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UNIVERSIDADE DE COIMBRA

Sirtuin 2 in hypothalamus: an emerging target in insulin resistance?

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob orientação do Professor Doutor Pedro Gomes (Centro de Neurociências e Biologia Celular) e da Professora Doutora Emília Duarte (Departamento de Ciências da Vida, Universidade de Coimbra)

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Abbreviations

AgRP	Agouti-related peptide
AMPK	AMP-activated protein kinase
ARC	Arcuate nucleus
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BCA	Bicinchoninic acid
BSA	Bovine serum albumine
CART	Cocaine- and amphetamine-regulated transcript
Cer	Ceramide
CPT1	Carnitine palmitoyltransferase I
CR	Caloric restriction
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
ERK	Extracellular-signal-regulated kinase
FAS	Fatty acid synthase
FBS	Fetal bovine serum
FFA	Free fatty acids
FOXO	Transcription factors forkhead box class O
Gab1	GRB2-associated-binding protein 1
GFAP	Glial fibrillary acidic protein
Glut4	Glucose transporter type 4
GRB2	Growth factor receptor-bound protein 2
HCl	Hydrogen chloride
IKKβ	IκB kinase β
IR	Insulin receptor
IRS	Insulin receptor substrates
JNK	c-Jun N-terminal protein kinase
LCFA-CoA	Long-chain fatty acyl-CoA
LH	Lateral hypothalamus
LPA	Lysophosphatidic acid

MAPK	Mitogen-activated protein kinase
MC	Melanocortin
MCH	Melanin-concentrating hormone
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NaF	Sodium fluoride
NAM	Nicotinamide
Nampt	Nicotinamide phosphoribosyltransferase
NeuN	Neuronal nucleic protein
NF-kB	Nuclear factor-kB
NPY	Neuropeptide Y
Orto	Sodium orthovanadate
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PIP	Phosphatidylinositol biphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
POMC	Proopiomelanocortin
PPARϒ	Peroxisome proliferator-activated receptor ϒ
PVN	Paraventricular nucleus
rDNA	Ribosomal deoxyribonucleic acid
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Shc	Src homology 2 domain-containing
Sir 2	Silent information regulator 2
SIRT	Sirtuin
SNS	Sympathetic nervous system
SOS	Son of sevenless

SS	Serum starvation
TZD	Thiazolidinedione
VMH	Ventromedial hypothalamus
WT	Wild type

Abstract

Obesity and type 2 diabetes constitute major public health problems worldwide. A central feature of these metabolic disorders is peripheral insulin resistance. Interestingly, recent studies suggest that insulin resistance also occurs in hypothalamus. This structure has an important role in the maintenance of homeostasis, by detecting metabolic changes and controlling neuroendocrine responses that regulate appetite, temperature, circadian control and release of hormones. It has been proposed that improving defective hypothalamic insulin signalling and neuroinflammation may restore insulin sensitivity in peripheral organs.

The sirtuin family (SIRT1-7) of NAD^+-dependent protein deacetylases have emerged as important regulators for a variety of cellular processes, including energy metabolism, stress response and possibly aging. SIRT2 has an important role in cell cycle regulation, neurodegeneration and tumor suppression. Data now emerging indicate that SIRT2 may also play a key role in metabolic regulation and neuroinflammation. Unpublished data from the host laboratory indicate that SIRT2 is downregulated in insulin-resistant hepatocytes and mouse livers as well as in blood cells from obese insulin-resistant human subjects. Importantly, SIRT2 overexpression in hepatocytes improves insulin sensitivity by reducing oxidative stress and improving mitochondrial dysfunction.

This study demonstrates that SIRT2 is expressed in the mouse hypothalamus, being particularly enriched in the VMH, and present in neurons. SIRT2 expression in the VMH is regulated by nutritional availability since its expression is increased under caloric restriction and markedly reduced in high-fat diet conditions. We also demonstrate that direct administration of palmitate, a saturated free-fatty acid, to the VMH results in reduced SIRT2 expression, similarly to what happens in high-fat diet. We found that exposure of cultured hypothalamic neurons to palmitate induces insulin resistance and downregulates SIRT2 expression. Importantly, serum starvation improves insulin sensitivity in normal conditions, and prevents the effect of palmitate on insulin resistance and SIRT2 downregulation.

This study suggest that SIRT2 may be in future an important target in obesity and insulin resistance treatment, by modulating its expression and/or activity.

Keywords: Sirtuin 2; Hypothalamus; Insulin signalling; Obesity; Type 2 Diabetes

Resumo

A obesidade e a diabetes tipo 2 representam atualmente dois dos maiores problemas de saúde pública a nível mundial. Uma das características centrais destes distúrbios metabólicos é a resistência à insulina periférica. Curiosamente, estudos recentes sugerem que a resistência à insulina ocorre também no hipotálamo. Esta estrutura cerebral tem um papel importante na manutenção da homeostase, através da deteção de alterações metabólicas, e no controlo das respostas neuroendócrinas, que regulam o apetite, temperatura, controlo circadiano e libertação de hormonas. Tem sido proposto que a melhoria da sinalização da insulina e da neuroinflamação no hipotálamo pode restaurar a sensibilidade à insulina em órgãos periféricos.

As sirtuínas (SIRT1-7), proteínas com atividade de desacetilação dependente de NAD^+, têm surgido como reguladores importantes numa variedade de processos celulares, tais como metabolismo, resposta ao stress e, possivelmente, envelhecimento. A sirtuína 2 (SIRT2) tem um papel importante na regulação do ciclo celular, neurodegeneração e supressão tumoral. Os dados agora emergentes indicam que a SIRT2 pode também desempenhar um papel importante na regulação metabólica e na neuroinflamação. Resultados obtidos previamente pelo nosso grupo, ainda não publicados, indicam que a SIRT2 é regulada negativamente em hepatócitos e fígados de ratinho insulino-resistentes e em células de sangue de humanos obesos resistentes à insulina. É importante salientar que a sobreexpressão de SIRT2 em hepatócitos insulino-resistentes melhora a sensibilidade à insulina, através da atenuação do stress oxidativo e de disfunção mitocondrial.

Este estudo demonstra que a SIRT2 é expressa no hipotálamo, encontrando-se particularmente enriquecida no VMH e presente em neurónios. A SIRT2 no VMH é regulada pela disponibilidade energética, uma vez que a sua expressão se encontra aumentada em restrição calórica e drasticamente reduzida em condições de dieta rica em gordura. Demonstramos ainda que, na presença de palmitato, um ácido gordo saturado, a SIRT2 no VMH também se encontra diminuída, de forma semelhante ao que acontece na dieta rica em gordura. Mostramos ainda que a exposição ao palmitato pode induzir resistência à insulina e diminuição da expressão de SIRT2. No entanto, a

privação de soro melhora a sensibilidade à insulina em condições normais, e evita o efeito da resistência à insulina induzida pelo palmitato, bem como a diminuição da SIRT2.

Este estudo sugere que a SIRT2 pode no futuro ser um alvo importante no tratamento da obesidade e da diabetes tipo 2.

Palavras-chave: Sirtuína 2; Hipotálamo; Sinalização da insulina; Obesidade; Diabetes tipo 2

Chapter I

Introduction

1. Metabolic Disorders

1.1. Obesity and Type 2 Diabetes

The increasing prevalence of obesity and type 2 diabetes represents one of the most important public health problems worldwide. The strong economic growth of developing countries in recent years has led to a raise in worldwide prevalence of overweight and obesity. Consequently, this leads to an increase in prevalence of type 2 diabetes, due to the close association between obesity and type 2 diabetes. The rising incidence of these disorders increases the costs associated with the treatment of this type of conditions (Scheen and Van Gaal, 2014).

Obesity is characterized by abnormal or excessive fat accumulation. Obese subjects have an increase in plasma FFA levels because metabolically stressed adipose tissue releases more FFA and its clearance is reduced. The elevation of FFA levels will inhibit insulin action, resulting in FFA release to circulation (Boden, 2011) . For these reasons, obesity is the major risk factor for noncommunicable diseases such as diabetes (Zimmet et al., 2001) , cardiovascular diseases (Poirier and Eckel, 2002) and some cancers, such as breast, colon and endometrial (Fader et al., 2009).

It is estimated that two thirds of adults worldwide are overweight and over 500 million are obese. These data become even more worrying when we verify that about 40 million children under the age of 5 are overweight or obese. It is currently estimated that around 3.4 million adults die each year as a result of obesity and overweight.

Type 2 diabetes is the most common form of diabetes, accounting for 90%-95% of all cases. About 382 million adults have this disorder and it is estimated that in 2035 this number will increase to 592 million adults. Type 2 diabetes is characterized by high levels of blood glucose (hyperglycemia) that results from defects in both insulin secretion and insulin action. The primary cause of hyperglycemia is an elevated rate of basal hepatic glucose production caused by hyperinsulinemia (DeFronzo, 1999) and circulating FFA, because elevations of plasma FFA inhibit insulin suppression of hepatic glucose production (Boden, 2011).

In most type 2 diabetes cases the body is able to produce insulin but is unable to respond to its effects. This condition is named insulin resistance.

Current therapies to treat type 2 diabetes, such as metformin and TZDs, promote accelerated pancreatic β cell exhaustion and apoptosis as the disease progresses, which ultimately renders the pancreas unable to produce insulin. These treatments have associated several side effects, such as weight gain, hypoglycemia, and/or liver and kidney insufficiency. Thus, there is an urgent need to improve the existing treatments making them capable of prolonging pancreatic function and diminishing insulin resistance with aging (Lavu et al., 2008).

2. Insulin Resistance

2.1. Insulin: biological effects and signalling pathways

Insulin is secreted in response to an increase in blood glucose concentration and promotes synthesis and storage of carbohydrates, lipids and proteins and inhibits their degradation and release back into the circulation. This potent anabolic hormone regulates glucose homeostasis by the suppression of hepatic glucose output and by increasing the rate of glucose uptake by the skeletal muscle and adipose tissue (Pessin and Saltiel, 2000).

Insulin signalling is a very complex process that involves multiple pathways and cascades of phosphorylation events (Hotamisligil, 2003). In the presence of insulin, the insulin receptor phosphorylates insulin receptor substrate (IRS) proteins that lead to activation of two main signalling pathways: the PI3K/Akt pathway, responsible for the majority of the metabolic actions of insulin, and the Ras/MAPK pathway, that regulates gene expression and cooperates with the PI3K pathway to control cell growth and differentiation (Taniguchi et al., 2006).

The PI3K/Akt pathway is highly conserved, and its activation is tightly controlled via a multistep process (Hemmings and Restuccia, 2012). Activation of the PI3K/Akt pathway elicits several cellular events such as stimulation of glycogen synthesis, inhibition of gluconeogenesis, and stimulation of glucose uptake (Könner and Brüning, 2012). The binding of PI3K heterodimers to phosphotyrosines on IRS proteins leads to the production of PIP3 from PIP2 at the plasma membrane. This phospholipid triggers activation of further downstream-signalling cascades that are mediated by Akt (Taniguchi et al., 2006). Activation of Akt leads to additional substrate-specific phosphorylation events in both the cytoplasm and nucleus, including inhibitory phosphorylation of the pro-apoptotic FOXO proteins. Fully active Akt mediates numerous cellular functions including angiogenesis, growth, proliferation, survival, protein synthesis, transcription, apoptosis and metabolic pathways such as glycogen synthesis, inhibition of gluconeogenesis and stimulation of glucose uptake (Hemmings and Restuccia, 2012).

Several mechanisms have been described for the activation of MAPK in response to insulin. Insulin stimulates tyrosine phosphorylation of the IRS proteins, Gab1, and Shc, allowing the binding of the adaptor molecule Grb2. This protein recruits SOS protein to the plasma membrane, where it can activate the G protein Ras that is able to induce the sequential phosphorylation and activation of the MAPK cascade via Ras/Raf/ERK1/2. MAPKs can phosphorylate transcription factors such as Elk-1 and are associated in particular with the proliferative effects of insulin (Biddinger and Kahn, 2006; Gehart et al., 2010; Könner and Brüning, 2012) (Figure 1). However, the view that insulin signalling is regulated by two different and independent pathways might be too simplistic taking into account recent studies that propose the interaction of these signalling pathways (Gehart et al., 2010).

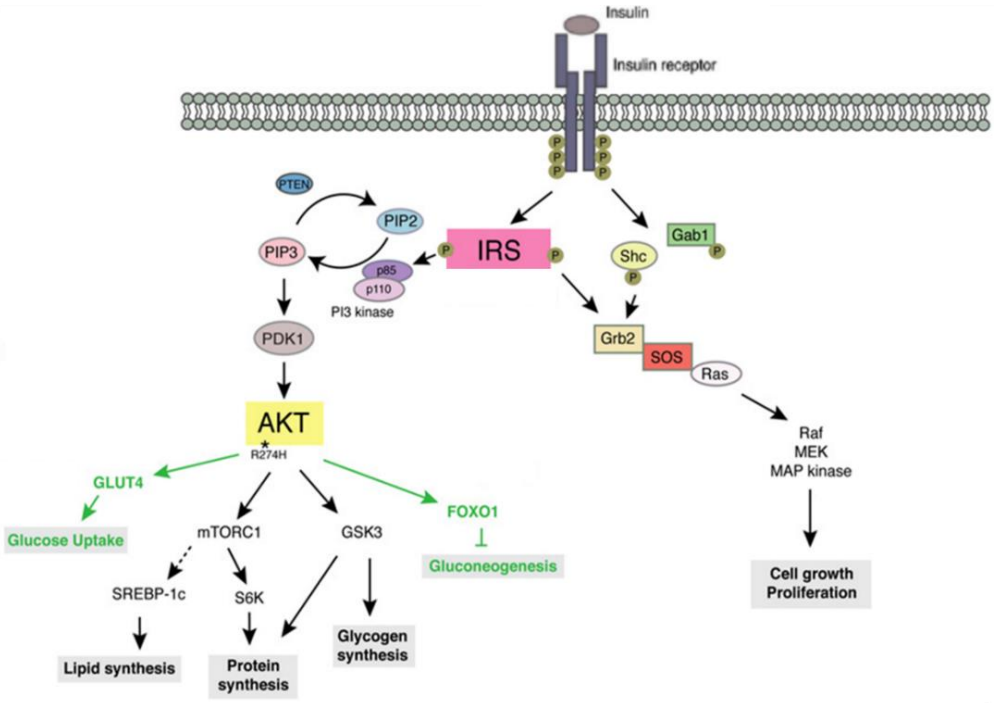

FIGURE 1 - INSULIN SIGNALLING BY PI3K /AKT AND RAS/MAPK PATHWAYS: Insulin stimulates phosphorylation of the IRS proteins, Gab1, and Shc, allowing the binding of the adaptor molecule Grb2. Grb2 recruits SOS protein that can activate the G protein Ras that induces phosphorylation and activation of the MAPK cascade via Ras/Raf/ERK1/2. MAPKs activation is associated with the proliferative effects of insulin. The binding of PI3K to phosphorylated IRS proteins leads to the production of PIP3 from PIP2. This phospholipid triggers activation of Akt that results in substrate-specific phosphorylation events, mediating numerous cellular functions including, metabolism, survival, protein synthesis, lipid synthesis, glycogen synthesis, transcription, and apoptosis. Adapted from: Könner and Brüning, 2012

A defect in signal transduction in these different pathways can lead to an abnormal regulation of the insulin processes, even in the presence of normal levels of insulin (Pessin and Saltiel, 2000). The pancreas compensates this defective regulation by secreting more insulin, and gradually the tissues become more insulin resistant (Taubes, 2009).

2.2. Principal inducers of Insulin Resistance

Insulin resistance is a state of reduced insulin sensitivity, an inability of insulin to lower plasma glucose levels through suppression of glucose production and stimulation of glucose utilization. This state occurs in different peripheral tissues, such as the pancreas, liver, adipose tissue and skeletal muscle, but also in brain (Kleinridders et al., 2013). In insulin resistance state, overnutrition leads to increased inflammation, changes in lipid metabolism and changes in gastrointestinal microbiota (Johnson and Olefsky, 2013). This condition has been associated with adverse outcomes, including obesity, glucose intolerance and hypertension (Biddinger and Kahn, 2006), that promote stroke, non-alcoholic fatty liver disease, polycystic ovary syndrome, asthma, some cancers and Alzheimer's disease (Taubes, 2009) . Several inducers have been proposed to explain the mechanisms of insulin resistance, in different experimental models.

High Glucose

In both animal models and humans, hyperglycemia impairs insulin-stimulated glucose utilization by peripheral tissues, such as muscle and fat, and decreases the ability of pancreatic β-cells to respond to hyperglycemia with acute insulin release, leading to the development of insulin resistance (Kawanaka et al., 2001; Nelson et al., 2000).

Glucose-induced insulin resistance has been studied in different experimental models. These include rats infused with high concentrations of glucose (Laybutt et al., 1999), isolated muscle preparations (Jensen et al., 1997; Kurowski et al., 1999), primary cultures of rat adipocytes (Lima et al., 1991), and cell lines, including 3T3-L1 adipocytes (Kawanaka et al., 2001). In isolated rat adipocytes, chronic exposure to high

glucose in the presence of insulin downregulates basal and acutely insulin-stimulated glucose transport. The effects of glucose and insulin appear to be associated with a post–insulin receptor defect (Lima et al., 1991). Although the concept of glucose-induced insulin resistance is well documented, the underlying mechanisms are not well understood (Nelson et al., 2000).

There are different hypotheses concerning the mechanism responsible for glucose-induced insulin resistance. One is that elevated intracellular glucose can lead to the activation of the PKC pathway (Campos, 2012; Kawanaka et al., 2001). Once activated, PKCs will increase phosphorylation of IRS proteins, resulting in insulin signalling inhibition and consequently insulin resistance (Kawanaka et al., 2001). Another possible hypothesis is that glucose-induced insulin resistance may be mediated by an increased flux of glucose into the hexosamine biosynthetic pathway, resulting in accumulation of UDP-N-acetylhexosamines (UDP-HexNAcs) (Kawanaka et al., 2001; Nelson et al., 2000), precursors for the synthesis of glycosyl side chains of proteins and lipids (Nelson et al., 2000).

Beyond the disagreement concerning the mechanism, there is also controversy about whether glucose-induced insulin resistance is mediated by high levels of both glucose and insulin or just by a high concentration of glucose (Kawanaka et al., 2001).

Chronic Insulin

Insulin resistance is also associated with chronic exposure to high levels of insulin. Defective insulin signalling at the β-cell impairs glucose-stimulated insulin release. At steady state, basal hyperinsulinemia can generate and maintain insulin resistance (Shanik et al., 2008). In studies with isolated rat adipocytes (Kobayashi and Olefsky, 1978), rats infused with high concentrations of insulin (Juan et al., 1999), and with different cell lines, the administration of insulin at high levels, similar to those found in insulin-resistant states, can induce insulin resistance, confirming that basal hyperinsulinemia can lead to insulin resistance.

The insulin pathway is regulated by numerous factors. The insulin receptor is a mediator of negative feedback. This negative regulation occurs due to the reduction in receptor affinity, reduction in the number of receptors in the surface of the cell, and

diminution of the effectiveness of the receptor as a transmitter of stimulatory signals. Each insulin receptor has two binding sites for insulin. First insulin molecule binds with high affinity, while the second insulin molecule binds with lower affinity. In the presence of increased levels of insulin, occupancy of receptor sites increases, and affinity diminishes (DeMeyts et al., 1976). The high concentration of insulin can also be responsible for a reduction in the cell surface receptor number by promoting internalization as well as degradation of receptors containing insulin (Gavin et al., 1974).

The insulin receptor activity is diminished under continuous exposure to insulin. The phosphorylation of insulin receptors activates downstream proteins, especially the IRS proteins to promote downstream activation. Phosphorylated IRS proteins have impaired ability to activate downstream elements and also act upstream to inhibit insulin receptor activity. Other molecules downstream in the insulin pathway, as mTOR, PI3K and Akt, transmit the activation process downstream and can also promote upstream negative feedback signals. In addition, chronic exposure to insulin can induce an alteration in downstream elements, including key components such as the IRS proteins (Hoehn et al., 2008; Zick, 2001). Hyperinsulinemia, impairment of glucose-stimulated insulin release and insulin resistance are intertwined biologically.

Free Fatty Acids (FFA)

Insulin resistant subjects normally present an abnormal lipid metabolism that results in increased circulating free fatty acid (FFA) concentrations. Several studies suggest that the lipid overload to peripheral tissues contributes to the development of insulin resistance by promoting the accumulation of fat-derived metabolites, that block an intracellular chain of events that normally trigger glucose transport into the cell, generally by inhibition of the translocation of a glucose transporter, Glut4, to the cell membrane (Holland et al., 2007). Insulin-stimulated glucose transport no longer works efficiently, and the cell becomes insulin resistant (Taubes, 2009).

The majority of these circulating fatty acids are assimilated into glycerolipids. In glycerolipid synthesis process, the acylation of LPA leads to the production of phosphatidic acid, which is dephosphorylated to produce diacylglycerol (DAG).

Different studies indicate that DAG is accumulated in peripheral tissues from insulin-resistant rodents (Turinsky et al., 1990), and can inhibit insulin action in various isolated tissues or cultured cells, including skeletal muscle (Montell et al., 2001), 3T3-L1 adipocytes and C2C12 myotubes (Chavez and Summers, 2003). DAG can be metabolized into triglyceride, the main form of stored fat in adipocytes, or into glycerophospholipids (Pickersgill et al., 2007). While these phospholipid molecules have no implication in insulin resistance, a number of less abundant glycerolipids have been identified as potential intermediates relating lipid overload to the impairment of insulin signalling and/or action (Holland et al., 2007).

Even though sphingolipids are less abundant than glycerolipids, numerous studies suggest that they have an important role in the regulation of insulin sensitivity during metabolic stress (Holland et al., 2007). Sphingolipid synthesis requires palmitoyl CoA, that together with serine work as the initial substrates for these lipid synthesis. To form palmitoyl CoA, the circulating palmitate has to be assimilated by cells where it is acylated (Hannun and Obeid, 2008). Saturated long-chain FFA palmitate (C16:0) is the most abundant FFA in circulation in obesity and type 2 diabetes conditions (20-30%). Excess of palmitate in circulation will lead to an increase in ceramide levels (Hannun and Obeid, 2008). Accumulation of ceramide in cells leads to suppression of Akt activation, which underlies its rapid effects on glucose uptake and anabolic metabolism. Ceramide appears to inhibit this signalling step through dephosphorylation of Akt and inhibition of the translocation and activation of Akt (Holland et al., 2007), leading to insulin resistance state. Excessive ceramide also activates PP1 phosphatase that can increase cytotoxic and apoptotic responses (Hannun and Obeid, 2008) (Figure 2).

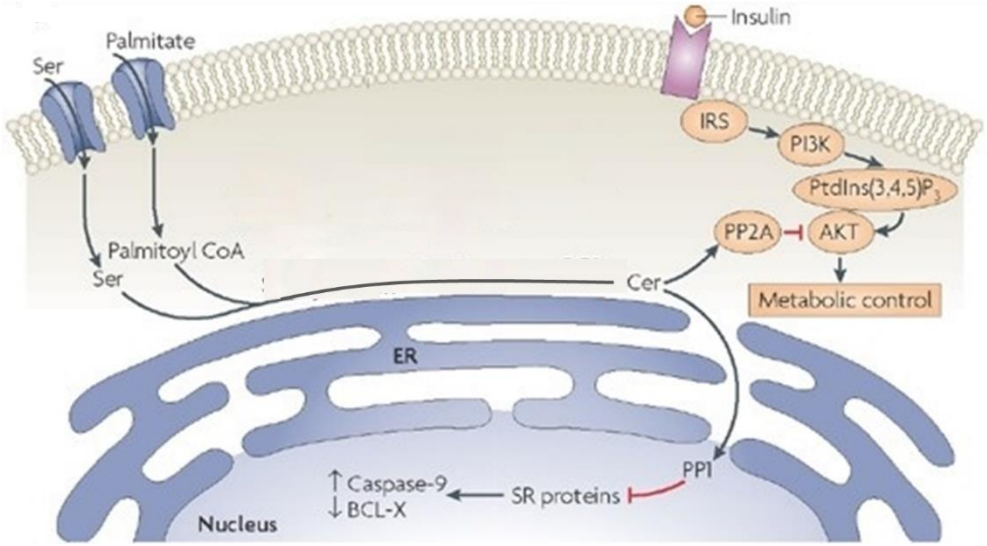

FIGURE 2 - INSULIN RESISTANCE AND PALMITATE SIGNALLING: Extracellular palmitate is assimilated by cells where it is acylated to form palmitoyl CoA, that with serine serve as the initial substrates for sphingolipid synthesis. Excessive palmitate leads to higher ceramide (Cer) levels. Accumulation of Cer activate protein phosphatase-2A (PP2A), resulting in inhibition of Akt, a key mediator of the metabolic effects of insulin. Excessive Cer also activates PP1 phosphatase that can increase cytotoxic and apoptotic responses. Adapted from: Hannun and Obeid, 2008

The levels of ceramides are elevated in muscle and liver from insulin-resistant rodents (Turinsky et al., 1990) as well as in humans (Adams et al., 2004). In several studies using different cell lines, such as C2C12 myotubes (Chavez et al., 2005; Chavez and Summers, 2003), 3T3-L1 adipocytes (Chavez and Summers, 2003), L6 myobtubes (Gao et al., 2009), mHypoE-44 neurons (Mayer and Belsham, 2010) and IVB hypothalamic cells (Benoit et al., 2009), palmitate was shown to induce insulin resistance, possibly by an increase in ceramide levels. However, incubating the same cells with unsaturated fatty acids, such as oleate, does not promote ceramide formation (Chavez et al., 2003; Holland et al., 2007).

The increased levels of FFA in plasma can also induce the release of inflammatory cytokines such as TNF-α, IL-1 and IL-6. When cultured adipocytes are exposed to these cytokines or stress signals, insulin-resistance is induced (Taubes, 2009). FFAs stimulate Toll-like receptor-mediated proinflammatory signalling, which activates IKKβ and JNK and stimulates production of cytokines. IKKβ and JNK are well-known serine kinases that phosphorylate IRS-1 at serine residues, leading to decreased metabolic signalling. JNK and IKKβ trigger activation of NF-kB, responsible for inflammatory cytokine

production (Figure 3). Inhibition of IKKβ or JNK with anti-inflammatory drugs or gene knockout improves insulin sensitivity concomitantly with reductions in serine phosphorylation of IRS proteins (Kim et al., 2008).

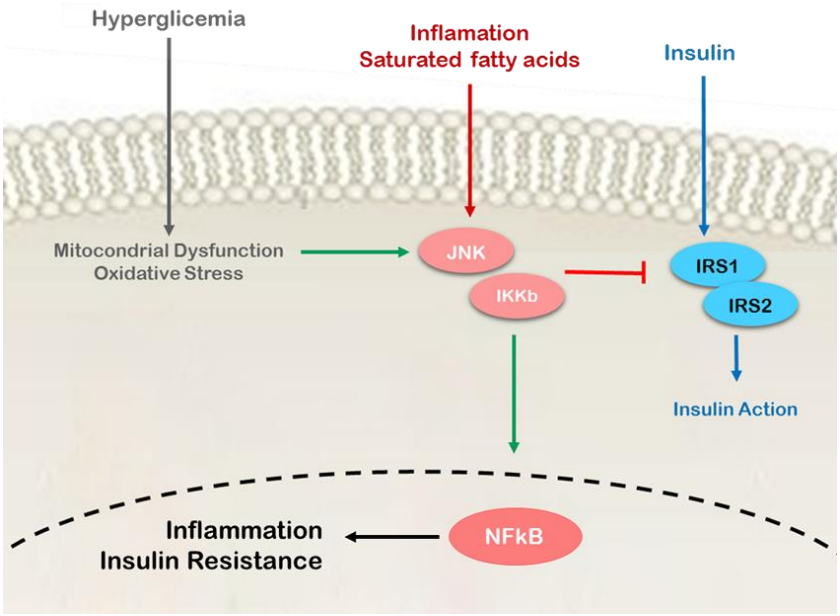

FIGURE 3 - INFLAMMATORY SIGNALLING IN INSULIN RESISTANCE: Cell surface insulin is transduced to cell by IRS phosphorylation. Hyperglycemia and FFA promote activation of IKKβ and JNK, serine kinases that phosphorylate IRS-1 leading to decreased insulin signalling. JNK and IKKβ activation triggers inflammatory cytokine production, by NF-kB activation, reinforcing insulin resistance.

Insulin resistance is also associated with mitochondrial dysfunction, which can result from genetic factors, oxidative stress, mitochondrial biogenesis, and aging. This impairment in mitochondrial function leads to ROS generation in peripheral tissues, such as liver, adipose tissue, and skeletal muscle, resulting in aggravated insulin resistance (Kim et al., 2008; Kleinridders et al., 2013). Increment in ROS concentrations leads to phosphorylation of IRS proteins, resulting in insulin resistance. Furthermore, ROS stimulate proinflammatory signalling by activation of IKKβ that phosphorylates IRS-1. Mitochondrial dysfunction results in accumulation of fatty acid metabolites, DAG, and long-chain fatty acyl-CoA (LCFA-CoA). Intracellular accumulation of DAG, allosteric activator of PKCs, activates PKCs that increase IRS proteins phosphorylation, leading to inhibition of insulin signalling and consequently to insulin resistance (Kim et al., 2008).

3. Hypothalamus and Insulin Resistance

3.1. Hypothalamus Nuclei and Neuropeptides

The brain, such as peripheral tissues, is vulnerable to metabolic stress (Kleinridders et al., 2013). The hypothalamus is a small brain structure located below the thalamus, capable to regulate appetite by detecting metabolic changes. This brain area is responsible to controlling neuroendocrine responses, temperature, circadian control and regulate hormonal cues such as insulin, leptin and ghrelin to regulate energy balance and glucose homeostasis, thus becoming a target of insulin resistance (Yue and Lam, 2012).

The hypothalamus consists of several interconnected nuclei: the arcuate nucleus (ARC), paraventricular nucleus (PVN), lateral hypothalamus (LH), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH) and suprachiasmatic nucleus (SCN) (Coll and Yeo, 2013).

The ARC of the hypothalamus has a defective blood-brain barrier (BBB). Thus, circulating hormones and nutrients can access the ARC without passing the BBB. Due to these anatomical features, the ARC is considered to be a hypothalamic area primarily sensing peripheral metabolic signals (Yu and Kim, 2012). The ARC has two distinct populations of neurons that have important effects on food intake, energy expenditure, and glucose homeostasis and are regulated by inputs from both hormonal and nutrient-related signals. One is a group of neurons coexpressing orexigenic neuropeptides, including neuropeptide Y (NPY) and agouti-related peptide (AgRP), that can stimulate food intake and reduce energy expenditure, and thereby promote weight gain. Consequently, a reduction in leptin or insulin allows an increase in NPY/AgRP neurons activity, causing insulin resistance and glucose intolerance (Schwartz and Porte, 2005; Yu and Kim, 2012). The other is a subset of neurons expressing anorexigenic neuropeptides, including proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) (Luthi-Carter et al., 2010; Yu and Kim, 2012) that innervate the same hypothalamic areas supplied by fibers from NPY/AgRP neurons. Unlike NPY/AgRP neurons, POMC neurons are stimulated by input from insulin and leptin and the binding of melanocortins to MC receptors inhibits food

intake and promotes weight loss (Figure 4). When neuronal input from leptin and insulin is reduced, POMC neurons are inhibited whereas NPY/AgRP neurons are activated, responses that in turn can cause hyperphagia, insulin resistance, and glucose intolerance (Schwartz and Porte, 2005).

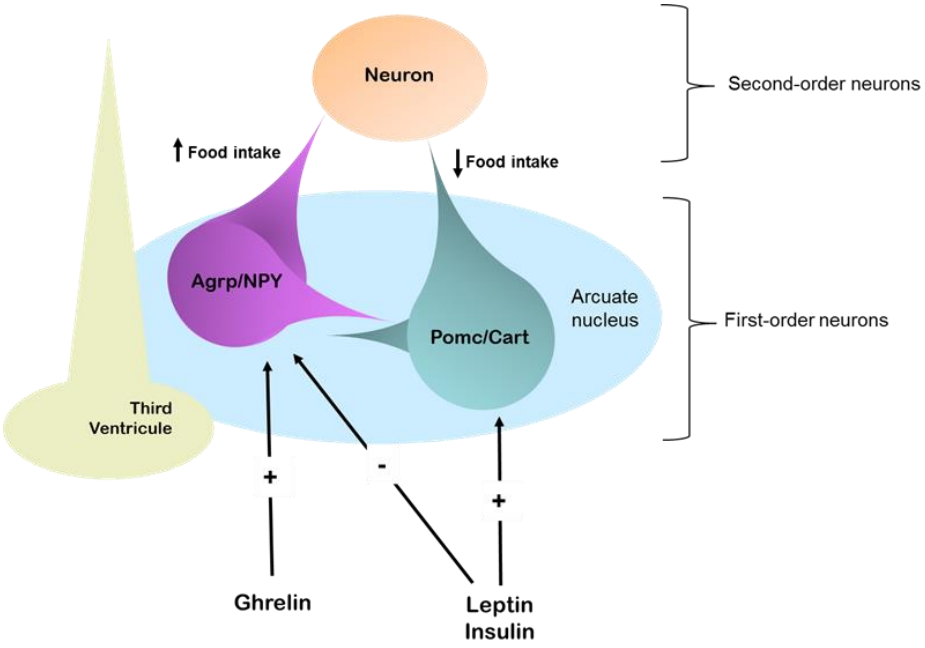

FIGURE 4 - NEURONAL CONTROL OF FOOD INTAKE: In arcuate nucleus of hypothalamus are present two different types of neurons to control energy homeostasis. One type produces neuropeptide Y and agouti-relate peptide, that stimulate food intake, and other type of neurons that produces proopiomelanocortin and cocaine- and amphetamine-regulated transcript protein, that inhibit food intake. Insulin and leptin are hormones that inhibit Agrp/NPY neurons and stimulates Pomc/Cart neurons. Thus, lower levels of insulin and leptin activate Agrp/NPY neurons and inhibit Pomc/Cart neurons. Ghrelin can activate Agrp/NPY neurons and stimulate food intake.

The PVN is located on the top of third ventricle in the anterior hypothalamus. This nucleus is an integrating center, where converge several neural pathways that influence energy homeostasis. It is supplied by axons projecting from the ARC-NPY/AGRP and POMC/CART neurons and from the orexin neurons. PVN contains terminals that have numerous appetite-modifying neurotransmitters. This nucleus is particularly sensitive to neurotransmitters effects on feeding and energy expenditures (Williams et al., 2001).

The suprachiasmatic nucleus (SCN) is considered the “biological clock” and is responsible to control circadian rhythms. This nucleus controls the sleep-wake cycle

and coordinates this with circadian rhythms in other brain areas and other tissues to enhance behavioural adaptation (la Fleur et al., 2001; Moore, 2007).

The lateral hypothalamus (LH) has a large and diffuse population of neurons, including neurons that express orexins and melanin-concentrating hormone (MCH), both involved in food intake stimulation. NPY terminals are abundant in the LH, in contact with orexin and MCH cells. Classically, LH was viewed as the ‘‘feeding center.’’ Stimulation of this nucleus increases food intake (Williams et al., 2001), while its destruction attenuates feeding and causes weight loss (Pimentel et al., 2014).

The DMH, located above the VMH, has direct connections with other hypothalamic nuclei such as the PVN and LH. The PVN and the DMH can cooperate functionally as a unit, which is involved in initiating and maintaining food intake. The DMH contains leptin receptors and some ARC-NPY/AGRP neurons also terminate in this nucleus (Williams et al., 2001).

The VMH, present in the mediobasal hypothalamus, is a bilateral cell group located above the median eminence (Choi et al., 2013). This nucleus is considered the primary ‘‘satiety center’’ in the hypothalamus (Williams et al., 2001), because it regulates body weight and energy homeostasis (King, 2006; Klöckener et al., 2011). Stimulation of the VMH inhibits feeding, whereas a lesion in this region causes overeating and weight gain (King, 2006). The VMH is a targeted site of leptin (Scott et al., 2009) and insulin (Choi et al., 2013) for the regulation of energy and glucose homeostasis. Previous studies suggest that direct application of leptin into the VMH, for instance, increases glucose uptake in skeletal muscle, heart, and brown adipose tissue (BAT), and this increased glucose uptake was reduced when the sympathetic nervous system (SNS) was denervated. This suggests that leptin signalling in the VMH plays a critical role in regulation of sympathetic tone from the VMH to peripheral tissues (Haque et al., 1999; Minokoshi et al., 1999; Toda et al., 2009).

3.2. Hypothalamic Insulin Resistance

Insulin mediates its biological effects through the insulin receptor (IR). This receptor is expressed in brain, with highest expression in the olfactory bulb, cortex, hippocampus, hypothalamus and cerebellum, and with relatively low levels in the

striatum, thalamus, midbrain, and brainstem (Fernandez and Torres-Alemán, 2012; Zhao et al., 2004).

Leptin/insulin crosstalk in the hypothalamus, especially in the VMH, impacts energy homeostasis in obesity and insulin-resistant states (Kleinridders et al., 2013). The fact that hyperinsulinemia and insulin resistance can be produced by hypothalamic lesions in VMH suggested a major role for the hypothalamus in regulation of insulin action (Kahn and Flier, 2000).

Previous studies indicate that insulin administration in rodents into the hypothalamic third ventricle can reduce food intake by a decrease in expression of the orexigenic neuropeptides NPY and AgRP, and by increasing the expression of anorexigenic neuropeptides proopiomelanocortin (POMC) (Schwartz et al., 2000). Neuron- specific loss of insulin receptors in brain leads to a modest increase in body fat (Brüning et al., 2000), and specific loss of insulin receptors in hypothalamic nuclei adjacent to the 3rd ventricle causes hyperphagia (Obici et al., 2002a). The role of hypothalamic nuclei in insulin sensing is still not fully determined. However, studies in rodent models suggest that central insulin signalling changes neural output to the liver and is required for the suppression of hepatic glucose production. Experiments manipulating the levels of insulin or its receptor in brain, particularly in hypothalamus, have demonstrated a role of central insulin signalling in the regulation of several peripheral tissues (Figure 5).

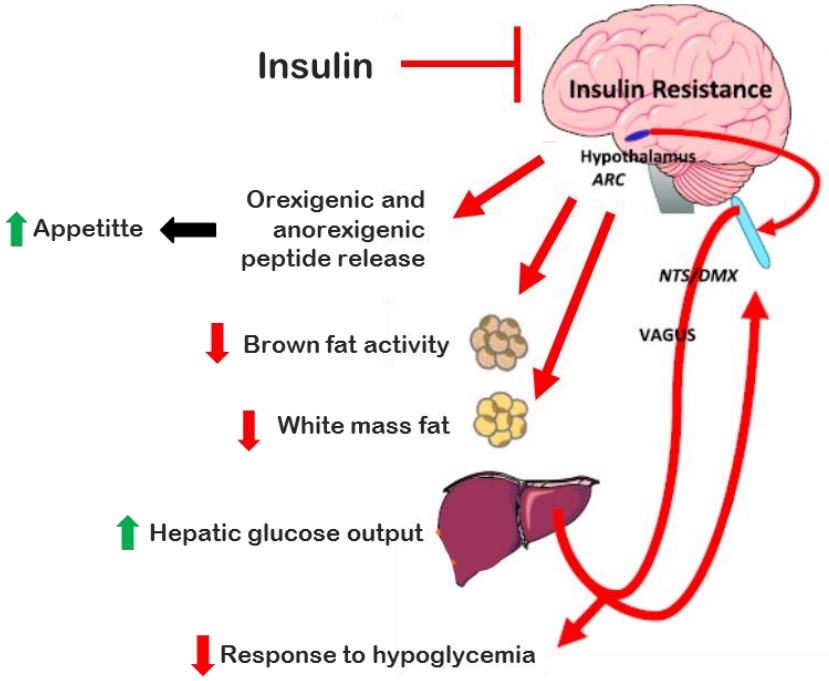

FIGURE 5 - EFFECTS OF INSULIN SIGNALLING IN HYPOTHALAMUS ON PERIPHERAL FUNCTIONS: Hypothalamic insulin resistance results in increased food intake, impaired of brown adipose tissue activity, decreased white fat mass, increased hepatic glucose output and impaired response to hypoglycemia. Adapted from: Kleinridders et al., 2014

For instance, the insulin suppression of glucose production in the liver (gluconeogenesis) is regulated by insulin receptors in liver and brain, mainly in hypothalamus, such that inactivation of IRs in each of these tissues causes a loss of insulin suppression of hepatic glucose production (Fisher and Kahn, 2003; Obici et al., 2002b). Similarly, injection of insulin into the hypothalamic third ventricle increases hepatic insulin sensitivity, through central pathways involving PI3K (Obici et al., 2002b).

It is known that obesity and type 2 diabetes, conditions normally associated with high-fat feeding, induce hypothalamic inflammation and insulin resistance, similar to what occurs in peripheral tissues and in other brain areas. Different studies indicate that hypothalamic lipid metabolism plays a role in energy homeostasis (López et al., 2008). The lipid overload, observed in obesity and type 2 diabetes, can induce insulin resistance in hypothalamus, similar to what occurs in peripheral tissues, due the accumulation of free fatty acid metabolites that impair insulin signalling (Yue and Lam, 2012).

It is also know that in fasting situations, hypothalamus has an important role in lipid metabolism. During fasting, ghrelin is released and activates hypothalamic AMP-

activated protein kinase (AMPK) resulting in deregulation of enzymes involved in fatty acid synthesis in hypothalamic nuclei, such as malonyl-CoA, CPT1 and FAS, indicating that modulation of hypothalamic fatty acid metabolism may be a possible player of energy homeostasis system, that integrates peripheral signals, such as ghrelin, with the central mechanisms regulating food intake (López et al., 2008).

Hypothalamic inflammation can also lead to resistance to anorexigenic hormones, such as leptin and insulin, and defective regulation of energy homeostasis. Prevention of hypothalamic inflammation restores leptin sensitivity and reduces adiposity.

Therefore, we can conclude that the hypothalamus plays an important role in insulin resistance states, showing that its effect goes much beyond the control of food intake and energy expenditure (Milanski et al., 2012). The hypothalamus-liver axis links insulin signalling in hypothalamus with autonomic innervation of the liver that plays a physiological role in the control of insulin sensitivity (German et al., 2009). This interaction occurs not only in physiological conditions but also in obesity. So, the hypothalamus has an important role in obesity and type 2 diabetes. Thus, it is possible that the improvement of insulin resistance in the brain can also lead to an improvement in peripheral insulin resistance (Milanski et al., 2012).

4. Sirtuins

4.1 Yeast Sir2

Studies of aging in yeast led to the discovery of a conserved family of NAD^+-dependent deacetylases known as the sirtuins (Haigis and Sinclair, 2010). These enzymes regulate metabolic pathways and sense alterations in energy status in mammals. Fluctuations of intracellular NAD^+ levels occur in response to nutrient availability. Consequently, sirtuins function as mediators to translate metabolic cues from the environment.

The founding member of the sirtuin family to be identified was the *Saccharomyces cerevisiae* NAD^+-dependent histone deacetylase *Sir2* (Chalkiadaki and Guarente, 2012). *SIR2* encodes a chromatin-silencing complex that represses gene transcription, silencing telomeres and recombinant DNA in yeast (Haigis and Sinclair, 2010). *SIR2* was capable to prolong lifespan in yeast by reducing the number of extrachromossomal rDNA. In nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster, SIR2* also functions to increase lifespan. Surprisingly, this capacity of *SIR2* is conserved in mammals, where mammalian sirtuins decelerate the decline in vital functions of tissues and organs associated with aging (Chalkiadaki and Guarente, 2012). The activation of these proteins contributes to prevention of type 2 diabetes, cancer, cardiovascular disease, inflammatory diseases and neurodegenerative conditions.

4.2 Mammalian Sirtuins

Sirtuins are the class III histone deacetylase family. Seven sirtuin isoforms (SIRT1-7) have been identified in mammalians which are characterized by their highly conserved central NAD^+-binding and catalytic domain named the sirtuin core domain (Haigis and Sinclair, 2010). Sirtuins regulate various cellular functions by deacetylating numerous substrates and modulating their activity. The deacetylation by sirtuins requires NAD^+ as a cofactor and involves an amide cleavage of NAD^+, and the formation of nicotinamide (NAM) (Figure 6) (Chalkiadaki and Guarente, 2012; Haigis and Sinclair, 2010).

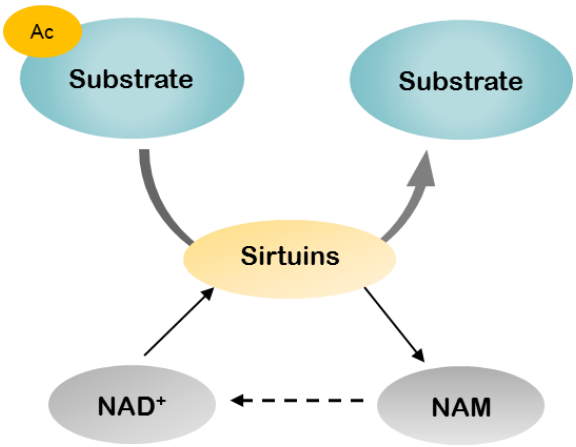

FIGURE 6 - SIRTUIN DEACETYLATION REACTION: Sirtuin deacetylases catalyze a biological reaction that consumes nicotinamide adenine dinucleotide (NAD^+) and releases nicotinamide (NAM), and the deacetylated substrate.

Increased NAD^+ levels, resulting from physiological changes induced by high energy demand states, including caloric restriction, fasting and exercise, lead to sirtuin activation that contributes to prevention of diabetes mellitus, cancer, cardiovascular disease, inflammatory diseases and neurodegenerative conditions (Chalkiadaki and Guarente, 2012).

Sirtuins are expressed in many tissues, such as the liver, kidney, adipose tissue, skeletal muscle and in several areas of the brain. Sirtuins are distributed in different cellular compartments. SIRT1, 6 and 7 are nuclear sirtuins and have a role in transcriptional regulation, through targeting of transcription factors, cofactors or histones. SIRT3, 4 and 5 are mitochondrial sirtuins and regulate the activity of enzymes and oxidative stress pathways. SIRT2, one of the least understood sirtuin isoforms, is the only cytoplasmic sirtuin member (Chalkiadaki and Guarente, 2012).

Based in their enzymatic activity, sirtuins might mediate the beneficial effects of caloric restriction (CR) since the levels of NAD^+ are elevated under this type of diet. CR is defined as a diet that supplies all essential nutrients with a lower content of calories (20-40% less calories) compared to *ad libitum* diet. CR is characterized by increased physical activity, improved glucose homeostasis, insulin sensitivity and extension of lifespan in several organisms (Chalkiadaki and Guarente, 2012). Recent studies propose that transgenic mice overexpressing sirtuins, especially SIRT1, show phenotypes resembling CR, including decreased insulin levels, lower body weight, reduction of fat

mass, lower levels of total cholesterol, improved glucose homeostasis, increased metabolic rate and higher oxygen consumption (Lavu et al., 2008). The expression level of SIRT1 increases upon CR in several rodent and human tissues such as white adipose, liver, skeletal muscle, brain and kidney (Haigis and Sinclair, 2010). In brain, SIRT1 is strongly expressed in the hypothalamus, especially in ARC and PVN, key nuclei involved in the control of energy balance (Satoh et al., 2010). CR increases significantly SIRT1 protein levels and induces neural activation in hypothalamus. In this brain area, SIRT1 functions as a key mediator of the central response to low nutritional availability, providing insight into the role of the hypothalamus in the regulation of metabolism and aging in mammals (Satoh et al., 2010).

There is also evidence that sirtuins can be involved in metabolic regulation. Metabolic abnormalities such as the increase in the levels of cholesterol, triglyceride and blood glucose, are normally associated with obesity and type 2 diabetes. There is evidence to support a relation between these metabolic disorders and sirtuins, in particular SIRT1. SIRT1 can inhibit PPARγ, the major regulator of adipogenesis, and by this way controls adipocyte differentiation and fat tissue accumulation. This sirtuin may also improve the secretion of insulin from pancreatic β cells by repressing the transcription of uncoupling protein 2 (Bordone et al., 2005; Moynihan et al., 2005). In addition, SIRT1 also mediates protection against hyperglycemia-induced toxicity and failure of pancreatic β cells by deacetylating FOXO1 in response to oxidative stress caused by hyperglycemia (Kitamura et al., 2005). Activation of SIRT1 by chemical activators, such as resveratrol, results in improved energy expenditure and increased metabolic phenotypes under obesity (Baur et al., 2006; Milne et al., 2007). SIRT1 overexpression in mice can also protect from the effects of high-fat diet. SIRT1 transgenic mice fed a high-fat diet are more glucose tolerant, leaner and present metabolic improvements than control mice.

High fat diet feeding can induce obesity and metabolic disorders in rodents that resemble the human metabolic syndrome, and for this reason it is considered a diet-induced obesity model. This type of diet contains about 32 to 60% of calories from fat. The majority of these diets contain saturated fat such as lard, beef tallow, or palmitic acid (Gajda, 2008). High fat diet allows the characterization of obesity development in an in vivo experimental setting that is pathophysiologically very similar to the human

disease. Due to this similarity, this experimental model is a huge contribution to the understanding of diet-induced obesity and insulin resistance and many pathophysiological concepts in the field, such as the importance of ectopic fat deposition, the interaction between inflammation and insulin resistance, and the mechanisms of lipotoxicity (Buettner et al., 2007).

The effects of SIRT1 on hepatic cholesterol, lipid metabolism and in skeletal muscle energy production possibly contribute to the increased insulin sensitivity and the improvement of metabolic phenotype in high fat diet animals.

Obesity and type 2 diabetes are characterized by a state of chronic low-grade inflammation that is tightly associated with the development of insulin resistance. SIRT1 regulates inflammation and energy balance by repressing the activity of NFκB, one of the main regulators of the inflammatory response (Schenk et al., 2008; Yeung et al., 2004). Interestingly, hypothalamic NFκB regulates central insulin and leptin signalling during obesity, and might be a SIRT1 target in this tissue (Purkayastha et al., 2011; Zhang et al., 2008b). SIRT6, other nuclear sirtuin, can also repress NFκB activity. SIRT6 also has a key role in glucose homeostasis, as shown in whole-body SIRT6-null mice, which demonstrate severe hypoglycemia and premature death (Mostoslavsky et al., 2006; Zhong et al., 2010). The metabolic defect in these mice is caused by increased glucose uptake by the liver and skeletal muscle (Zhong et al., 2010).

Although SIRT1 is the most studied sirtuin isoform, as illustrated above, it is possible that other sirtuins can also regulate important biological processes, such as metabolism and energy balance, through the deacetylation of substrates that are important to maintain metabolic homeostasis.

4.3 Sirtuin 2 (SIRT2)

SIRT2 has an important role in cell cycle regulation (North et al., 2003), neurodegenerative diseases (Outeiro et al., 2007) and tumor suppression (Kim et al., 2011). This sirtuin isoform is localized along the microtubule network and shows striking preference for acetylated tubulin peptide as a substrate, establishing SIRT2 as bona fide tubulin deacetylase (North et al., 2003).

Although SIRT2 displays ubiquitous expression, it is a highly abundant protein in the adult brain. Within the mouse brain, SIRT2 is expressed in different cellular types, including oligodendrocytes (Li et al., 2007), microglia (Pais et al., 2013), and neurons (Maxwell et al., 2011). SIRT2 expression has been reported in several regions of the central nervous system of the mice, including the cortex, hippocampus and spinal cord (Maxwell et al., 2011; Pais et al., 2013). No information is currently available regarding hypothalamic SIRT2 expression. As this brain area is a target of insulin resistance, it would be interesting to investigate if SIRT2 is present in the hypothalamus. This enzyme, due to its deacetylase capacity, may be involved in pathophysiology of insulin resistance.

SIRT2 is the most abundant sirtuin in adipose tissue (Jing et al., 2007), being downregulated in adipose tissue of obese subjects (Krishnan et al., 2012) and induced by caloric restriction. Adipose tissue plays an important role in metabolism regulation by storing excess energy and mobilizing the stored lipids for energy supply in case of need. Adipose tissue mass is regulated according to nutritional and physiological conditions. Many factors can be involved in regulation of adipose tissue formation. FOXO family of transcription factors regulate metabolism and stress resistance. FOXO1 transcription factor is a key component of insulin signalling cascade, important in controlling organism metabolism, growth and regulation of lifespan. FOXO1 inhibits adipogenesis through deacetylation, negatively regulating $PPAR_{\gamma}$ transcriptional activity (Wang and Tong, 2009). SIRT2 has the capacity to deacetylate FOXO1 and consequently reduces $PPAR_{\gamma}$ transcriptional activity (Jing et al., 2007). Besides the regulation of adipocyte differentiation, due to its deacetylase activity SIRT2 can also regulate sterol and fatty acid biosynthesis, gluconeogenesis and lipogenesis, (Jing et al., 2007; Krishnan et al., 2012; Wang and Tong, 2009). SIRT2 can also mitigate inflammation by deacetylating NF-kB (Pais et al., 2013). Both conditions are intimately intertwined with insulin resistance (Houstis et al., 2006).

Previous studies relate SIRT2 expression with insulin resistance in C2C12 skeletal muscle cells (Arora and Dey, 2014). However, a causal link between hypothalamic SIRT2 and insulin resistance has not yet been reported. Unpublished data from our laboratory indicate that SIRT2 is downregulated in insulin-resistant hepatocytes and mouse livers as well as in blood cells from obese insulin-resistant human subjects, and

this is mechanistically linked to increased oxidative stress and mitochondrial dysfunction. Importantly, SIRT2 overexpression in hepatocytes improves insulin sensitivity by reducing oxidative stress and improving mitochondrial dysfunction. These results suggest that SIRT2 activation may be a potential therapeutic approach in insulin-resistant states (Jing et al., 2007).

5. Hypothesis and Objectives

Assuming that SIRT2 is downregulated in hepatic insulin resistance and overexpressing SIRT2 restores hepatic insulin sensitivity, and that improving hypothalamic insulin signalling is able to partly restore glucose homeostasis in diabetes and obesity, we thus developed the hypothesis that hypothalamic SIRT2 activation may restore general metabolic homeostasis and improve central and peripheral insulin resistance in the setting of obesity and type 2 diabetes. Therefore, this project will test the possibility that SIRT2 may be expressed and regulated by energy availability in brain sites governing energy balance. It is thus important to confirm that SIRT2 is involved in the regulation of insulin signalling and sensitivity in the hypothalamus, since the link between hypothalamic SIRT2 and the regulation of insulin sensitivity has not been investigated.

This study is aimed to characterize the localization of SIRT2 in hypothalamic nuclei and to determine whether hypothalamic SIRT2 expression is regulated by changes in energy availability, specifically under caloric restriction and high fat diet-induced obesity and insulin resistance. We will also study different insulin resistance inducers in an attempt to find the inductor that promotes insulin resistance and investigate its effect in SIRT2 expression levels. We want to investigate the relation between the expression of this protein and insulin resistance development.

Chapter II

Materials and Methods

Experimental Animals

Male C57BL/6 mice were purchased from Charles Rivers Laboratories (Wilmington, MA, USA). Mice were housed under a 12 h light/dark cycle in a temperature/humidity controlled room. Control mice were maintained in standard chow diet having *ad libitum* access to water and food. Mice in caloric restriction diet had the food reduced by 30% compared with control mice. Mice in high-fat diet were fed with a diet containing 40% more fat than chow diet. These animals had *ad libitum* access to water and food. The dietary protocols were performed over a period of 8 weeks. After the diet period animals were sacrificed. All experimental procedures were performed in accordance with the European Union Directive 86/609/EEC for the care and use of laboratory animals. In addition, animals were housed in a licensed animal facility (international Animal Welfare Assurance number 520.000.000.2006). Moreover, people coordinating the animals studies have received appropriate education (FELASA course) as required by the Portuguese authorities.

Cell Culture

Clonal mouse hypothalamic mHypoE-N42 neuronal cells were obtained from CELLutions Biosystems. Cells were cultured in 100 mm culture plates and maintained at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) growth media (Glucose, 4.5 g/L; and sodium bicarbonate, 3.7 g/L) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, in a humid atmosphere with 37ºC, 5% CO_2.

Conjugation of fatty acids to bovine serum albumin

Stock solution of 10 % FFA- free BSA was prepared join free-fatty acids bovine serum albumin with serum-free culture medium, at 37ºC. After the FFA- free BSA be completely dissolved, the solution was filtered in a laminar flow hood, using a 0.2mm filter, and was stored at -20ºC. Stock solutions (100 mM) of palmitate (C16:0) (Sigma Aldrich; St. Louis, MO) were prepared in 0.1 M NaOH (70ºC for 30 minutes). Concentrated stock solutions of fatty acids (FFA) were added to a 10% fatty-acid-free bovine serum albumin (Sigma Aldrich; St. Louis, MO), to a concentration of 5 mM. The

solution was left at 37ºC for 1 hour, to allow FFA:BSA conjugation, and pH was adjusted to 7.4. After, FFA:BSA conjugation was filtered in a laminar flow hood, using a 0.2mm filter, and was stored at -20ºC.

Cell Treatments

mHypoE-N42 cells were cultured on 100 mm culture plates until near confluency (~3 days). To serum starvation experiments, 10 %FBS medium was replaced by a 0 %FBS medium 24 hours before the treatment. In all experiments the treatments were performed in serum-free medium. To determine the effect of different stimuli on insulin signalling and SIRT2 expression, mHypoE-N42 cells were treated for 24 hours with vehicle (BSA), palmitate (0.25 and 0.5 mM), insulin (100 nM) and glucose (25 mM).

Insulin Stimulation

Following chronic treatment with palmitate, insulin or glucose, the medium was rapidly removed and cells were acutely stimulated with recombinant human insulin (100 nM) in PBS (pH 7.4), at 37ºC during 15 minutes. After this period, plates were placed on ice and cells were rapidly rinsed with ice cold PBS. This solution was removed and cells were harvested by scraping in 200 µl of protein extraction buffer.

Protein extraction and quantification

Cells or tissue samples were prepared in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% (v/v) Triton X-100; 0.5 % (w/v) deoxycholate; 0.1 % (w/v) sodium dodecyl sulphate (SDS); Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Basel, Switzerland); 1 mM sodium orthovanadate (Orto); 1 mM dithiothreitol (DTT); 10 mM sodium fluoride (NaF); 200 µM phenylmethylsulfonylfluoride (PMSF)). After being sonicated (4 bursts of 5 seconds each), lysates were centrifuged (13.000 rpm; 20 min; 4°C) and protein concentration was determined by the bicinchoninic acid (BCA) method. The samples were denatured with 6x concentrated electrophoresis sample buffer (0.5 M Tris-HCl, pH 6.8; 30% (v/v)

glycerol; 10.4 % (w/v) SDS; 0.6 M DTT; 0.012% (w/v) bromophenol blue), heated at 95ºC for 5 min and stored at -20ºC until use.

Western Blotting

Proteins (30 µg) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10 % gels until an appropriate separation of the molecular weight standards was achieved. The electrophoresis was run on a Tris-Bicine buffer (25 mM Tris; 25 mM Bicine; 1% (w/v) SDS; pH 8.3), at 70 V in the first 10 min and after at 120 V. Proteins were electro-transferred to PVDF membrane using a transfer tank kept at 4ºC, with a constant current of 750 mA during 3 hours in CAPS transfer buffer (10 mM CAPS, pH 11.0; 10% (v/v) methanol). Membranes were blocked for 1 h at room temperature with blocking buffer (5% (w/v) non-fat dry milk or 5% (w/v) bovine serum albumin in Tris buffered saline (20 mM Tris; 137 mM NaCl; pH7.6) with 0.1% TWEEN-20. Membranes were then incubated overnight at 4ºC with the following primary antibodies: rabbit polyclonal anti-SIRT2 (1:1000; SIGMA S8447); rabbit polyclonal anti-pAKT (Ser 473) (1:1000; Cell Signalling 9271); rabbit polyclonal anti-Akt (1:1000; Cell Signalling 9272); mouse monoclonal anti-Gapdh (1:5000; ABCAM ab8245); mouse polyclonal anti-Actin (1:5000). After washing in TBS-T (four times, 10 minutes each), the membranes were incubated for 1 hour at room temperature with the respective alkaline phosphatase-linked secondary antibodies: anti-rabbit (1:10 000) and anti-mouse (1:10 000). Membranes were washed four times and antibody detection was performed with ECF substrate. Scanning was performed using VersaDoc Imaging System (Bio Rad) and quantification of the bands was performed using Quantity One Software (Bio Rad).

Stereotaxic surgeries

BSA or BSA/palmitate were injected bilaterally into the hypothalamus (VMH) of male mice. For that mice were anesthetized with an intraperitoneal injection of avertin. Scalp fur was shaved and prepared using povidone-iodine USP and alcohol 70% pads. Mice were placed into a stereotaxic device (Kopf Instruments) with lambda and bregma oriented at the same vertical coordinate. A small midline incision was

made over the dorsal scalp to provide access to the cranium, and the cranial surface was cleaned. Coordinates for needle placement to target the VMH were 1.4 mm posterior to bregma (AP = –1.4) and 0.45 mm lateral to the midline (L = ±0.45). A skull window was outlined with a fine drill, and a 26-gauge stainless steel needle was lowered until it reaches the meninx to establish "0". The needle was subsequently placed at –5.3 mm for the dorsoventral coordinates.

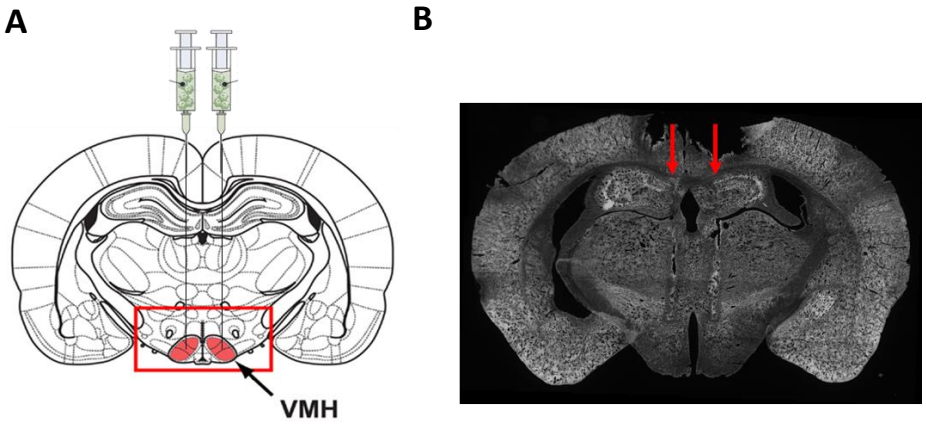

FIGURE 7 – STEREOTAXIC SURGERIES IN VMH OF MICE: A) Schematic illustration and B) immunohistochemistry image of stereotaxic surgery performed in male mice to injected BSA and BSA/Palmitate in VMH. The coordinates used in this approach were 1.4 mm posterior to bregma (AP = –1.4), 0.45 mm lateral to the midline (L = ±0.45), and -5.3 mm for dorsomedial coordinates.

Then, 2 µl of vehicle (1% BSA) or 2 µl of 1 mM palmitate (complexed with BSA) were injected through the needle over a 0.25 µl/min period (total time = 8 min). The needle was kept in place for an additional 5 minutes to allow the drug to diffuse away from the needle tip. After the recovery period (48h), mice were anesthetized, sacrificed, perfused with PFA and the brain was removed and processed for immunohistochemistry.

Immunohistochemistry

Mice were anesthetized with avertin before sacrifice. Animals were perfused with PBS followed by 4% paraformaldehyde (PFA). Brains were fixed with 4% PFA overnight and placed into 25% sucrose. Brain coronal sections from mouse were cut to 25 µm using a cryostat at -21ºC. For each brain were used two brain sections, the first section around bregma -0.82 mm, for the PVN and SCN, and the second brain section around

bregma -1.58 mm, to cover the Arc, VMH, DMH, and LH. Sections were incubated in a blocking and permeabilization solution, containing 10% normal goat serum, 3% bovine serum albumin and 0.3% Triton X-100. Sections were then incubated overnight at 4ºC with rabbit polyclonal anti-SIRT2 (1:750; SIGMA S8447) used to investigate protein localization and to quantify the expression of SIRT2. For double immunostaining sections were also incubated with mouse monoclonal antibodies: anti-glial fibrillary acid protein (GFAP, 1:500) used to visualize astrocytes, anti-Neuronal Nuclei (NeuN, 1:500) used to visualize neurons and anti-CD11b (1:500), used to visualize microglia. After, sections were washed in PBS (three times, 15 min each) and incubated for 2 h at room temperature with respective secondary antibodies, both from Live Technologies: Alexa-Fluor 488-conjugated goat anti-rabbit IgG (1:200), Alexa-Fluor 594-conjugated goat anti-mouse IgG (1:200), and also with Hoechst 33342 (1:1000). Sections were washed in PBS (two times, 15 min each), mounted in slices and observed in the inverted widefield microscope Carl Zeiss Axio Observer Z1, using the digital CMOS camera (ORCA Flash 4.0), and the 40x objective and in Carl Zeiss Cell Observer Spinning Disk microscope, using the 20x objective, to investigate SIRT2 distribution in hypothalamus and in confocal microscope, to assess the co-localization of SIRT2 with markers of different cell types.

SIRT2 protein signals were quantified by measuring the intensity per area in each hypothalamic nucleus using Image J software.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analysed using Student's unpaired t-test with two-tailed p value, as indicated in figure legends. A value of $p < 0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism Software.

Chapter III

Results

1. SIRT2 is expressed in the mouse hypothalamus and enriched in the VMH

To investigate the presence of SIRT2 in the mouse hypothalamus, we performed western blot analysis using different tissues where SIRT2 expression has been previously reported, including the liver and the cerebral cortex, in comparison with the hypothalamus. We found that SIRT2 immunoreactivity is also present in the mouse hypothalamus as well as in mHypoN42 hypothalamic neurons. The anti-SIRT2 antibody did not produce any significant positive signals on SIRT2-deficient brain lysates, assuring its specificity (Figure 8).

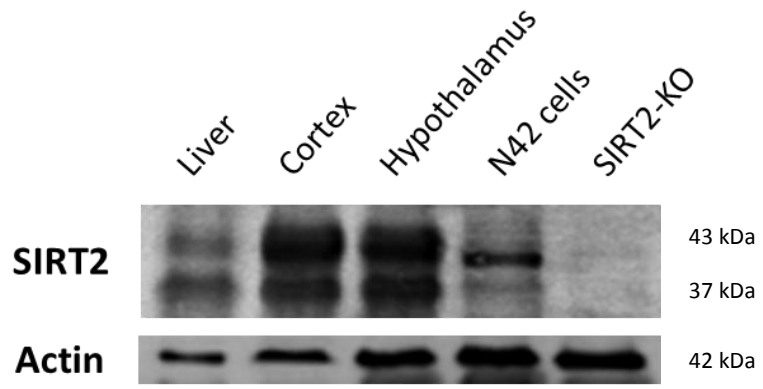

FIGURE 8 - SIRT2 IMMUNOREACTIVITY IN THE MOUSE HYPOTHALAMUS: Western blot showing SIRT2 in extracts of liver, cortex and hypothalamus from WT mice and from hypothalamic mHypoN42 cells. SIRT2-KO mice (whole brain) were used as a negative control. These results are representative of 3 independent experiments.

In order to confirm the previous results and to investigate the anatomical distribution of SIRT2 in the different hypothalamic nuclei, brain sections of WT mice fed a chow diet were stained with anti-SIRT2 antibody and the intensity of the signal was quantified. In an attempt to evaluate SIRT2 expression in the most relevant hypothalamic nuclei, two different brain coronal sections were used, bregma -0.82 mm, for the PVN and SCN, and bregma -1.58 mm, to cover the Arc, VMH, DMH, and LH. Our data reveal the presence of SIRT2 in the mouse hypothalamus and the expression of this protein in major hypothalamic nuclei (PVN, SCN, Arc, DMH, LH and in VMH). Interestingly, SIRT2 expression is enriched in the VMH (Figure 9).

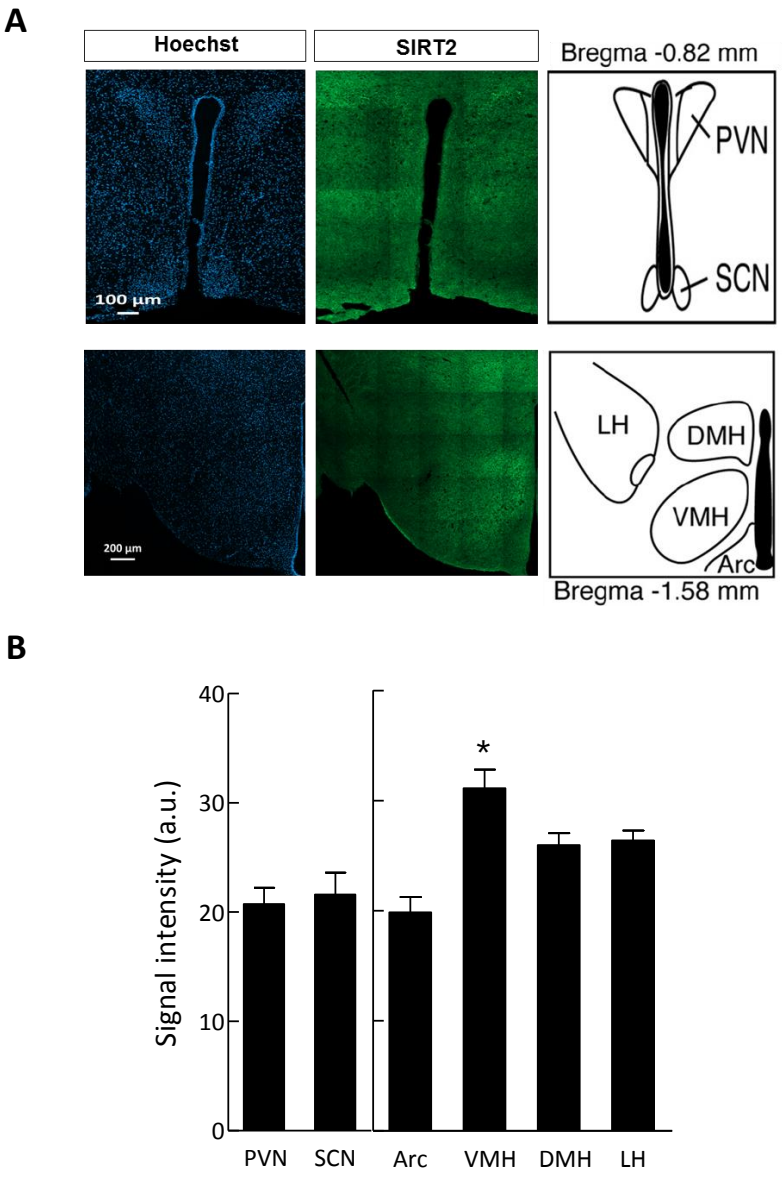

FIGURE 9 – SIRT2 IMMUNOREACTIVITY IN MOUSE HYPOTHALAMIC NUCLEI: (A) Immunofluorescent staining of SIRT2 (green) in major hypothalamic nuclei, including the PVN, SCN, Arc, VMH, DMH, and LH. Nuclei are counterstained by Hoechst (blue). (B) Signal intensities of SIRT2 staining in hypothalamus of mice fed a regular chow diet ad libitum (n=6; 2 sections per hypothalamic nucleus). Results are shown as mean ± SEM; n=6-9 mice per group. *p<0.05, significantly different from all the other hypothalamic nuclei, as determined by unpaired Student's t-test.

2. SIRT2 is a neuronal protein

In order to characterize the type of cells in the hypothalamus containing SIRT2 in WT mice under chow diet, we performed double immunofluorescent staining with SIRT2 and the neuron specific marker NeuN, the astrocyte specific marker GFAP, and activated microglia marker CD11b. We did not detect a significant co-localization

between SIRT2 and markers specific to either astrocytes or microglia. In contrast, we observed a significant co-localization between SIRT2 and the neuronal marker NeuN, suggesting that SIRT2 is expressed in hypothalamic neurons (Figure 10).

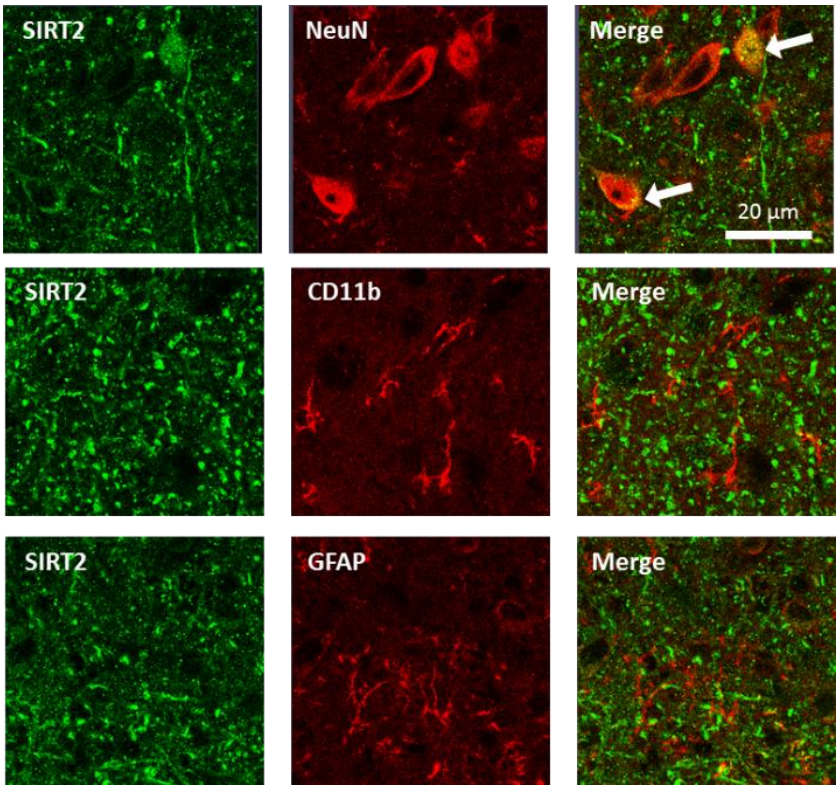

FIGURE 10 - SIRT2 IS A NEURONAL PROTEIN: Immunofluorescent staining of SIRT2 (green), NeuN (neurons, red), GFAP (astrocytes, red) and CD11b (activated microglia, red) in the VMH of WT mice under chow diet.

3. SIRT2 protein levels in VMH are downregulated upon HFD feeding

In order to investigate the role of SIRT2 as a nutrient sensor, we performed immunohistochemistry analysis using brain coronal sections of mice fed a chow diet, a caloric restriction diet and a high-fat diet. Sections were stained with anti-SIRT2 antibody and the intensity of the signal was quantified. Because SIRT2 is enriched in the VMH, we decided to investigate the expression of the protein specifically in this hypothalamic nucleus. SIRT2 immunoreactivity in the VMH of caloric-restricted mice showed no significant difference compared to VMH of mice under chow diet. However, the levels of SIRT2 immunoreactivity in the VMH of mice fed a high-fat diet were significantly lower (57.7%± 7.9%), compared to VMH of control (chow) diet (Figure 11). These results indicate that SIRT2 protein levels in VMH are downregulated in HFD.

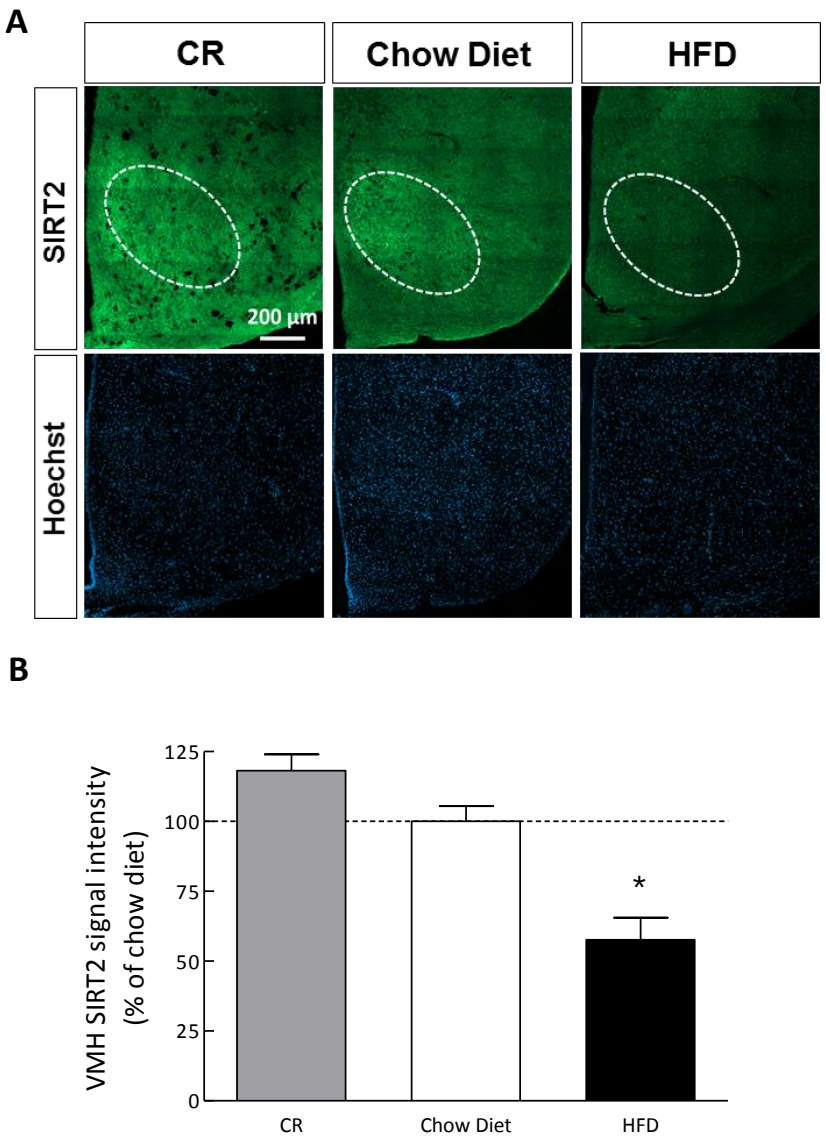

FIGURE 11 – VMH SIRT2 PROTEIN EXPRESSION IS REDUCED BY CHRONIC HIGH FAT DIET FEEDING: A) Images and B) quantification of SIRT2 immunoreactivity in the ventromedial nucleus of the hypothalamus (VMH) in mice fed a regular chow, a high fat diet (HFD) or under calorie restriction (CR). Results are shown as mean ± SEM; n=3-6 animals per group. *p<0.05, significantly different from Chow diet mice, as determined by unpaired Student's t-test.

4. Palmitate attenuates insulin signalling and concomitantly downregulates SIRT2 expression

Given that SIRT2 is a neuronal protein we decided to use a mouse embryonic hypothalamic neuronal cell line, mHypoN42, in subsequent experiments in an attempt to understand the role of SIRT2 in insulin sensitivity. To assess insulin sensitivity we investigated the levels of phosphorylated Akt, a protein involved in insulin signalling

pathway, by western blot. In normal conditions, this protein is phosphorylated when cells are stimulated with insulin. However, under insulin resistant conditions, insulin fails to induce Akt phosphorylation and based in this alteration it is possible to investigate insulin resistance status of the cell. To identify the time and concentration of insulin capable to promote an response in Akt phosphorylation, mHypoN42 hypothalamic neurons were stimulated with insulin, at different time points (0-60 minutes) under a fixed concentration of insulin (100 nM) and different concentrations (0-1000 nM), during a period of 15 minutes. We observed an increase in Akt phosphorylation in a time- and dose- dependent manner (Figure 12). Based in these results we decided to use in subsequent experiments 100 nM of insulin, during 15 minutes, to stimulate Akt phosphorylation and thus to be able to assess insulin resistance status of mHypoN42 hypothalamic neurons.

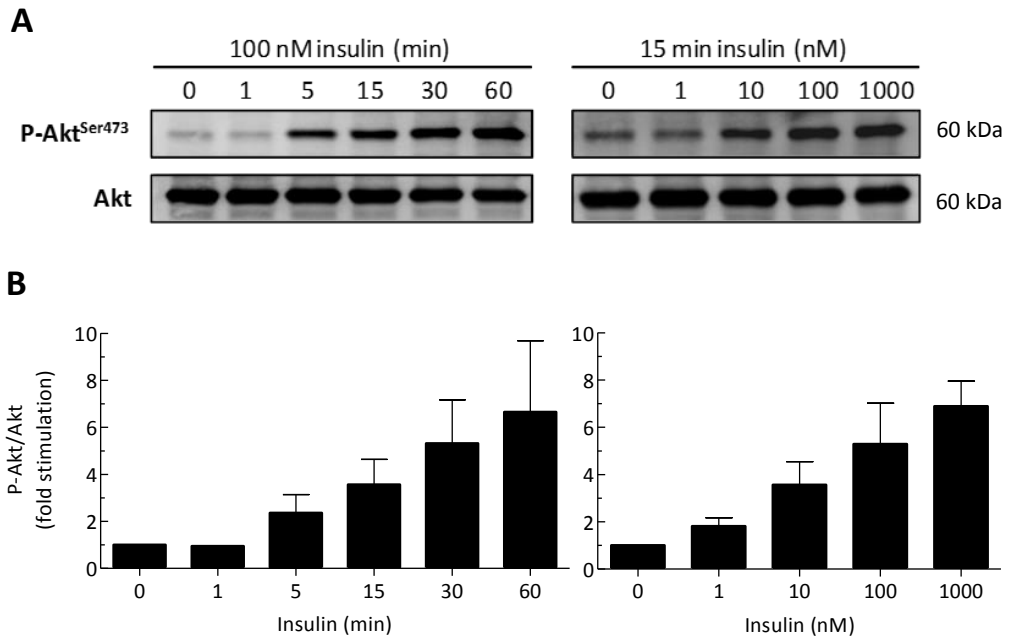

FIGURE 12 – INSULIN TIME COURSE AND CONCENTRATION: A) Immunoblot and B) quantification of the levels of p-Akt after insulin stimulation at different time points (0-60 min) and with different concentrations (0-1000 nM). Results are shown as mean ± SEM; n=4 independent experiments.

Hyperglycemia, hyperinsulinemia and lipid overload are characteristic of obesity and type 2 diabetes. We decided to investigate the contribution of each of these factors to insulin resistance in hypothalamic neurons. In an attempt to create an in vitro model of insulin resistance, mHypoN42 hypothalamic neurons were treated with

25 mM of glucose, 100 nM of insulin or 500 µM of palmitate for 24 hours. After this period, cells were exposed to 100 nM of insulin for 15 minutes. Cells treated with 1%BSA, the vehicle needed to palmitate enter in the cell, were used as control. Through the levels of Akt phosphorylation we observed that in control, the levels of p-Akt after insulin stimulation undergo a 4.13 ± 0.55 fold increase. Glucose treatment had no effect in insulin-induced Akt phosphorylation levels (4.59 ± 0.43 fold increase). The exposure to 100 nM of insulin promoted a decrease in in insulin-enhanced p-Akt levels (2.23 ± 0.15 fold increase), an indicative factor of insulin resistance. Similarly, palmitate exposure (500 µM; 24 hours) led to a reduction in p-Akt levels, an indicative factor of insulin resistance (1.12 ± 0.19 fold increase) (Figure 13).

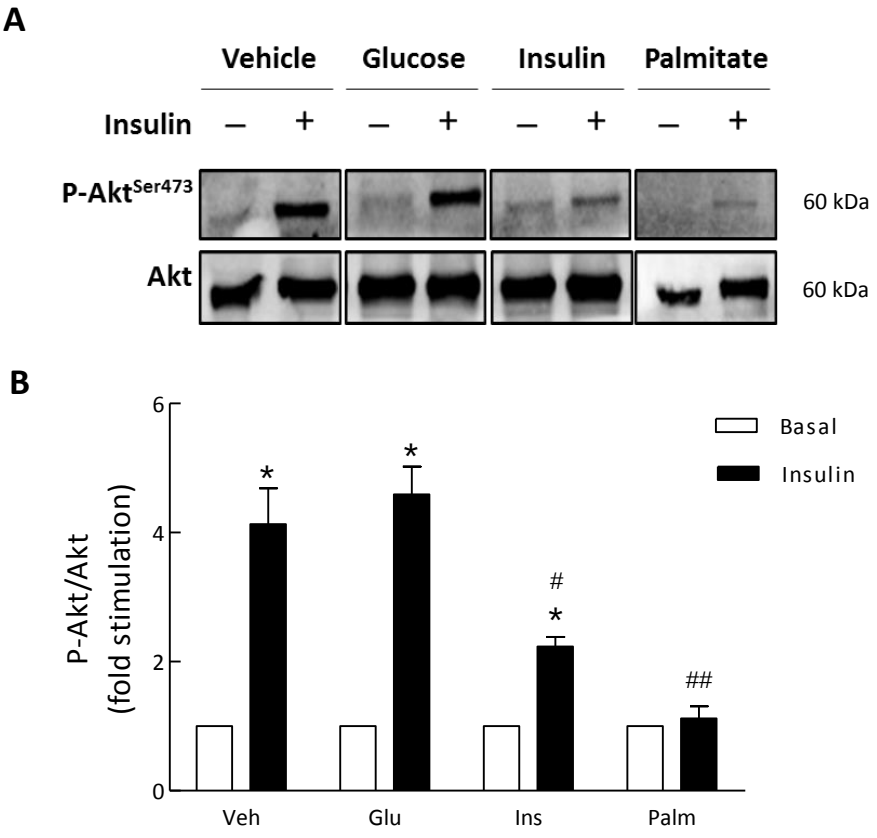

FIGURE 13 – PALMITATE AND CHRONIC INSULIN INDUCE INSULIN RESISTANCE IN MHYPON42 CELLS: A) Immunoblot and B) quantification of the levels of p-Akt after 24 hours of exposure to high glucose (25 mM; Glu), chronic insulin (100 nM; Ins) and palmitate (500 µM; Palm), and after insulin stimulation (100 nM; 15 min). Results are shown as mean ± SEM; n=3-4 independent experiments. *p<0.05 significantly different from cells in basal conditions with the same treatment; #p<0.05, ##p<0.01, significantly different from control cells treated with BSA and stimulated with insulin, as determined by unpaired Student's t-test. Veh- vehicle.

To investigate the role of hypothalamic SIRT2 in insulin resistance conditions, we decided to assess the levels of this protein in mHypoN42 hypothalamic neurons, in the same experimental conditions used in the previous experiment. In this experiment we only use the basal conditions to investigate the protein expression. Cells treated with 1% BSA (vehicle) were again used as control, and in this group the expression of SIRT2 was normalized to 100%. We observed that in high glucose (79% ± 1.8%) and chronic insulin (75.6% ± 4.3%) exposure occur a slight decreased in SIRT2 expression, when compared with SIRT2 expression in vehicle (control). However, SIRT2 expression decreased significantly (30.7% ± 1.5%) in mHypoN42 hypothalamic neurons treated with palmitate (Figure 14).

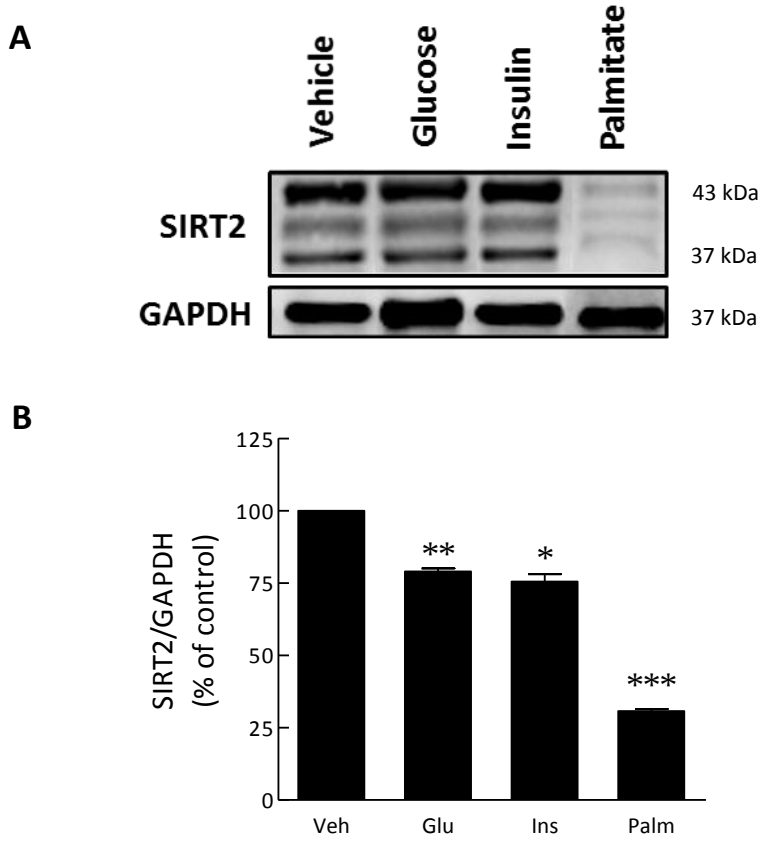

FIGURE 14 - PALMITATE DOWNREGULATES SIRT2 EXPRESSION IN MHYPON42 CELLS: A) Immunoblot and B) quantification of SIRT2 levels after 24 hours of exposure to high glucose (25 mM; Glu), chronic insulin (100 nM; Ins) and palmitate (500 µM; Palm), in basal conditions. Results are shown as mean ± SEM; n=3-4 independent experiments. *p<0.05, **p<0.01, ***p<0.001, significantly different from control cells treated with BSA, as determined by unpaired Student's t-test. Veh-vehicle.

5. Serum starvation ameliorates insulin sensitivity under normal conditions in N42 cell line

In an attempt to mimic the effect of caloric restriction, mHypoN42 hypothalamic neurons were serum starved for 24 hours prior to palmitate treatment. To investigate the effect of serum starvation in insulin sensitivity we investigated the levels of p-Akt in normal and serum starvation conditions. In both conditions one group of cells were maintained in basal conditions and another were stimulated with insulin (100 nM; 15 minutes). We observed that in control cells the levels of insulin-induced p-Akt stimulation increased 4.13 ± 0.55 fold. However, after 24 hours of serum starvation the levels of Akt phosphorylation nearly doubled (8.76 ± 0.44 fold increase) (Figure 15). These results suggest that serum starvation promotes an increase in insulin sensitivity in mHypoN42 hypothalamic neurons.

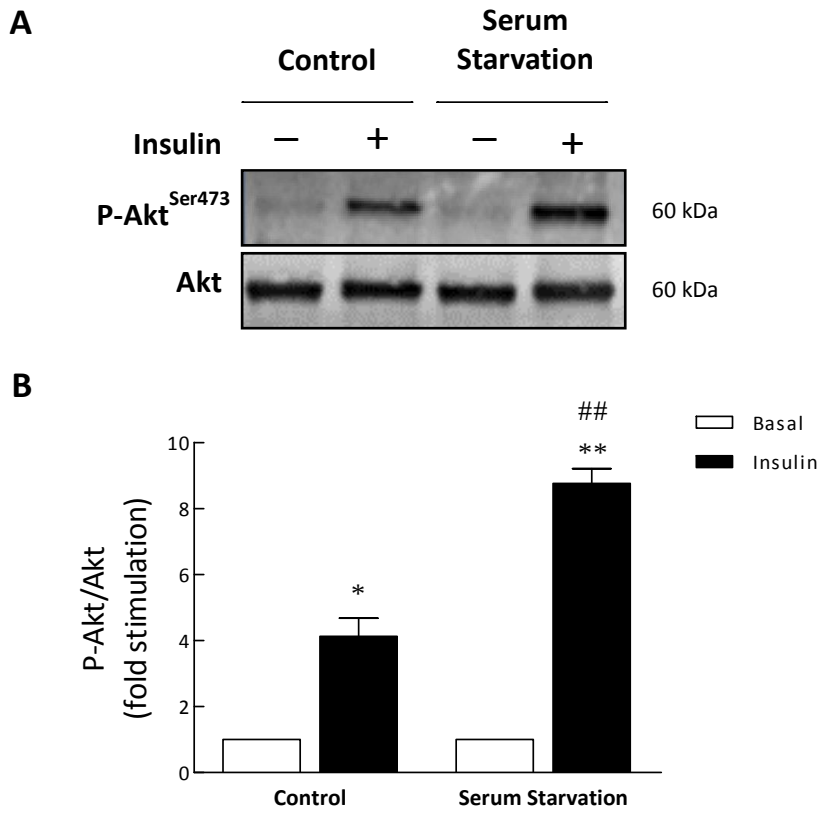

FIGURE 15 - SERUM STARVATION PROMOTES INSULIN SENSITIVITY IN MHYPON42 CELLS: A) Immunoblot and B) quantification of p-Akt levels after 24 hours of exposure to palmitate (500 µM) and after acute insulin stimulation (100 nM; 15 min). Results are shown as mean ± SEM; n=3-4 independent experiments. * p<0.05, ** p<0.01, significantly different from basal cells in the same treatment; ##p<0.01, significantly different from control cells treated with BSA 1% in serum presence and stimulated with insulin (100 nM; 15 min), as determined by unpaired Student's t-test.

6. Serum starvation prevents palmitate-induced insulin resistance and SIRT2 downregulation

Having determined that serum starvation increases insulin sensitivity under control conditions, we decided to evaluate the effect of serum removal on palmitate-induced insulin resistance. For that was tested two different concentrations of palmitate, 250 µM and 500 µM, for 24 hours. After treatments cells were stimulated with insulin (100 nM; 15 minutes). The experiments were performed in normal and serum starvation conditions. Cells treated with 1% BSA (vehicle) and stimulated with insulin were used as control, in both situations, and in this group the expression of p-Akt was normalized to 1. In the presence of serum the lower concentration of palmitate had no effect in insulin-induced Akt phosphorylation (0.94 ± 0.08 fold increase). However, in the higher palmitate concentration (500 µM), p-Akt levels decreased significantly (0.35 ± 0.04 fold increase), an indication of insulin resistance. Under serum starvation, palmitate had no effect in insulin-stimulated Akt phosphorylation (Figure 16). These results suggest that 24 hours of serum starvation can prevent the effect of the saturated fatty-acid palmitate in the decrease of Akt phosphorylation, and consequently inhibited the induction of insulin resistance by this FFA in mHypoN42 hypothalamic neurons.

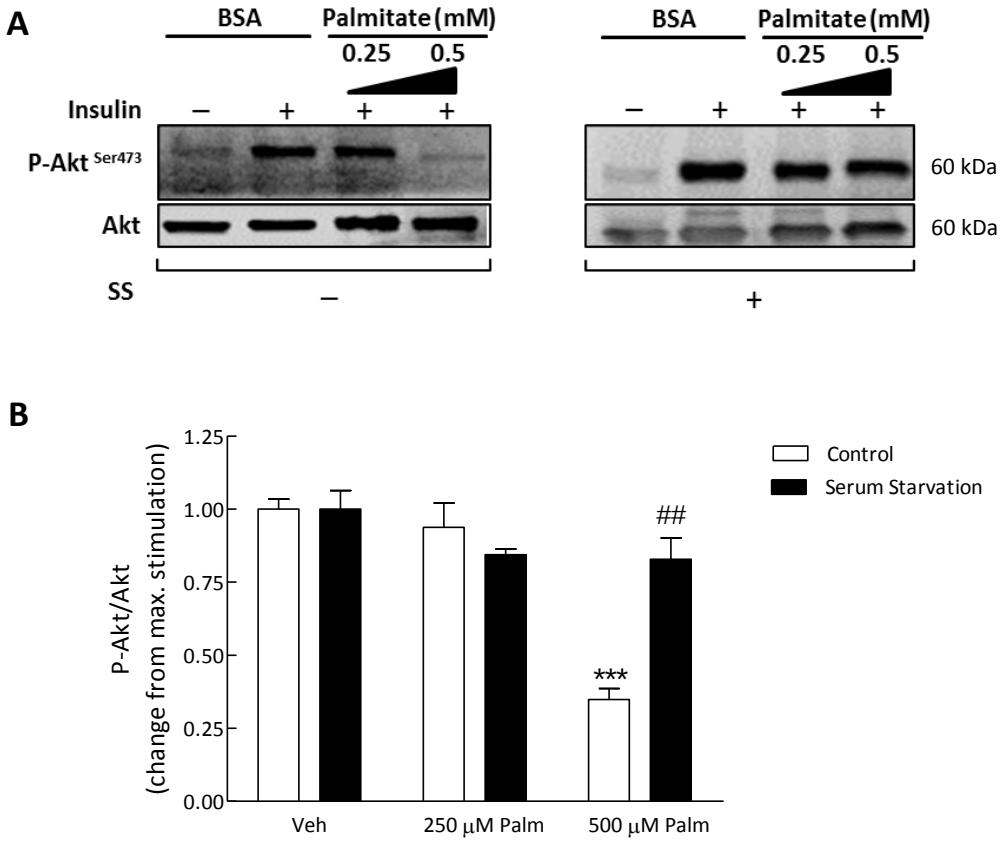

FIGURE 16 – SERUM STARVATION PREVENTS PALMITATE-INDUCED INSULIN RESISTANCE: A) Immunoblot and B) quantification of p-Akt levels after 24 hours of exposure to vehicle or palmitate (250 and 500 μM), and after insulin stimulation (100 nM; 15 min). Cells were serum starved during a period of 24 hours. Results are shown as mean ± SEM; n=4-6 independent experiments. ***p<0.001 significantly different from BSA treatment; ## p<0.01, significantly different from cells treated with 500 μM of palmitate in serum presence, as determined by unpaired Student's t-test.

In an attempt to investigate the behaviour of SIRT2 expression in serum starvation conditions we decided to check the levels of the protein in the same conditions used in the previous experiment. We observed that in normals conditions the levels of SIRT2 decrease in a dose-dependent manner. The lower dose of palmitate promotes a significant decrease in SIRT2 levels (71% ± 4.2%). In the higher dose of palmitate, SIRT2 expression presents a strong decrease (30% ± 2.1%). After 24 hours of serum starvation palmitate had no effect in SIRT2 expression (Figure 17). These results suggest that 24 hours of serum starvation can also prevent the decrease of SIRT2 levels that is normally induced by palmitate.

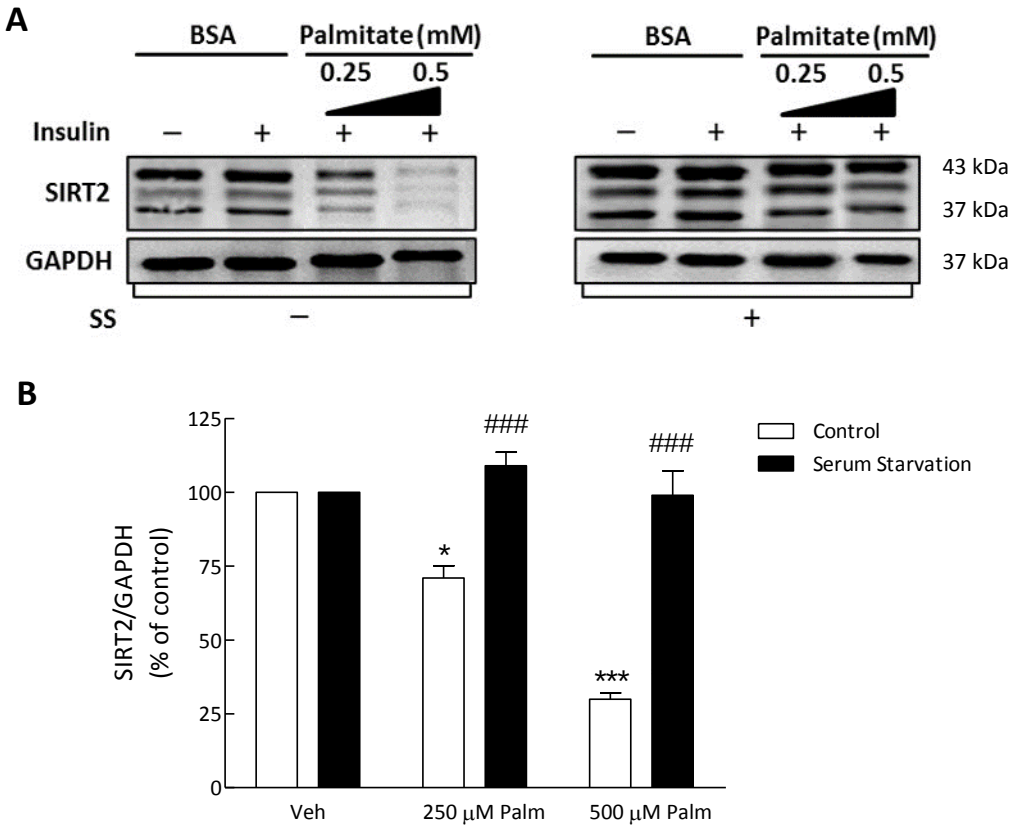

FIGURE 17 - SERUM STARVATION PREVENTS PALMITATE-INDUCED SIRT2 DOWNREGULATION: A) Immunoblot and B) quantification of SIRT2 levels after 24 hours of exposure to vehicle or palmitate (250 and 500 μM), and after insulin stimulation (100 nM; 15 min). Cells were serum starved during a period of 24 hours. Results are shown as mean ± SEM; n=3-6 independent experiments. *p<0.05, ***p<0.001 significantly different from BSA treatment; ###p<0.001 significantly different from control cells in the same treatment, as determined by unpaired Student's t-test.

7. Palmitate induces SIRT2 downregulation in mice VMH

Our previous results indicated that SIRT2 is downregulated in the VMH of mice fed a HFD, and that treatment with saturated fatty acid palmitate induces insulin resistance and concomitantly downregulates SIRT2 expression in mHypoN42 hypothalamic neurons. It is known that palmitate is the most abundant FFA in high fat diet. Based in these observations we decided to investigate the effect of palmitate administration directly into the VMH of adult mice. For that we injected bilaterally 1 mM of palmitate into the VMH of WT adult mice fed a standard chow diet. Animals were sacrificed 48 hours after the surgery. We also injected a group of mice with BSA, normally used as a vehicle that allows palmitate to enter in cells, to confirm that the effect occurs due to palmitate and not to vehicle. Mice that did not receive any

injection were used as controls in this experiment. Assuming that SIRT2 expression in non-injected mice is 100%, we do not observe any alteration in protein expression in mice injected with BSA (103.0% ± 7.9%), indicating that BSA is not responsible for any alteration in SIRT2 expression. However, when we analysed mice injected with palmitate (1 mM), we saw a significant decrease in SIRT2 expression (59.6% ± 4.6%) (Figure 18). This effect seems restricted to the injected area. These results suggest that palmitate may be a major contributor to SIRT2 downregulation in the VMH in HFD adult mice.

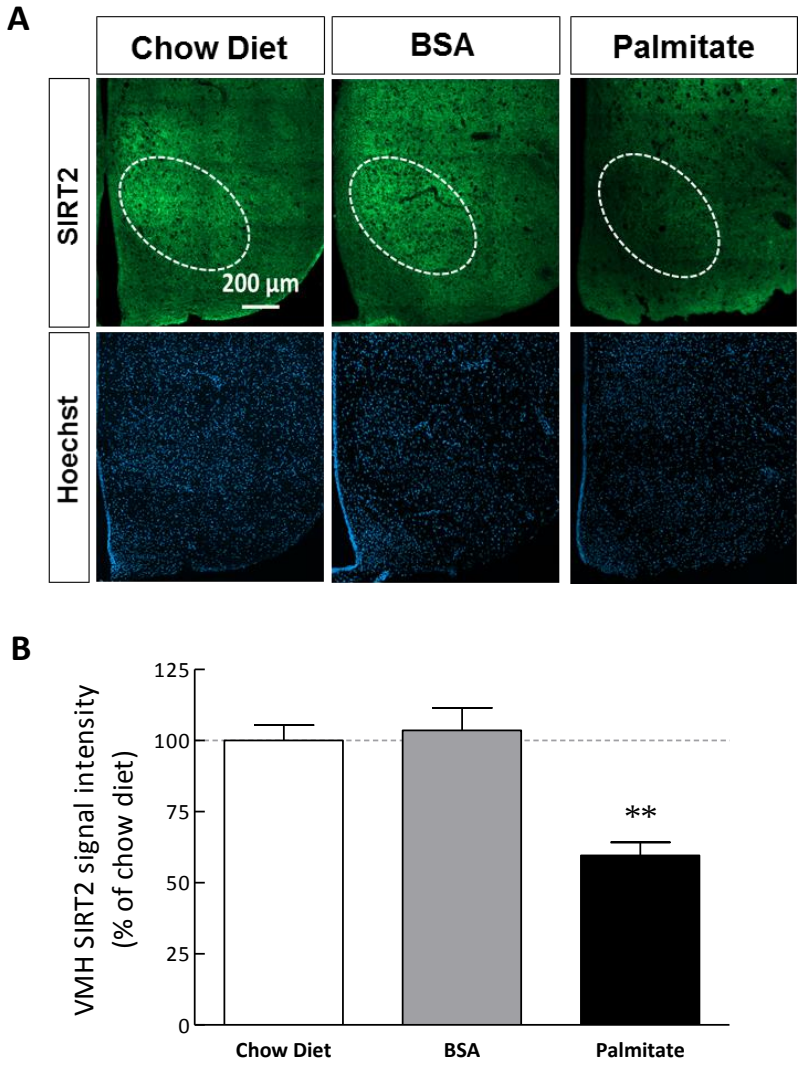

FIGURE 18 – PALMITATE DOWNREGULATES SIRT2 EXPRESSION IN VMH OF MICE FED A CHOW DIET: A) Images and B) quantification of SIRT2 immunoreactivity in the ventromedial nucleus of the hypothalamus (VMH) of chow diet fed mice no-injected, injected with BSA (1%) and injected with palmitate (1 mM), during 48 hours. Results are shown as mean ± SEM; n=3-6 animals. **p<0.01, significantly different from Chow diet fed mice, as determined by unpaired Student's t-test.

Chapter IV

Discussion

SIRT2 is a NAD^+ - dependent deacetylase that has an important role in cell cycle regulation (North et al., 2003), neurodegenerative diseases (Outeiro et al., 2007) and tumor suppression (Kim et al., 2011). SIRT2 is the most abundant sirtuin in adipose tissue (Jing et al., 2007) and is also present in different regions of the rodent brain, such as the cortex and hippocampus (Maxwell et al., 2011; Pais et al., 2013). However, there are currently no studies reporting SIRT2 expression in hypothalamus. This brain area is a target of insulin resistance and a causal link between hypothalamic SIRT2 and insulin resistance has not been established. Our studies demonstrate that SIRT2 is expressed in the mouse hypothalamus, particularly enriched in the VMH, and present in neurons. SIRT2 in VMH is regulated by nutritional availability since its expression is markedly reduced in high-fat diet conditions. Similar to high-fat diet, we also demonstrate that administration of the saturated free-fatty acid palmitate directly to the VMH also decreases SIRT2 expression. In addition, mHypoN42 hypothalamic neurons exposed to chronic insulin or palmitate causes insulin resistance, confirmed by p-Akt levels, whereas high glucose does not alter insulin sensitivity of the cells. However, palmitate is the only treatment that induces a marked decrease in SIRT2 expression. Importantly, serum deprivation for 24 hours improves insulin sensitivity under normal conditions, and prevents palmitate-induced insulin resistance and SIRT2 downregulation.

Hypothalamic neurons have the capacity to detect alterations in circulating hormones and substrates and transmit this information into adaptive outputs in an attempt to maintain the normal energy and glucose/insulin homeostasis (Coppari et al., 2005; Hill et al., 2010; Parton et al., 2007; Vianna and Coppari, 2011). Previous studies demonstrate that VMH neurons have this important ability. The VMH is one of the majors hypothalamic nucleus and is considered the “satiety center” (Williams et al., 2001), because of its role in body weight regulation and energy homeostasis (King, 2006; Klöckener et al., 2011). Previous studies demonstrate that stimulation of the VMH inhibits feeding, whereas a lesion in this region causes overeating and weight gain (King, 2006). The VMH is composed by several cell types (McClellan et al., 2006), however steroidogenic factor 1 (SF1)-expressing neurons are exclusively expressed in the VMH. In normal metabolic conditions, these neurons are active. Its activation is dependent on leptin and insulin that induces a signalling cascade that culminates in SF-

1 activation in the nucleus (Blind et al., 2012). Several studies suggest that SF-1 neurons in the VMH play a critical suppressive role against body weight gain, an important role in the regulation of energy and glucose metabolism (Choi et al., 2013). For instance, the loss of leptin receptor (LEPR) in SF-1 neurons significantly predisposes to developing (Dhillon et al., 2006) and insulin resistance (Bingham et al., 2008) while an enhanced in LEPR signalling in this neurons promotes an increase in insulin sensitivity (Zhang et al., 2008a). Similarly, the reduction of PI3K/Akt signalling in SF-1 neurons decreases autonomic responses to hypercaloric feeding and consequentially causes hypersensitivity to diet-induced obesity (Xu et al., 2010). Sirtuins are present in liver, kidney, skeletal muscle, adipose tissue and in several regions of the brain, where they participate in metabolic functions. The most studied sirtuin, SIRT1, is present in hypothalamus and until now is the only sirtuin described in the VMH. In this study we show for the first time that SIRT2 is expressed in the mouse hypothalamus. We also demonstrate that this protein is present in major hypothalamic nuclei, but enriched in the VMH and present in VMH neurons. Considering the role of the VMH in body weight regulation and energy homeostasis, and the exclusively presence of SF-1 neurons in this hypothalamic nucleus, our results suggest that SIRT2 may be expressed in these neurons and consequently be involved in metabolic functions, as regulation of body weight and energy homeostasis.

The loss of SIRT1 in SF-1 neurons predisposes to diet-induced energy and glucose/insulin imbalance and impairs insulin action in skeletal muscle. However SIRT1 overexpression in SF-1 neurons prevents diet-induced obesity and insulin resistance due to increased energy expenditure and enhanced skeletal muscle insulin sensitivity, demonstrate the important protective roles of SIRT1 in SF-1 neurons against dietary metabolic imbalance (Ramadori et al., 2011). Our study demonstrates that SIRT2 in VMH is regulated by HFD, since its expression decreases significantly upon HFD feeding. These results are in accordance with the studies performed in SIRT1, where the downregulation of the protein are related with an increase in body weight and an impairment in energy homeostasis and insulin sensitivity. When we infused palmitate, one of the major component in high-fat diet, directly into the VMH, the expression of SIRT2 decreases in this nucleus, to levels similar to that found in HFD. These results indicate the possibility that palmitate is the major responsible for SIRT2

downregulation in HFD, and also sustain the theory that SIRT2 in VMH is regulated by nutritional levels, working as a nutrient sensor mainly in obesity and consequently in type 2 diabetes conditions.

It would be interesting in future studies to evaluate whether SIRT2 is expressed in SF-1 neurons and after delete and overexpress SIRT2 in this neurons in an attempt to investigate the role of this protein in energy homeostasis and insulin sensitivity.

The role of hypothalamic SIRT2 in insulin resistance conditions has not been established; however, it is already known that due to its deacetylase activity SIRT2 is involved in important metabolic functions, such as adipocyte differentiation, regulation of sterol and fatty acid biosynthesis, gluconeogenesis and lipogenesis, (Jing et al., 2007; Krishnan et al., 2012; Wang and Tong, 2009), and also alleviates inflammation by deacetylating nuclear factor-kB (NF-kB) (Pais et al., 2013). These conditions are closely related with insulin resistance (Houstis et al., 2006). Knowing that SIRT2 is expressed in VMH neurons, we used a mouse hypothalamic neuronal cell line (mHypoE-N42) in an attempt to investigate the link between hypothalamic SIRT2 and insulin resistance. Insulin resistance can be induced by different stimuli. Hyperglycemia, hyperinsulinemia and lipid overload are characteristic insulin resistance conditions. High concentrations of glucose, mimicking hyperglycemia, can induce alterations in insulin pathway (Petersen and Shulman, 2006), possibly by the activation of the PKC pathway that increases phosphorylation of IRS proteins and inhibits insulin signalling (Campos, 2012; Kawanaka et al., 2001), and an increase in the flux of glucose into the hexosamine biosynthetic pathway (Kawanaka et al., 2001; Nelson et al., 2000). These effects were already studied in different in vivo and in vitro experimental models (Jensen et al., 1997; Kawanaka et al., 2001; Kurowski et al., 1999; Laybutt et al., 1999; Lima et al., 1991). In this study, exposure of mHypoE-N42 hypothalamic neurons to high glucose does not induce insulin resistance. These cells are cultured in a growth medium containing high glucose (25 mM) and a further increase in the concentration of glucose (25 mM) does not promote significant metabolic stress capable to induce alterations in insulin sensitivity and consequently in insulin signalling, that remain unaltered. However, these findings do not rule out the possibility that exposure to high levels of glucose for longer periods may cause insulin resistance.

Chronic treatment with high levels of insulin, a situation that mimics hyperinsulinemia, promotes a negative regulation of insulin receptors that occurs due to the reduction in receptor affinity (DeMeyts et al., 1976), reduction in the number of receptors in the surface of the cell (Gavin et al., 1974) and the diminution of the effectiveness of the receptor as a transmitter of stimulatory signals. The impairment in insulin receptor activity under conditions of chronic insulin exposure occurs because the phosphorylation of insulin receptors activates downstream proteins such as IRS proteins that impair the ability to activate downstream elements and also act upstream to inhibit insulin receptor activity. Other molecules downstream in the insulin pathway, such as mTOR, PI3K and Akt, transmit the activation process downstream and can also promote upstream negative feedback signals (Hoehn et al., 2008; Zick, 2001). Previous studies performed in *in vivo* (Juan et al., 1999; Kobayashi and Olefsky, 1978) and *in vitro* models demonstrate that the administration of insulin at high levels can lead to insulin resistance. In agreement with the literature, in our study using mHypoE-N42 hypothalamic neurons the chronic exposure to insulin (100 nM; 24 hours) induced a decrease in p-Akt levels after acute insulin stimulation. This alteration was likely promoted by a decrease in insulin receptor activity, responsible for the reduction in expression and activity of downstream molecules such as Akt, used as an index of insulin resistance.

Another common inducer of insulin resistance is palmitate, a saturated free-fatty acid very abundant in western diet. The excess of this fatty acid in circulation is the responsible for an increase in ceramide levels (Hannun and Obeid, 2008). The accumulation of ceramide in cells inhibits insulin signalling through dephosphorylation of Akt and inhibition of the translocation and activation of Akt (Holland et al., 2007), leading to an insulin resistance state. Excessive ceramide levels can also increase cytotoxic and apoptotic responses (Hannun and Obeid, 2008) and promote the release of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, that trigger activation of NF-kB. These cytokines can also phosphorylate IRS-1, leading to decreased metabolic signalling (Taubes, 2009). Palmitate is a well-established inducer of insulin resistance, having already been used in different *in vitro* studies (Benoit et al., 2009; Chavez et al., 2005; Chavez and Summers, 2003; Mayer and Belsham, 2010). Since that palmitate is the most abundant fatty acid in high fat diet we decided to use this insulin resistance

inducer. Palmitate inhibits insulin-induced Akt phosphorylation, only in the higher concentration tested (500 µM). The presence of this FFA probably promotes an increase in ceramide levels responsible for an impairment in insulin signalling by a reduction in Akt phosphorylation. This fact corroborates the hypothesis that palmitate enters in the cell and promotes an increase in ceramide levels, resulting in insulin resistance.

Previous studies indicate that there is a cross-talk between the PI3K/Akt pathway and sirtuins (Houtkooper et al., 2012; Nogueiras et al., 2012). Recent studies propose that SIRT2 is an Akt binding partner critical for Akt activation by insulin. In normal conditions Akt and SIRT2 form a binding complex, being this protein essential for an optimal Akt activation (Ramakrishnan et al., 2014). We assessed SIRT2 expression after treatment with high glucose, chronic insulin and palmitate. Cells exposed to high glucose show unaltered SIRT2 expression and probably this is related with the normal activation of p-Akt in these conditions. After the exposure to chronic insulin the levels of SIRT2 are also similar to the control although the levels of p-Akt are diminished. Since that high levels of insulin promote the receptor desensitization, insulin stimulation does not promote insulin signalling activation, so the activity of Akt is not compromised. This fact is probably the explanation for the maintenance of SIRT2 levels after chronic insulin exposure. On the other hand, the exposure to palmitate reduces the Akt phosphorylation and promotes downregulation of SIRT2 expression. A previous study concluded that SIRT2 is required for insulin-induced Akt activation, and that SIRT2 inhibition blocks Akt phosphorylation whereas SIRT2 overexpression increases the activation of Akt and its downstream targets (Ramakrishnan et al., 2014). In our study the decrease of insulin sensitivity in the presence of palmitate (500 µM) is probably related to the impairment in SIRT2 expression. Interestingly, although 250 µM palmitate does not promote significant alterations in p-Akt levels, it induces a decrease in SIRT2 expression. This can indicate that SIRT2 downregulation anticipates palmitate-induced insulin resistance. It would be important in future to silence as well as to overexpress SIRT2 in mHypoE-N42 hypothalamic neurons, both in insulin resistance and in control situations, in an attempt to investigate the importance of SIRT2 in Akt activation in hypothalamic neurons, since hypothalamus plays an important role in insulin resistance.

It is well known that serum starvation increase the activity of AMPK (Ramakrishnan et al., 2014; Wang et al., 2012). This increase is responsible for an amelioration of insulin sensitivity promoted by the increase in Akt phosphorylation. The increase in AMPK can also lead to the activation of nicotinamide phosphoribosyltransferase (Nampt), promoting an increase in NAD^+ levels (Wang et al., 2012) and consequently to the activation of some sirtuins such as SIRT1 (Cohen et al., 2004) and SIRT2 since the activity of sirtuins is dependent on NAD^+ as a cofactor. In our study, serum starvation enhanced the insulin-stimulated Akt response. Based in previous studies that implicated SIRT2 in insulin-induced Akt activation (Ramakrishnan et al., 2014), probably this improvement in Akt activation is related with the increase in SIRT2 activity after serum starvation. It would be important in future to block SIRT2 activity in hypothalamic neurons in order to investigate the role of SIRT2 activity in Akt activation. Having shown that palmitate can induce insulin resistance in mHypoE-N42 hypothalamic neurons, and since serum starvation can ameliorate insulin sensitivity, we checked the effect of nutrient deprivation in palmitate-induced insulin resistance. Serum starvation prevented palmitate effects in both p-Akt activation and in SIRT2 expression. The increase in SIRT2 activity after serum starvation may also be the explanation for these findings. In future it would be important confirm the increase in NAD^+ levels in serum starvation conditions. Serum starvation is a strong inducer of autophagy. This mechanism has a protective role in stress conditions. For this reason it would be interesting to investigate the potential role of autophagy in insulin resistance under starvation conditions. FFA can induce inflammatory responses, based in the release of cytokines that can be involved in alterations of metabolic signalling. It would be interesting to check alterations in inflammation in starvation conditions.

Collectively, this study suggests that SIRT2 may be a target in insulin resistance, and in future its modulation may be important in the development of new therapies for obesity and type 2 diabetes treatment.

Chapter V

Conclusions

The results presented in this work allowed us to draw the following main conclusions:

- SIRT2 is present in the mouse hypothalamus and enriched in the VMH;
- In this nucleus SIRT2 is expressed in neuronal cells;
- HFD decreases SIRT2 protein levels in the VMH;
- Palmitate attenuates insulin signalling and downregulates SIRT2 levels;
- Serum starvation increases insulin sensitivity and prevents the effects of palmitate in insulin signalling and in SIRT2 expression;
- Palmitate administration to the VMH caused a decrease in SIRT2 expression.

This study suggests that SIRT2 may be in future an important target in obesity and insulin resistance treatment, by modulating its expression and/or activity.

In further *in vivo* experiments it would be interesting to confirm that SIRT2 is expressed in SF-1 neurons, and to silence or overexpress SIRT2 in these neurons as an attempt to investigate the role of this protein in energy homeostasis and insulin sensitivity.

In further *in vitro* studies it is worth testing the importance of SIRT2 in Akt activation by inhibiting or overexpressing this sirtuin in insulin resistance and control situations, and to block SIRT2 activity in order to investigate the role of SIRT2 activity in Akt activation and confirm the increase in NAD^+ levels in serum starvation conditions. It would be also interesting to investigate autophagy and inflammation in serum starvation conditions.

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