have poor control of plasma glucose levels and are slow to clear a glucose load (Peres, Goncalves et al. 1999; Enes, Peres et al. 2011). However, it is unclear if this reflects a limited capacity for hepatic glycogen synthesis from glucose, a limited uptake of glucose by other tissues, or both. Hepatic glycogen can be synthesized from glucose via the so called “direct pathway” or from gluconeogenic precursors by the indirect pathway (Newgard, Hirsch et al. 1983), as depicted in Fig. 38. Indirect pathway precursors can be derived from glucose metabolism (i.e. lactate) or from other sources, for example glycerol from triglyceride hydrolysis or dietary amino acids. Carnivorous fish such as seabass that are fed with a high protein/low carbohydrate diet synthesize the bulk of liver glycogen via the indirect pathway (Viegas, Rito et al. 2012). In contrast, humans and rodents typically synthesize the majority of glycogen via the direct pathway reflecting a higher proportion of dietary carbohydrate (Jones, Fagulha et al. 2006; Delgado, Silva et al. 2009; Soares, Viega et al. 2009; Soares, Carvalho et al. 2012). Alterations in direct and indirect pathway activities can reflect changes in hepatic insulin actions, such as in insulin-dependent diabetes (Bischof, Bernroider et al. 2002) as well as adaptation to different dietary regimes (Obeid, Bittar et al. 2005; Obeid, Boukarim et al. 2006). Deuterated water (^2H_2O) is an inexpensive and versatile tracer for the study of hepatic glycogen synthesis under both natural and artificial feeding conditions (Delgado, Silva et al. 2009; Soares, Viega et al. 2009; Barosa, Silva et al. 2012; Soares, Carvalho et al. 2012). Analysis of glycogen positional ^2H-enrichments provides estimates of the fractional synthetic rate as well as direct and indirect pathway contributions to glycogen synthesis (Soares, Viega et al. 2009; Soares, Carvalho et al. 2010). These estimates rely on two key assumptions concerning exchange of metabolite and water hydrogens. First, the position 2 hydrogen of glucose-6-phosphate (G6P) is fully exchanged with body water such that the enrichment of hepatic G6P position 2 is equivalent to that of body

water. When this assumption holds, the fractional synthetic rate of glycogen from G6P can be calculated from the ratio of glycogen position 2 to body water ^{2}H-enrichments (Soares, Viega et al. 2009). To the extent that exchange between position 2 and water is incomplete, the fractional synthetic rate is underestimated (Soares, Carvalho et al. 2010). Second, the position 5 hydrogen of G6P is assumed to be enriched by indirect pathway activity. When this assumption holds, the fractional contribution of the indirect pathway can be calculated from the ratio of glycogen position 5 to body water enrichment.

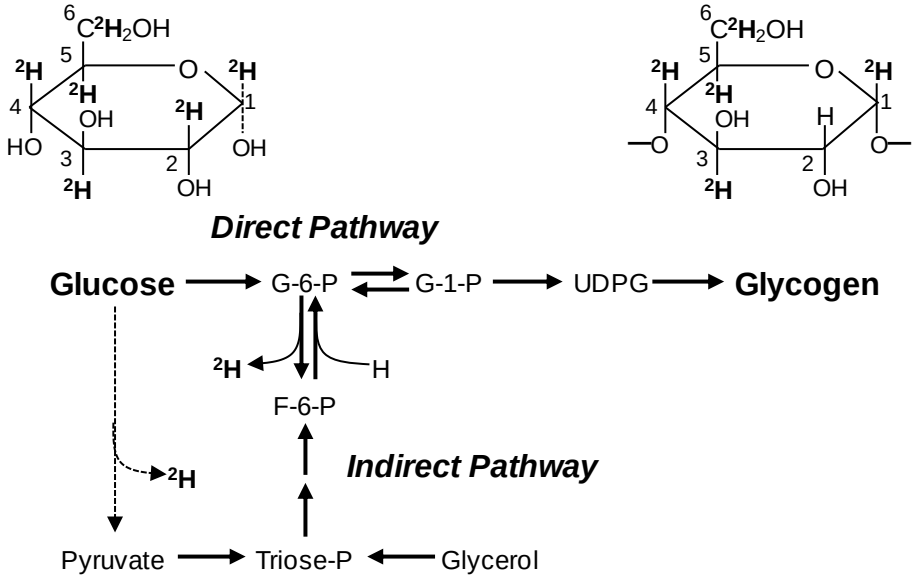

Figure 38: Depiction of direct and indirect pathways and deuterium/hydrogen exchanges during conversion of [U-$^2H_7$] glucose to glycogen. As a result of interconversion of glucose-6-phosphate and fructose-6-phosphate, the deuterium (^{2}H) of G6P position 2 is exchanged with a proton (H) from water. Some metabolic intermediates are omitted for clarity. F-6-P = fructose-6-phosphate, G-6-P = glucose-6-phosphate, G-1-P = glucose-1-phosphate, Triose-P = triose phosphates, UDPG = uridinediphosphoglucose.

To the extent that G6P position 5 is enriched by processes other than indirect pathway flux, such as transaldolase exchange (Delgado, Silva et al. 2009), the indirect pathway contribution is overestimated and that of the direct pathway is correspondingly under evaluated.

These assumptions have been tested in humans given an oral glucose tolerance test (OGTT) as well as a regular breakfast meal by monitoring the fate of the deuterium labels of [U-$^2H_7$]glucose following its conversion to UDP-glucose, sampled as urinary glucuronide.

Analysis of UDP-glucose ^{2}H-enrichment revealed that a), exchange of G6P position 2 with body water was essentially complete (Delgado, Silva et al. 2009; Barosa, Silva et al. 2012) and b), ~15–20% of G6P derived from the direct pathway underwent transaldolase exchange activity, resulting in a modest overestimation of indirect pathway and underestimation of direct pathway contributions (Delgado, Silva et al. 2009; Barosa, Silva et al. 2012).

The goal of this study was to apply the same methodology to quantify hepatic glycogenic G6P–fructose-6-phosphate (F6P) and transaldolase exchanges in rats and seabass following a glucose challenge. Aside from the interest of interspecies comparison of these parameters, these studies are also important for the following reasons. The rat is widely used as a small animal model of diabetes and liver carbohydrate metabolism, hence there is widespread interest in studying hepatic glycogen fluxes in this animal, (Soares, Carvalho et al. 2012). The seabass is a widely cultivated carnivorous saltwater fish and there is currently high interest in studying its capacity to utilize carbohydrate-based substrates as a cheaper and more sustainable alternative to fishmeal-based diets. The disposal of glucose into hepatic glycogen is a widely used parameter for evaluating carbohydrate utilization in this and other carnivorous fish species (Enes, Peres et al. 2011).

Methods

Fish handling and sampling

For the present study, 8 European seabass (D. labraxL.) provided by a local farm were transported to the lab and acclimated at 20 °C and 30‰ salinity in a 200 L tank supplied with well aerated filtered seawater from a recirculation system equipped with a central filtering unit and UV unit. Tank water temperature, salinity, pH, and dissolved oxygen were continuously monitored and NH_4^+, $NO^-$and NO^{2-}were assessed every 7 days and maintained within optimal ranges. During acclimation, fish were provided with food until satiated once a day (Dourasoja Ultra 5, SORGAL, S.A.; 44% protein, 18% fat, 2.2% carbohydrates, 9.2% ash, 5 mm standard pellet). Following acclimation, the fish were fasted for 21 days. At the end of this period, fish were anesthetized in a 30 L tank of saltwater containing 0.1 $g.L^{-1}$ of MS-222 for approximately 2 min. Fish were measured (21 ± 0 cm), weighed (88 ± 7 g) and sampled for blood from the caudal vein with heparinized syringes. Blood was kept on ice, centrifuged (3000 g for 10 min) to separate plasma and stored at −20 ºC for posterior assessment of ^{2}H-

enrichment of body water and glucose quantification with a commercial assay kit (Invitrogen, Spain). Fish were then injected intraperitoneally (10 mL.kg^{-1}) with saline solution with 200 mg/mL of glucose enriched to 49% with [U-$^2H_7$]glucose for a glucose load of 2000 mg·kg^{-1}. Fish were allowed to recover in the tank for 48 h after which were once again anesthetized, measured, weighed and sampled for blood as previously described. This time, the maximum possible volume of blood was withdrawn. Fish were then sacrificed by cervical section; livers were excised, weighed, freeze-clamped in liquid N_2 and stored at −80 °C until further analysis. Experimental procedures complied with the Guidelines of the European Union Council (86/609/EU).

Rat studies

Six male Wistar rats (Rattus norvegicus) at 8 weeks of age were studied. Animal handling followed the ethical proceedings for scientific experiments of Institutional Animal Care and Use Committee. Animals were maintained in a 12 h dark–light cycle (19:00/07:00) with ad libitum access to food and water. After 24 h of fasting, awake animals were submitted to an intraperitoneal glucose tolerance test (2000 mg·kg^{-1}), using 98% [U-$^2H_7$]glucose. Glucose was monitored from tail tip samples 15 min before the load and at 120 min after the load, using a One-Touch Vita glucometer (LifeScan). At this point animals were sacrificed by cervical dislocation and the abdominal cavity was opened. Blood was collected from the vena cava and immediately centrifuged (1000 g for 5 min) to obtain plasma samples, which were stored at −20 °C for later assessment of ^2H-enrichment of body water. Livers were excised, weighed, freeze-clamped in liquid N_2 and stored at −80 °C until further analysis.

Blood glucose and liver glycogen extraction

Plasma was mixed with 0.3 N $ZnSO_4$ and 0.3 N $Ba(OH)_2$ (1.5 mL of each solution per mL of blood) and protein was removed by centrifugation (3500 g for 15 min.). The supernatant was desalted by passage through sequential columns containing Dowex-50-H^+ and Amberlite, then lyophilized and stored at −20 °C. Glycogen was extracted by alcoholic precipitation after alkaline tissue hydrolysis as previously described (Soares, Viega et al. 2009). Briefly, frozen liver powder was treated with 30% KOH (2 mL per gram of liver) at 70 °C for 30 min. After vigorous vortex, the mixture was treated with 6% Na_2SO_4 (1 mL per g of liver) and 99.9% ethanol (to a

final concentration of 70%, 7 mL per gram of liver) and left overnight at 4 °C to precipitate glycogen. After centrifugation, the upper liquid phase was discarded and the solid residue was dried. The residue was resuspended in 5 mL acetate buffer (0.05 M pH = 4.5) and 20 µL of an aqueous solution containing 16 U of amyloglucosidase from Aspergillusniger (glucose-free preparation, Sigma-Aldrich, Germany) was added to hydrolyze glycogen to its glucosyl units. Samples were incubated overnight at 55 °C and centrifuged. The supernatant was collected and a 100 µL aliquot was stored separately so glucose derived from glycogen enzymatic hydrolysis could be quantified with standard assay kit (Invitrogen, Spain). The remainder was lyophilized.

Derivatization of glucose to monoacetone glucose (MAG)

Each lyophilized extract was vigorously mixed with 5 mL acetone containing 4% sulphuric acid (v/v), both enriched with deuterium to 2%. Anhydrous ^{2}H-enriched acetone was prepared as previously described (Jones, Barosa et al. 2006; Nunes and Jones 2009). The mixture was stirred overnight at room temperature to yield diacetone glucose. The acetonation reaction was quenched by adding 5 mL of water (also enriched with deuterium to 2%) and the pH was adjusted to 8 with 1 M $NaHCO_3$ followed by acidification of the solution to pH 2.0 with 1 M HCl. The newly formed diacetone glucose was hydrolyzed to MAG by incubation at 40 °C for 5 h. The solution pH was then increased again to 8.0 with 1 M $NaHCO_3$ and the samples were dried by rotary evaporation under vacuum. MAG in the residue was extracted with 4 mL of boiling ethyl acetate. Following evaporation of ethyl acetate, the residue was dissolved in 0.6 mL mixture of 1:10 water/acetonitrile (v/v) buffered with $NaHCO_3$ (9 mg·mL^{-1}) for ^{2}H NMR analysis.

^{2}H NMR analysis

Proton-decoupled ^{2}H NMR spectra were acquired at a temperature of 50 °C with a Varian VNMRS 600 MHz NMR (Agilent, SantaClara, CA, USA) spectrometer equipped with a 3 mm broadband probe. Acquisition parameters included a 90° hard pulse angle, a sweep width of 10 ppm, 1.6 s acquisition time, 0.1 s pulse delay and continuous WALTZ-16 broadband ^{1}H decoupling. Up to 2000 scans were collected per sample, corresponding to ~1 h of collection

time. The summed free induction decays were processed with 0.5–1.0 Hz line-broadening before Fourier transform. MAG ^{2}H-enrichments were quantified from the ^{2}H NMR spectra by measuring the intensity of each signal of the MAG hexose moiety relative to the mean intensity of the two intramolecular methyl reference signals at 1.28 and 1.40 ppm (Nunes and Jones 2009). Spectra were analyzed with the NUTS PC-based NMR spectral analysis software (Acorn NMR Inc., USA).

Quantification of deuterium–hydrogen exchanges and glycogen synthesis pathway contributions:

- **Quantifying G6P–F6P exchange with [U-$^2H_7$] glucose**

When [U-$^2H_7$] glucose is metabolized to glycogen via the direct pathway, the position 3 deuterium (H3) is fully retained, while the position 2 deuterium (H2) is exchanged for a solvent proton during exchange of G6P and F6P. Hence, the ratio of glycogen H2 to H3 informs the fraction of direct pathway G6P that exchanged with F6P as follows:

$$\text{Percent of direct pathway G6P exchanged with F6P} = 100 \times (1 - H2/H3) \quad (1)$$

- **Quantification of transaldolase exchange activity from analysis of glycogen and glucose ^{2}H-enrichments**

Transaldolase (TA; EC 2.2.1.2) exchange catalyzes the exchange of the 4, 5 and 6 carbon fragments of F6P and glyceraldehyde-3-phosphate (G3P) independently of oxidative pentose phosphate pathway flux (see Fig. 1). Thus, TA exchange activity results in a loss of ^{2}H-enrichment in positions 4, 5 and 6 relative to position 3 hence the mean enrichment of these hydrogens relative to that of position 3 ($H456_m$/H3) reflects the fraction of G6P that participated in TA exchange.

$$\text{Percent of G6P participating in TA exchange} = 100 \times (1 - H456_m/H3) \quad (2)$$

- **Quantification of glucose load contribution to hepatic glycogen synthesis via the direct pathway**

When [U-$^2H_7$]glucose is metabolized through the indirect pathway, the ^2H-label in position 3 is assumed to be quantitatively removed following conversion of [U-$^2H_7$]glucose-6-phosphate to triose phosphates and pyruvate. Hence, glycogen that is subsequently synthesized via indirect pathway is not enriched with ^2H in this position. Meanwhile, H3 is retained during direct pathway metabolism of [U-$^2H_7$]glucose to glycogen. Therefore, the direct pathway contribution of the load to hepatic glycogen synthesis can be estimated as the ratio of glycogen H3 to load glucose H3 ($H3_{GLY}/H3_{GLC}$):

$$\text{Percent of glycogen derived from the load via direct pathway} = 100 \times H3_{GLY}/H3_{GLC} \quad (3)$$

- **Estimation of whole-body glucose pool size**

For rats, the glucose pool size before and after the load was estimated from the product of the plasma glucose levels, (expressed as mg/100 g) and 0.25 times the body mass, where 0.25 is the reported glucose space for rats (Smadja, Morin et al. 1988). For seabass, the same calculation was performed but with a glucose space of 0.13 (Garin, Rombaut et al. 1987).

- **Load glucose contribution to hepatic glycogen synthesis**

The amount of hepatic glycogen derived from the glucose load via the direct pathway was estimated as the product of the hepatic glycogen level and fractional direct pathway contribution to glycogen synthesis. Values are expressed as $mg \cdot kg^{-1}$ body mass. The fraction of the glucose load that was converted to hepatic glycogen via the direct pathway was estimated as the ratio of hepatic glycogen synthesized from the load via the direct pathway to the quantity of the glucose load.

Statistical analysis

Values are presented as mean ± standard error of the mean (S.E.M.). Analysis of variance (ANOVA) was used to test the existence of significant differences between basal and post-load states for various parameters. Student's two-tailed unpaired t-test was used to compare means between rat and seabass parameters. Differences were considered statistically significant at $p < 0.05$.

Results

For rats, the glucose load resulted in a transient doubling of plasma glucose levels by 30 minutes (data not shown) followed by a return towards basal values by the sampling period of 120 minutes, as shown in Table 3. For the seabass, plasma glucose levels remained significantly elevated at 48 hours after the injection and are characteristic of the inferior glucose tolerance of carnivorous marine fish compared to mammals. Plasma glucose ^{2}H-enrichments at the endpoints are shown in Table 4.

Table 3: Characteristics of glucose dosage and plasma glucose excursion in fasted rats and seabass following an intraperitoneal glucose load.

Parameter	Rat	Seabass
Mass	358 ± 5 g	88 ± 7 g
Fasting interval	24 h	21 days
Glucose load	2000 mg·kg^{-1}	2000 mg·kg^{-1}
Basal plasma glucose	4.4 ± 0.1 mmol·L^{-1}	3.3 ± 0.7 mmol·L^{-1} ^{ns}
Endpoint plasma glucose	5.8 ± 0.3 mmol·L^{-1}	14.6 ± 1.8 mmol·L^{-1} **
Basal plasma glucose pool size	198 ± 5 mg·kg^{-1}	79 ± 15 mg·kg^{-1} ***
Endpoint plasma glucose pool size	262 ± 12 mg·kg^{-1}	342 ± 41 mg·kg^{-1} ^{ns}
Endpoint liver glycogen pool size	108 ± 18 mg·kg^{-1}	659 ± 56 mg·kg^{-1} ***
Load disposed into liver glycogen via direct pathway	12 ± 1 mg·kg^{-1}	249 ± 54 mg·kg^{-1} ***

Mean values ± S.E.M. are presented. Differences from correspondent value in rats are indicated by asterisks (*t*-test, $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

Table 4: Blood glucose and liver glycogen ^{2}H-enrichments, in percentage, following administration of a glucose load enriched with [U-$^2H_7$]glucose to a group of rats and a group of seabass. Metabolite enrichments are normalized for differences in [U-$^2H_7$]glucose load enrichments between the different groups.

Metabolite	Species	Glucose and glycogen positional ^{2}H-enrichments						
		1	2	3	4	5	6_R	6_S
Blood glucose	Rat (n = 6)	$16.8 \pm 0.5^{a,c}$	11.9 ± 0.5^{b}	18.5 ± 0.4^{c}	$18.1 \pm 0.2^{a,c}$	16.8 ± 0.3^{a}	$18.9 \pm 0.3^{a,c}$	$17.5 \pm 0.6^{a,c}$
	Seabass (n = 8)	$41.9 \pm 2.7^{a,***}$	$22.3 \pm 2.2^{b,**}$	$50.1 \pm 2.8^{a,***}$	$42.1 \pm 2.5^{a,***}$	$40.7 \pm 2.7^{a,***}$	$47.9 \pm 2.7^{a,***}$	$45.7 \pm 2.6^{a,***}$
Liver glycogen	Rat (n = 6)	10.2 ± 1.1^{a}	4.4 ± 0.5^{b}	11.5 ± 1.2^{a}	10.6 ± 1.1^{a}	10.5 ± 1.1^{a}	11.4 ± 1.1^{a}	11.2 ± 1.2^{a}
	Seabass (n = 8)	$30.4 \pm 5.0^{a,**}$	$18.3 \pm 3.5^{b,**}$	$33.7 \pm 5.2^{a,**}$	$29.4 \pm 4.8^{a,**}$	$29.0 \pm 4.8^{a,**}$	$32.2 \pm 4.8^{a,**}$	$31.2 \pm 4.9^{a,**}$

Mean values ± S.E.M. are presented. Within each set of blood glucose and liver glycogen enrichments, values not sharing a common superscript letter are significantly different from each other (one-way ANOVA followed by Tukey test, $p < 0.0001$ for seabass blood glucose; $p < 0.05$ for rat and seabass liver glycogen). Differences from correspondent value in rats are indicated by asterisks (t-test, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.0001$).

For rats, the enrichment of glucose position 3 was ~25% that of the load, indicating that approximately one-quarter of plasma glucose at 120 minutes was derived from the load. The remaining fraction was accounted for by endogenous production and/or pre-existing unlabeled glucose. The difference between basal and postload whole body glucose pool was ~100 mg/kg, equivalent to ~5% of the glucose load. For the seabass, plasma glucose position 3 enrichment was significantly higher and indicated that about one-half of the plasma glucose was derived from the load (see Table 5). The change in whole body glucose pool size between pre- and postload was ~260 mg/kg, equivalent to ~13% of the glucose load. For both rat and seabass, the position 2 enrichment of plasma glucose was significantly depleted compared to all other sites. This indicates futile cycling activity between plasma glucose and hepatic G6P, where the deuterium in position 2 is exchanged with unlabeled water hydrogen as a result of G6P-F6P interconversion, while the other deuterium labels remain bound to the hexose skeleton. There was no significant depletion of position 4, 5 and 6 enrichments relative to position 3 indicating that the plasma glucose pool had not undergone any significant transaldolase exchange activity.

Table 5: Contribution of the glucose load, in percentage, to blood glucose and hepatic glycogen levels at the endpoint and the fraction of G6P hydrogen 2 that was removed by exchange with F6P via G6P-isomeraseand the fraction of G6P hydrogens 4, 5 and 6 that were removed via a combination of G6P-isomerase and transaldolase exchanges.

Species	Load contributions		Glucose-6-phosphate exchanges	
	Blood glucose	Hepatic glycogen via direct pathway	G6P-isomerase	Transaldolase
Rat (n = 6)	19 ± 1	12 ± 1	61 ± 1	5 ± 1
Seabass (n = 8)	51 ± 3**	34 ± 5*	47 ± 3*	10 ± 1*

Mean values ± S.E.M. are presented. Differences from correspondent value in rats are indicated by asterisks (*t*-test, $^*p < 0.01$ and $^{**}p < 0.0001$).

Hepatic glycogen enrichments were systematically lower than that of plasma glucose for both rat and fish (Table 4). This is consistent with the dilution of the ^{2}H-enriched glucosyl units by a residual amount of pre-existing unlabeled glycogen and by unlabeled glycogen that was synthesized via the indirect pathway. As for plasma glucose, hepatic glycogen showed a significant depletion of position 2 enrichment relative to all other sites, indicative of exchange between this hydrogen and that of body water by glucose-6-phosphate isomerase. Compared to humans, where ~90% of the position 2 deuterium is exchanged at the level of hepatic G6P, this fraction was considerably lower for the rat (~64%) and even more so for seabass (~47%), as shown in Table 5. For the rat, enrichment of positions 4, 5 and 6 were essentially equivalent to that of position 3 and the ratio of positions 4, 5 and 6 enrichment to that of position 3 were ~1.0 indicating that transaldolase-mediated exchange of glycogenic G6P was negligible. For the seabass, while there was no significant difference between enrichment of positions 4, 5 and 6 to that of position 3, the ratio of positions 4, 5 and 6 to that of position 3 was systematically less than 1.0, consistent with a minor amount of transaldolase exchange activity (see Table 5).

In seabass, hepatic glycogenesis via the direct pathway played a significant role in sequestering the glucose load, accounting for about one-eighth of the load at the time of sampling. In contrast, the direct pathway accounted for an insignificant fraction of the glucose load for the rat (see Table 3). This indicates that essentially the entire glucose load must have been disposed into peripheral tissues such as skeletal muscle and adipocytes.

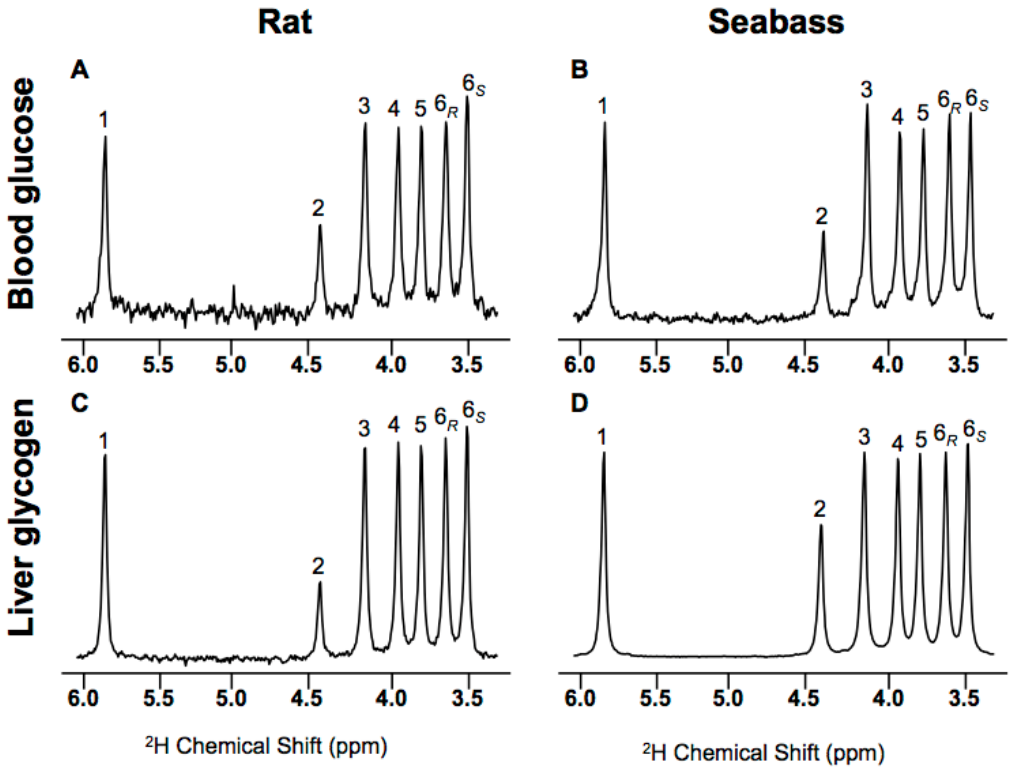

Figure 39: ^2H NMR spectra of monoacetone glucose samples derived from rat blood glucose (A) and seabass (B) and liver glycogen of rat (C) and seabass (D) following administration of a glucose load enriched with[U-$^2H_7$]glucose. The number above each signal indicates its glucosyl positional origin.

Discussion

The disposal of a deuterated glucose load into hepatic glycogen was examined in fasted rats and seabass. The initial fasting conditions (24 h for rats and 21 days for seabass) were chosen as to deplete hepatic glycogen to residual levels so that the opportunity for net hepatic glycogen deposition from the glucose load was maximized for both species. The relatively slow rates of glucose turnover and hepatic glycogenolysis in seabass compared to rat necessitates a longer fasting period to achieve similar degrees of hepatic glycogen depletion (i.e. ≤10% of postprandial levels). Likewise, the longer post-load interval chosen for hepatic glycogen sampling in seabass (48 h) compared to the rat (2 h) reflects the inherently slower disposal and metabolism of a glucose load in seabass compared to rat. Given that seabass intermediary metabolic fluxes are fundamentally governed by ambient water temperature, it is almost certain that glucose disposal rates will be accelerated at higher temperatures. Our measurements were performed at 18 °C, representing the median of the temperature range

for cultivating seabass, but they have maximum feed utilization and growth rates at 24 °C and 26 °C, respectively (Person-Le Ruyet, Mahe et al. 2004).

Our study provided insights on hepatic disposal of glucose into glycogen following a glucose load, as well as exchanges between the hydrogens of glucose and body water during this process. Our studies indicate that for rats, hepatic glycogen synthesis via the direct pathway does not have a significant role in disposal of a glucose load in the setting of a glucose tolerance test. We have previously shown that in rats subjected to the same protocol, direct pathway synthesis of hepatic glycogen accounted for only ~1% of the glucose load while gluconeogenesis from endogenous substrates accounted for the large majority of glycogen that was synthesized (Soares, Carvalho et al. 2010). This suggests that for rats, the restoration of euglycemia following a glucose load is almost entirely mediated by clearance into peripheral tissues such as the skeletal muscle and adipocytes. For seabass, hyperglycemia persisted at 48 h post-load while a significantly higher fraction of hepatic glycogen was synthesized via the direct pathway. This suggests that hepatic glycogenesis may have a more prominent role for glucose disposal in fish compared to rats. It also demonstrates that the seabass has a significant capacity for up regulation of direct pathway fluxes in response to a glucose load .In comparison, when fasted seabass are reefed with standard fish meal, the direct pathway contribution is insignificant (Viegas, Rito et al. 2012). Despite this, the clearance of the glucose load is much slower in comparison to the rat suggesting that glucose uptake by peripheral tissues has a much more limited role for clearing a glucose load in seabass compared to the rat. The fraction of the glucose load disposed into urine is likely to be negligible for non-diabetic rats, but it is possible that in the seabass, a portion of the load was cleared in this manner. In rainbow trout, the plasma glucose threshold for glucosuria is about 22 mM or 400 mg/dL (Bucking and Wood 2005). Above these levels about 0.5–2% of renal glucose flux is lost to the urine. Assuming that seabass have a similar threshold and given that the endpoint glucose was around 340 mg/dL, it is likely that earlier glucose values would have exceeded the 400 mg/dL threshold. Under these conditions, it is possible that a portion of the glucose load was cleared via urine.

The analysis of hepatic glycogen synthesis with labeled water is a versatile approach that provides information about the fractional replacement of glycogen during feeding as well as informing the substrates that contribute to glycogen synthesis (Postle and Bloxham 1980; Jones, Fagulha et al. 2006; Soares, Carvalho et al. 2012; Viegas, Rito et al. 2012; Delgado,

Martins et al. 2013). The analysis relies on certain assumptions concerning exchange of hydrogens between sugar phosphates and body water. These include the complete exchange of G6P hydrogen2 and body water via G6P-isomerase so that G6P that is synthesized from unlabeled glucose becomes enriched in position 2 to the same extent as body water while G6P derived via the indirect pathway is also quantitatively enriched in this position. Thus, the fraction of hepatic glycogen derived from all G6P sources is equivalent to the ratio of position 2 enrichment to body water (Jones, Fagulha et al. 2006; Soares, Viega et al. 2009; Barosa, Silva et al. 2012). In humans, exchange of G6Phydrogen 2 and body water is 90% complete (Jones, Fagulha et al. 2006; Delgado, Silva et al. 2009; Barosa, Silva et al. 2012). In comparison, the data of this report indicate that this exchange is considerably less extensive in both rat and seabass. There are two mechanisms that can contribute to incomplete exchange of G6P hydrogen 2 and body water. First, if G6P derived from glucose is recruited for glycogenesis before it has the opportunity to be converted to F6P and back by G6P-isomerase, then it will not become enriched in position 2. The second mechanism is isotopic discrimination against ^{2}H incorporation by G6P-isomeraseduring conversion of F6P to G6P. This latter process becomes significant when the rate of F6P and G6P exchange is relatively slow compared to G6P uptake by glycogenesis and results in a reduced enrichment of position 2 relative to body water regardless of the G6Psource (i.e. either direct or indirect pathways). Conversely, when G6P–F6P exchange is fast compared to glycogenic G6P utilization, this isotopic discrimination is abolished.

For rats, there is evidence suggesting that isotopic discrimination of G6P position 2 enrichment is not significant. Under extended fasting conditions, where essentially all G6P is derived via gluconeogenesis, the enrichment of G6P position 2 approaches that of body water and is also equivalent to position 5, a site that is also enriched during gluconeogenesis but is not subject to isotopic discrimination. However, under postprandial conditions there is evidence for limited G6P–F6P exchange during the conversion of glucose to glycogen via the direct pathway due to the channeling of glycogenic G6P into the glycogen synthesis pathway (Agius, Peak et al. 1996; Agius, Centelles et al. 2002). If this is the mechanism that accounts for the retention of position 2 enrichment following direct pathway conversion of [U-$^{2}H_{7}$]glucose to glycogen, then our data suggest that ~40% of the G6P intermediate did not undergo G6P–F6P exchange (Table 5). Therefore, in the $^{2}H_{2}O$ experiment, glycogen units derived via the direct pathway would be enriched in position 2 to only ~60% of body water while the remaining 40% would be unlabeled. Thus for rats, the direct pathway contribution measured

from the enrichment of glycogen from $^{2}H_{2}O$ (Soares, Viega et al. 2009) is an underestimate. Based on the present data, these estimates need to be corrected with a factor of 1.64 (i.e. 1 + 39/61). Meanwhile, enrichment of glycogen position 5 from $^{2}H_{2}O$ via the indirect pathway is not limited by exchange or isotope effects hence the indirect pathway contribution as read by the enrichment of position 5 relative to body water does not need any correction. For seabass, the retention of position 2 enrichment following metabolism of [U-$^{2}H_{7}$] glucose to glycogen was even greater than that of the rat, with less than one-half of the position 2 deuterium removed via F6P–G6P exchange. On this basis, the calculated direct pathway contribution based on the enrichment of glycogen from $^{2}H_{2}O$ needs to be corrected by a factor of 2.11 (1 + 52.7/47.3). For the seabass, the limited ^{2}H–^{1}H exchange at position 2 of G6P is at least partially contributed by a kinetic isotope effect mediated by G6P-isomerase, as described in plants (Schleucher, Vanderveer et al. 1999). Under conditions of low dietary carbohydrate and residual direct pathway activity, hepatic glycogen synthesis is almost entirely mediated by the indirect pathway (Viegas, Rito et al. 2012) and enrichment of glycogen positions 2 and 5 from $^{2}H_{2}O$ via this pathway is theoretically equivalent. We observed a significantly lower enrichment of glycogen position 2 compared to position 5 that is consistent with the isotopic discrimination of ^{2}H-incorporation into position 2 of G6P via G6P-isomerase (Viegas, Rito et al. 2012). Hydrogens 4, 5 and 6 of G6P can also exchange by a combination of G6P-isomerase and transaldolase activities (Bock, Schumann et al. 2008; Basu, Chandramouli et al. 2009; Basu, Barosa et al. 2010). In overnight-fasted humans, this exchange accounts for ~20% of hepatic G6P following a glucose tolerance test (Delgado et al., 2009) or a mixed meal (Barosa, Silva et al. 2012). Transaldolase exchange results in the incorporation of $^{2}H_{2}O$ or other gluconeogenic tracers into G6P and glycogen without any net indirect pathway flux and therefore results in an overestimation of the indirect pathway contribution by these methods. Accordingly, if direct and indirect pathway fluxes are measured with a glucose tracer labeled in positions 4, 5 or 6, such as [5-^{3}H]glucose, transaldolase activity will remove this label at the level of G6P (Stingl, Chandramouli et al. 2006). This results in an apparently higher dilution of the label by indirect pathway flux and an underestimation of the direct pathway contribution (Stingl, Chandramouli et al. 2006). Our data indicate that transaldolase mediated exchange of hepatic G6P is insignificant in rat and is of minor importance in seabass, at least under the conditions of a glucose load. Therefore, estimations of indirect pathway contributions from the enrichment of glycogen position 5 from $^{2}H_{2}O$ do not need to be corrected for this process.

Finally, our study has highlighted some key differences between a carnivorous fish and an omnivorous rodent in the role of hepatic glycogenesis during clearance of a glucose load. Paradoxically, for a species that is considered to be glucose intolerant, the seabass liver exhibited "textbook behavior" in that it sequestered a significant fraction of the load as glycogen via the direct pathway. In contrast, this mechanism played a very minor role in glucose disposal by the rat, in agreement with previous studies (Soares, Carvalho et al. 2010). Instead, the vast majority of the load was likely disposed into insulin-sensitive peripheral tissues including the skeletal muscle and adipocytes, and this process was able to counteract hyperglycemia within a relatively short interval (~1–2 h). In contrast, the seabass remained hyperglycemic 48 h after the load despite the higher amount of load sequestered into hepatic glycogen. This indicates that glucose uptake by peripheral tissues (principally muscle) plays a much less prominent role in restoring euglycemia following a glucose load in seabass compared to rat.

Acknowledgements

The authors acknowledge the valuable assistance of all colleagues who participated in any of the stages of this work. The authors acknowledge the financial support from Fundação para a Ciência e a Tecnologia (FCT) in the form of a Ph.D. Fellowship to F.O.M.: SFRH/BD/51194/2010, and Research Grants to J.G.J.: PTDC/EBB-BIO/098111/2008 & PTDC/SAU-MET/111398/2009. The NMR spectrometer is part of the National NMR Network and was purchased in the framework of the National Programme for Scientific re-equipment, contract REDE/1517/RMN/2005, with funds from POCI 2010 (FEDER) and FCT.

CHAPTER 5 - GENERAL DISCUSSION

5. General Discussion

The ability to regulate glucose and lipid levels over the daily feeding and fasting cycles depends in large part on insulin bioavailability and actions. The appropriate levels of plasma insulin in relation to the inflow of glucose and lipids is crucial for fuel homeostasis in living organisms and dysfunctions in insulin bioavailability and actions contribute to metabolic dysfunction in diseases such as T2D. The balance between insulin secretion and insulin clearance is critical for the precise coupling of insulin bioavailability to systemic glucose and lipid availability.

The liver, situated directly downstream from the pancreas, plays a crucial role in first pass insulin clearance. Hepatic insulin is cleared by the actions of two principal enzymes: IDE and PDI (Duckworth, Bennett et al. 1998).

In rats that were studied before and after an intraenteric glucose load, we confirmed that changes in hepatic IDE activity explained the observed alterations of insulin clearance between fasted and fed states. Moreover, IDE activity and hepatic NO levels have an inverse relationship throughout this glucose challenge. This confirmed, *in vivo*, the inhibition of IDE activity by NO that was already demonstrated with purified IDE *in vitro* (Cordes, Bennett et al. 2009). Also, when hepatic NO levels were artificially increased by the infusion of a NO donor substrate, we confirmed a corresponding reduction in hepatic IDE activity and insulin clearance.

Regarding the PDI enzyme, less is known about its contributions to insulin clearance and the regulation of systemic insulin levels either under normal feeding-fasting states or under pathophysiologic conditions. PDI converts insulin into its two component chains by cleavage of the disulfide bonds, leading to the release of A and B chains to the circulation (Lambert and Freedman 1983; Tang and Tsou 1990). With our results, we can speculate that the activity of this enzyme is stimulated under conditions where insulin bioavailability/sensitivity needs to be higher, such as in the post-prandial state, by forwarding internalized insulin to A and B chain formation, which are known to have systemic biological activity. Further confirmation of this hypothesis would require measurement of plasma A and B chain concentrations. Our studies suggest that NO levels are not involved in regulating PDI activity and expression, but other factors may be involved. We hypothesize that GSH may be one such factor since it has been described that PDI activity is sensitive to the GSSG/GSH ratio. Moreover, it has been

previously shown that GSH levels are increased in the post-prandial state and this associated with an increase in insulin sensitivity (Guarino, Afonso et al. 2003; Raturi and Mutus 2007).

In the first part of this work we established that insulin clearance is involved in the control of insulinemia under normal physiological conditions. From the fasted to fed state there is a concerted increase on insulin secretion and decrease on insulin clearance. This results in an increased insulin bioavailability in the fed state -matching the period of maximal systemic glucose appearance via absorption. At the same time, other alterations are promoted that further tune insulin bioavailability and actions. The detected rapid increase of hepatic NOS activity and consequent hepatic NO levels during feeding that was previously reported (Lautt, Macedo et al. 2001; Guarino, Afonso et al. 2003) represents one such process that through our work is now linked to the regulation of insulin bioavailability (Figure 40). Moreover, we have previously shown that GSNO, a product of GSH nitrosylation by NO, when infused in fasted animals increases their insulin sensitivity to that of postprandial animals (Fernandes, Guarino et al. 2012). Hence, in addition to increasing insulin bioavailability through reducing its clearance, high hepatic NO levels can also increase insulin sensitivity thereby potentiating insulin actions.

As for many other aspects of metabolic control, we demonstrated that liver plays an important role in insulin clearance. However, when we began our studies, it was unclear if NO signaling in other tissues could also be involved in regulating insulin clearance. We observed in previous studies that the infusion of L-NAME directly in the portal vein promoted a lesser increase in insulin clearance in comparison to systemic infusion of a comparable amount. Therefore, NO signaling in other tissues could also be involved in regulating hepatic insulin clearance.

Thus, we showed that the PVN-NO signal contributes to the regulation of hepatic insulin clearance. The depletion of NO in this hypothalamic region led to an increase in insulin clearance and in part explains the greater of systemic over hepatic NOS inhibition on insulin clearance (unpublished results). It remains to be determined whether or not this effect is mediated via alterations in neuronal activity and ANS modulation (Figure 40).

It is known that after meal ingestion, there is activation of the parasympathetic nervous system and consequent stimulation of NO release in the liver. There is also the stimulation of insulin secretion from the pancreas which also promotes an increase in hepatic NO levels. By the described inhibitory effects of hepatic NO on IDE activity and insulin clearance, there is an

increase in plasma insulin levels. The mechanisms that explain the increase in hypothalamic NO levels are much less certain. We speculate that vagal stimulation as well as increased systemic insulin levels may stimulate hypothalamic NO production (Figure 40). It is already known that insulin *per se* promotes the stimulation of the NO production in the hypothalamus (Cabou, Cani et al. 2007), but the relationship between this activity and those of hepatic NO production and IDE inhibition has not been established. Our studies suggest that alteration in postprandial hepatic and hypothalamic NO levels act in concert to promote the inhibition of hepatic IDE activity, decrease hepatic insulin clearance and promote insulin bioavailability (Figure 40).

In contrast, for pathophysiological states such as T2D, obesity or hypertension - that incidentally also feature chronic low-grade inflammation - the increase in iNOS expression and activity results in higher than normal NO levels, which in turn may inhibit IDE activity to such an extent that peripheral hyperinsulinemia is established. This appears to be the case for the Wistar rats fed a high sucrose diet that show high expression and activity of iNOS concurrent with high NO levels and hyperinsulinemia (unpublished results). Meanwhile, in obese subjects, the central actions of insulin are impaired thereby diminishing peripheral tissue insulin sensitivity. As an example, Heni *et al* demonstrated that that brain insulin resistance altered ANS balance and they suggested that this may contribute to consequent hepatic metabolic dysfunctions (Heni, Wagner et al. 2014).

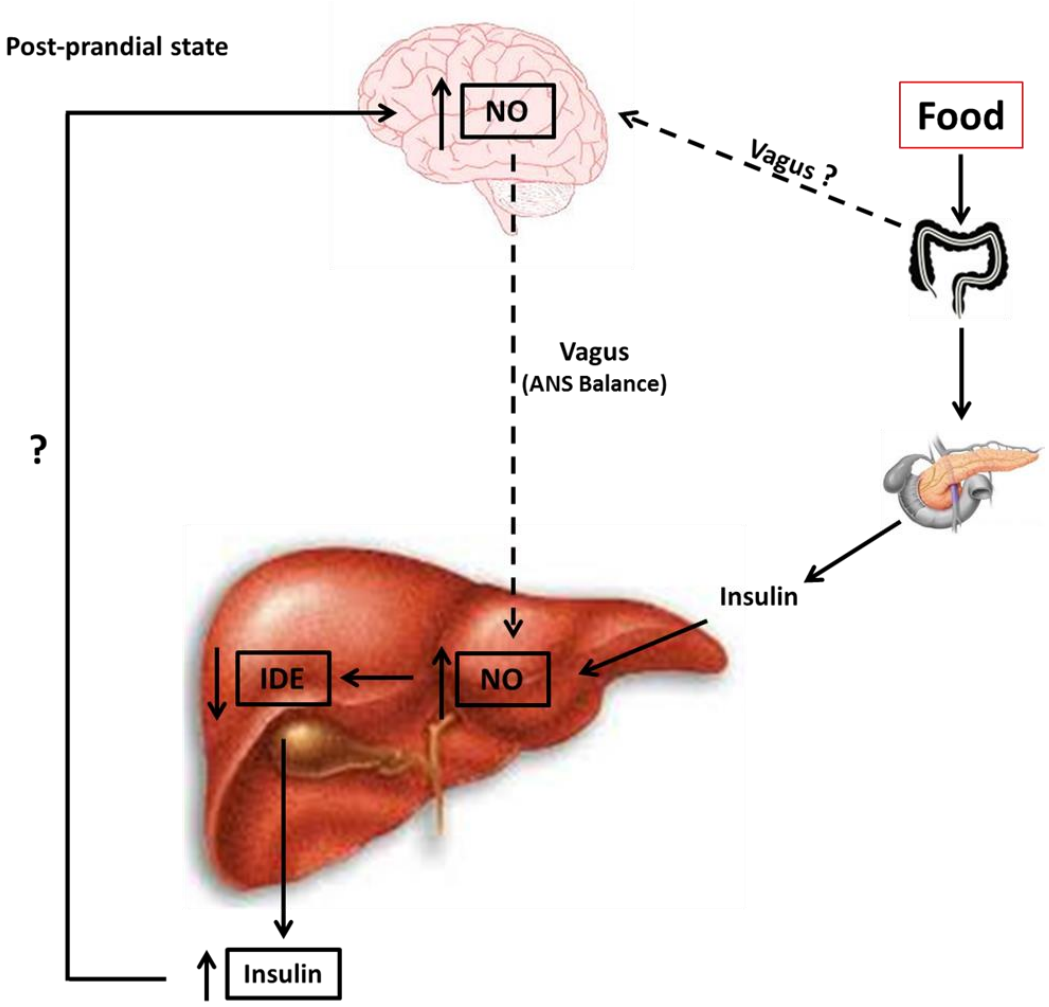

Figure 40: Model/Hypothesis for the role of NO over insulin bioavailability in the post-prandial state: In the post-prandial state there is the activation of the parasympathetic system that stimulates the release of hepatic NO. At the same time, there is stimulation of pancreatic insulin release that is released directly on the portal-vein. The increase of hepatic NO levels inhibits hepatic IDE activity, with a consequent decrease of insulin clearance and increase of plasma insulin levels. It remains to be answered if hypothalamic ANS modulation (by vagus activation) acts isolated or in concert with plasma insulin in the stimulation of hepatic and hypothalamic NO production, in order to further increase insulin bioavailability in the post-prandial state.

Our results with acute NOS inhibition showed that short-term systemic NO depletion increased hepatic insulin signaling and glucose transporter expression, suggesting that NO decrease may in part explain the acute decrease in meal-induced glucose excursions. However, long-term NOS inhibition, while also increasing insulin clearance, did not result in decreased plasma insulin levels because of a compensatory increase in insulin secretion. So, we conclude that NOS inhibition can lead to an increase in insulin clearance that is also associated with

increased hepatic activation of the insulin signaling pathway and improved glucose homeostasis.

We also observed that altering NO levels in the PVN resulted in changes of hepatic metabolites, notably hepatic TGs. Therefore, alterations in hypothalamic NO may contribute to diet-induced changes in hepatic TG levels, since systemic NO levels are also modified by different diets (Perreault and Marette 2001). Previous studies already showed that the NO signal from the hypothalamus is involved in lipid metabolism regulation, with different impacts between normal and pathophysiological states. Under normal conditions, hypothalamic NO levels contribute to a decrease in hepatic TG levels. However, in pathophysiologic conditions, the overproduction of NO from iNOS in the hypothalamus, like observed in obesity, can be a not yet known factor involved on hepatic TGs accumulation and consequent fatty liver (Dallaire and Marette 2004). Although there is also some evidence for the central NO-dependent modulation of ANS and consequent alteration of hepatic lipid metabolism (Nogueiras, Wiedmer et al. 2007; do Carmo, Bassi et al. 2011), it remains to be determined if these effects can be attributed to the direct alteration in central nerve signaling secondary to altered hypothalamic NO levels or if it is a consequence of the changes in hepatic insulin clearance.

Given that over the last few decades, T2D rates have rapidly increased in association with lifestyle changes, urbanization and aging (Chen, Magliano et al. 2011; International Diabetes Federation 2013), improved knowledge on the complex processes of metabolic dysfunction and insulin impairment involved in T2D etiology is crucial to prevent or ameliorate the effects of the disease. Insulin clearance is highly correlated with insulin resistance and T2D incidence (Rudovich, Rochlitz et al. 2004; Erdmann, Mayr et al. 2009). The initial impairment of insulin actions and consequent disorders of glucose and lipid metabolism promotes the establishment of chronic hyperinsulinemia. Among other things, this provokes impairment of β-cell insulin secretion (Duckworth, Bennett et al. 1998). Insulin clearance connects these two important parameters, hence its study opens new perspectives to assess the loss insulin-dependent regulation of fuel homoeostasis and identify novel therapies that may act in part by modifying insulin clearance. At the same time the actions of existing interventions may be seen in a new light by studying their effects on insulin clearance (Wei, Ke et al. 2014). Thus, we uncovered a hitherto overlooked action of troglitazone - a putative PPAR-α agonist - on hepatic insulin clearance. We showed that troglitazone ameliorated fatty liver in rats fed with high sucrose diets, in part through suppression of *de novo* lipogenesis. Unexpectedly, we also showed that

the reversal of hyperinsulinemia in these animals was entirely attributable to increased insulin clearance. We confirmed that as in our previous studies, this increased insulin clearance was correlated with both a stimulation of hepatic IDE activity and decreased hepatic NO levels.

Finally, changes in hepatic glucose and glycogen metabolism are key early outcomes of insulin resistance and T2D and methodologies. Quantify these pathways that are directly translatable from animal models to humans are essential components for evaluating perturbation and restoration of hepatic carbohydrate metabolic regulation. The deuterated water (^2H_2O) method for quantifying sources of hepatic glucose and glycogen synthesis is highly translatable, but to date has been based on assumptions about metabolite deuterium exchanges that have been tested in humans (Jones, Fagulha et al. 2006; Basu, Barosa et al. 2010), but not in animal models. We describe important species-specific differences in deuterium exchange at the level of hepatic glucose-6-phosphate and triose phosphate and based on this information, correction factors that allow more accurate determination of glucose and glycogen synthesis fluxes in rats were obtained. In turn this permits better translation of hepatic metabolic flux measurements between T2D animal models and T2D patients thereby improving our understanding of the metabolic consequences of impaired insulin bioavailability.

CHAPTER 6 - FINAL CONCLUSIONS

6. Final Conclusions

The overall conclusions of the studies presented in this document are that hepatic NO is an important signaling molecule for the *in vivo* regulation of IDE and, consequently, insulin clearance and peripheral insulin bioavailability. Also, hypothalamic NO, particularly in the PVN region, has a role in regulating hepatic insulin clearance. Its chronic depletion promotes an increase in hepatic insulin signaling and glucose transporter expression. The stimulation of the lipogenic pathway and accumulation of hepatic TGs is an overt metabolic consequence of PVN NO depletion. However, it remains unclear if PVN-NO signal impacts directly on liver function or if it impacts indirectly on liver metabolism by ANS modulation. In insulin resistant states, treatment by TZDs drugs insulin clearance was shown to have a relevant role with troglitazone protecting against fatty liver by decreasing hepatic DNL and NEFAs levels. Moreover, troglitazone increased insulin clearance, and consequently decreased plasma insulin levels, by stimulation of hepatic IDE activity. This novel attributed action of TZD opens therapeutic strategies for reversing the onset of non-alcoholic fatty liver disease and countering hyperinsulinemia in T2D subjects. Finally, we identified significant species-specific differences in the enrichment of liver glycogen from $^{2}H_2O$. These observations allow improved translation of hepatic carbohydrate fluxes between experimental animal models and human subjects.

CHAPTER 7- REFERENCES

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