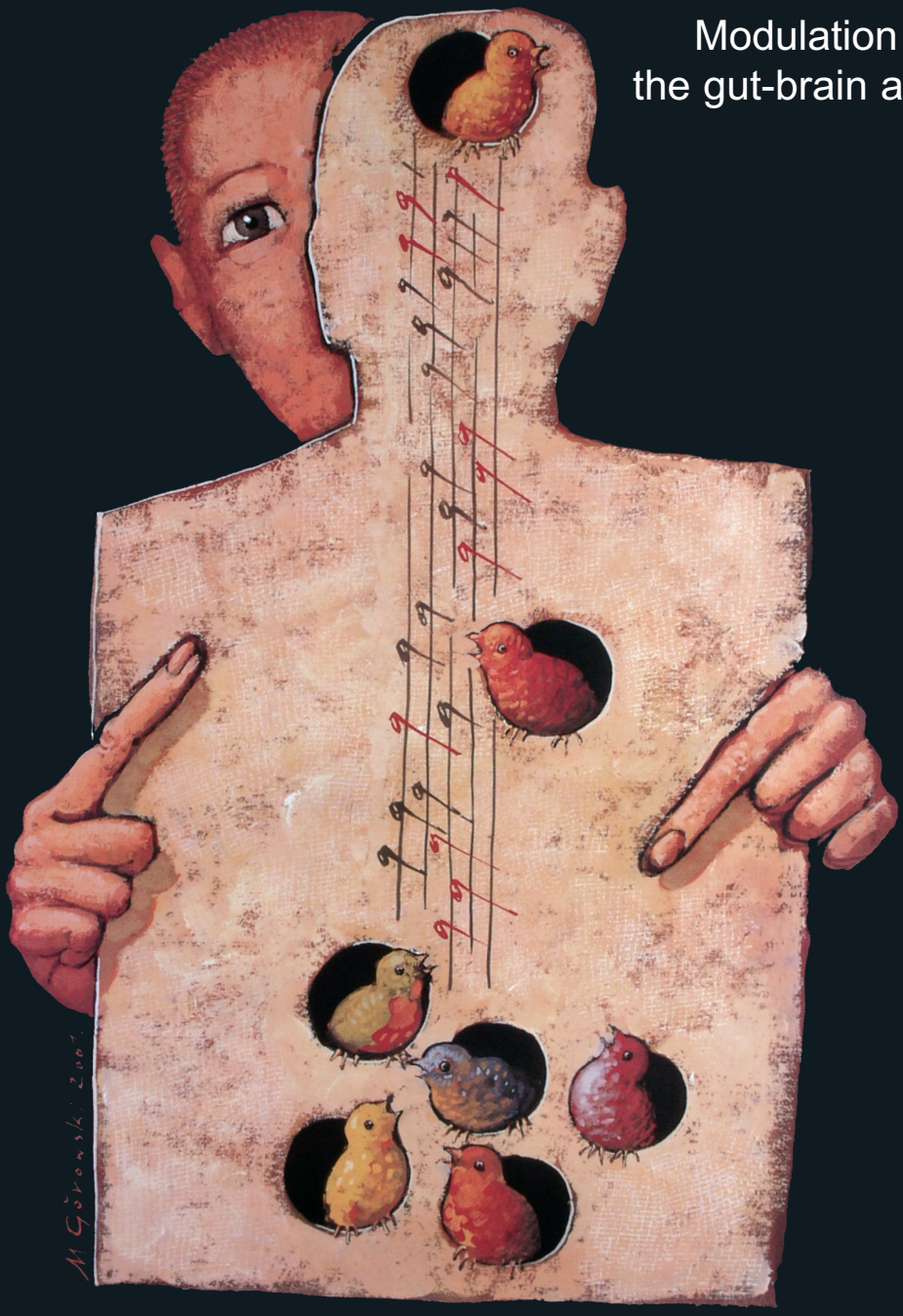
Insulin Sensitivity

Modulation by
the gut-brain axis



Annemieke Heijboer

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General introduction

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1. Introduction

Maintenance of plasma glucose concentration is highly important for normal body physiology. Glucose is under normal circumstances the only energy source for the brain. The brain is unable to store glucose and is therefore dependent on glucose derived from the circulation. In the control of glucose homeostasis, insulin is an important hormone. Insulin stimulates glucose uptake by tissues like skeletal muscle and adipose tissue, and inhibits glucose production by the liver. The extent of action of insulin on glucose uptake and glucose production is determined by tissue insulin sensitivity. Physiologically, insulin sensitivity can be influenced by many factors, like obesity, FFA concentrations, glucoregulatory hormones, etc. Pathophysiological changes in insulin sensitivity are seen in obesity and type II diabetes mellitus.

The studies in this thesis were performed to investigate the role of feeding status in crosstalk with the gut and the brain in the modulation of insulin sensitivity. In this chapter, a brief review is given of the involved diseases, obesity and type II diabetes mellitus (section 2), and of regulation of glucose metabolism (section 3). In this latter part, glucose homeostasis, nutritional status, insulin resistance and therapies for insulin resistance are discussed. In section 4, the current knowledge of gut-brain interactions and food intake is summarised. This chapter ends with the outline of the present thesis.

2. Obesity and type II diabetes mellitus

Evolution has provided humans with physiological mechanisms to survive times of scarcity of food. The purpose of these mechanisms is to conserve energy, seeking food in times of scarcity and storing energy in times of abundance. Hence, this system leads towards storage of fat and weight gain in conditions of caloric excess. During the last few decades, unique circumstances and lifestyle alterations have developed from an evolutionary perspective in industrialised countries. In contrast to previous eras there is plenty of food and physical activity is reduced. This maladaptive combination of genes to survive periods of scarcity and an environment with abundant dietary calories has led to an increased incidence of overweight and obesity.

Overweight and obesity are commonly assessed by using body mass index (BMI), defined as the quotient of weight in kilograms and the square of height in meters (kg/m^2). A BMI over 25 kg/m^2 is defined as overweight and a BMI over 30 kg/m^2 as obese.

Globally, obesity has reached epidemic proportions, with more than 1 billion overweight adults (more than 300 million are obese among them). Childhood obesity is already epidemic in some areas and on the rise in others. According to the US Surgeon General, in the USA the number of overweight children has doubled and the number of overweight adolescents has trebled since 1980 (1). Recent data show that in the Netherlands, 46.5% of the population is overweight and 10.9% of the population is obese. Dramatically increasing percentages of obese youngsters are seen as well (2).

Obesity is a major risk factor for developing chronic diseases, including cardiovascular disease, hypertension and stroke, certain forms of cancer, and type II diabetes mellitus. Ninety percent of the patients with type II diabetes mellitus are obese or overweight. Type II diabetes mellitus now affects obese children even before puberty. Retinopathy, kidney failure, heart disease, neuropathy and foot diseases are major complications of diabetes. These complications decrease quality of life, and increase the risk for premature death. Diabetes mellitus is the sixth leading cause of death with 3.2 million deaths world-wide every year (1;3).

3. Regulation of glucose metabolism

1. Glucose homeostasis

It is highly important for normal body physiology to keep a constant blood glucose level. As the brain has no endogenous glucose supply and is a major consumer of glucose, it is dependent on glucose derived from the circulation. Plasma glucose concentration is maintained within narrow limits by a fine balance between endogenous (hepatic) glucose production and peripheral glucose utilisation. During fasting, glucose is the obligatory fuel that provides more than 90% of energy needed for brain function (4;5). The liver produces this obligatory amount of glucose by glycogenolysis and gluconeogenesis (6). Glycogenolysis is the process of breakdown of glycogen via glucose-6-phosphate to free glucose, gluconeogenesis is the process of generating new molecules of glucose from intermediates derived from the catabolism of glycerol and some amino acids (7). Glucose is also taken up by peripheral tissues, like skeletal muscle, adipose tissue and heart tissue. A small amount of glucose can be stored in skeletal muscle and the liver, as the polysaccharide glycogen, to provide a reserve supply of energy.

Glucose balance is tightly regulated by the interaction of different regulatory mechanisms, such as the classical glucoregulatory hormones, like insulin, glucagon, catecholamines, cortisol and growth hormone. Insulin inhibits endogenous glucose

production and stimulates glucose uptake in skeletal muscle and adipose tissue. Insulin inhibits gluconeogenesis by inhibiting the transcription of the main gluconeogenic enzyme, phosphoenolpyruvate caboxykinase and by increasing the transcription of the main glycolytic enzyme, pyruvate kinase (8) (9). In addition, insulin decreases hepatic uptake of precursor amino acids and their availability from muscle (10). Insulin stimulates glucose uptake by binding to insulin receptors in the plasma membrane of skeletal muscle or adipose tissue. This binding triggers a variety of signal transduction pathways, which ultimately results in fusion of glucose transporter-4 (GLUT-4) with the plasma membrane. The increased number of plasma-membrane glucose transporters causes a higher rate of glucose movement from the extracellular fluid into the cells (11).

In addition to these effects on peripheral tissues, insulin affects neuropeptides in the hypothalamus involved in regulating food intake and energy expenditure (see also paragraph *'brain and glucose metabolism')*. More than 140 years ago, Claude Bernard (12;13) punctured the fourth ventricle in rabbits, which resulted in glucosuria. Although these striking findings suggested a key role for the brain in glucose homeostasis, its importance was largely neglected after the discovery of insulin in 1922. However, new findings have revived interest in the role played by the brain, in particular the hypothalamus, in both glucose metabolism and the mechanism linking obesity to type II diabetes mellitus (14;15).

2. Nutritional status

With regard to nutritional status, there are two functional states: the absorptive state, during which ingested nutrients are entering the blood from the gastrointestinal tract, and the postabsorptive state, during which the gastrointestinal tract is empty of nutrients and energy must be supplied by the body's own stores.

During the absorptive state, glucose is the major energy source of the body. During this phase, glucose taken up by skeletal muscle is in part oxidised and in part stored as glycogen. In adipocytes, the most important fate of glucose in the absorptive state is the transformation to fat (triglycerides (TG)) for storage. The transformation of glucose to TG is called lipogenesis. The liver takes up glucose as well and stores it either as glycogen or transforms it to TG. Most of this liver-TG is secreted as very low density lipoproteins (VLDL) into the blood. However, *de novo* lipogenesis contributes to only ~5 percent of VLDL-TG, whereas the major part of VLDL-TG is derived from reesterification of fatty acids derived from adipose tissue. VLDL-TG are taken up by peripheral tissues depending on tissue specific activity of lipoprotein lipase. Ingested TG will directly be transported as chylomicrons to

peripheral tissues, especially adipose tissue, for storage and in other tissues, like the heart, for oxidation. During the absorptive state insulin levels are increased, thereby stimulating glucose uptake and glycogen synthesis and inhibiting glucose production. Insulin also stimulates lipogenesis and inhibits lipolysis (catabolism of TG into glycerol and fatty acids) and VLDL production. In this way, insulin lowers plasma glucose levels and promotes the storage of FFA/TG in fat, liver and skeletal muscle. When the absorptive state ends, synthesis of glycogen and fat stops and net catabolism occurs.

In the postabsorptive state and during prolonged fasting, the gastrointestinal tract is empty, resulting in cessation of glucose absorption from the intestine. However, glucose concentrations must be maintained within narrow limits to preserve normal functioning of the body. When glucose concentrations decrease to low values, alterations of neural activity ranging from slight impairment of mental function to coma and even death may occur (16). There are two ways to keep glucose concentrations at a constant level, stimulation of glucose production and inhibition of glucose uptake. During the postabsorptive state glucose is produced by the liver through glycogenolysis and gluconeogenesis (6). The increase in gluconeogenesis is facilitated by low insulin concentrations present during fasting. This also results in a decrease in glucose uptake by insulin dependent tissues such as skeletal muscle and adipose tissue. Consequently, glucose is available for non-insulin dependent tissues such as the brain (4;5). In addition, lipolysis (catabolism of TG into glycerol and fatty acids) increases in adipose tissue, resulting in increased release of fatty acids from adipose tissue, which can be used by muscle and other tissues for energy supply. The liver can transform these fatty acids into ketone bodies by β-oxidation, and release them into the blood or convert them in VLDL-TG (see above). During prolonged fasting, ketone bodies are an important energy source for many tissues, including the brain (5).

3. Obesity, insulin resistance, and type II diabetes

Energy intake, which the body derives from food, is required to match energy expenditure, necessary for physical activity and other body functions. When energy intake exceeds expenditure, energy is stored in the form of adipose tissue to be utilised in conditions of food scarcity or increased energy demand. Overweight and obesity are outcomes of long-term excess of energy intake relative to energy expenditure. Particularly fat and energy intake are strongly and positively associated with body weight gain. A high fat (energy-dense) diet is an independent risk factor for overweight (17). At the metabolic level, the imbalance between energy intake and

energy expenditure, which leads to energy deposition in form of adipose tissue, can be seen as an imbalance between fat deposition and fat oxidation. Fat oxidation occurs predominantly during the postabsorptive state, whereas fat deposition is stimulated during the absorptive state. Obesity is an important determinant of insulin resistance and represents the most important risk factor for the development of type II diabetes mellitus (18-20).

Insulin resistance reflects a condition with reduced biological effects of insulin (21). Different tissues may have different tissue-specific sensitivities to the actions of insulin. As insulin normally inhibits endogenous glucose production, hepatic insulin resistance is characterised by diminished inhibition of glucose production by insulin. In peripheral tissues, especially skeletal muscle and adipose tissue, insulin resistance is characterised by decreased insulin-mediated glucose uptake. With regard to lipid metabolism, the inhibitory effects of insulin on lipolysis and VLDL production are decreased and insulin-mediated lipogenesis is also decreased. Both genetic and environmental factors, such as dietary habits, are involved in tissue-specific insulin sensitivity. Up till now, with the exception of rare monogenic variants, the inherent susceptibility to type II diabetes mellitus is considered to be attributable to complex interacting genetic determinants.

Insulin resistance is a major determinant of the pathophysiology of type II diabetes mellitus. Insulin resistance results in the inability of circulating insulin to properly suppress hepatic glucose production and to stimulate the disposition of glucose (and other metabolic fuels). This leads to progressive hyperglycaemia, and therefore more prolonged stimulation of pancreatic β-cells. When β-cell compensation ultimately fails, glucose levels rise even more, leading to either impaired glucose tolerance or overt diabetes.

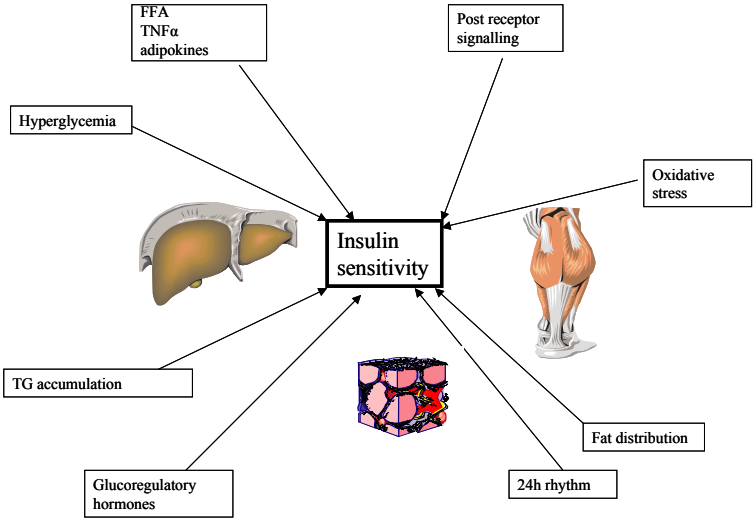

Figure 1. Mechanisms that are involved in regulating insulin sensitivity.

The interaction between overweight and insulin resistance is complex and involves several epidemiological associations (figure 1). Briefly reviewed, these are:

- Fat distribution: Patients with central adiposity have higher insulin levels and are more insulin resistant than subjects with similar weight but with a peripheral type of obesity (22-24).
- Plasma FFA levels: The extent of (direct) exposure of liver and muscle cells to FFA concentrations might be involved in mediating tissue specific insulin resistance. For instance, experimental elevation of FFA induces insulin resistance (25-27). At the cellular level, FFA and their metabolic products can reduce insulin signalling in muscle and liver (27).
- Ectopic triglyceride accumulation: TG content of skeletal muscle and liver correlates directly with insulin resistance (27-31). These observations suggest that accumulation of fat in liver and muscle tissue might (partly) mediate obesity-induced insulin resistance.
- Adipokines: Another major mechanism linking obesity to insulin resistance is a group of peptides, made by fat cells that alter insulin sensitivity. Adiponectin has been shown to reduce insulin resistance and reduced levels of adiponectin are found in progressive obesity (32;33). Tumor necrosis factor-alpha (TNFα), interleukin-6, resistin and leptin increase insulin resistance (34). Elevated levels of these adipocytokines are observed with obesity (34;35). Various adipose tissue beds produce different amounts of these peptides, perhaps adding to the regional differences in the contribution of these adipose depots to insulin resistance.
- Hyperglycaemia: Hyperglycaemia itself is known to induce insulin resistance (36). This partially reversible phenomenon is known as glucose toxicity. In β-cells, oxidative glucose metabolism will always lead to production of reactive oxygen species, normally detoxified by catalase and superoxide dismutase. Because these enzymes are present in low amounts in β-cells, hyperglycaemia can result in the production of large amounts of reactive oxygen species in β-cells, with subsequent damage to cellular components.
- Number of insulin receptors, post-receptor signalling by insulin and synthesis and translocation of GLUT4: It is suspected that alterations in de expression and/or function of these factors underlie insulin resistance in obesity as well as in type II diabetes. Among the many molecules involved in the intracellular processing of the signal provided by insulin, insulin receptor substrate (IRS)-2, the protein kinase B (PKB)-beta isoform and the forkhead transcription factor Foxo1a (FKHR) are of particular interest in this context as recent data

have provided strong evidence that dysfunction of these proteins results in insulin resistance in vivo (37;38).

- Glucoregulatory hormones: Glucocorticoids (39), sex steroids (40), growth hormone (41), and catecholamines (42;43) influence tissue insulin sensitivity.
- Oxidative stress and vascular reactivity: These factors have also been suggested to be involved in the development of insulin resistance (44-46). However, oxidative stress, vascular reactivity, inflammation and insulin resistance seem to be interrelated and more research is needed to elucidate this relationship.
- Diurnal rhythms: It is recently shown in healthy humans that insulin sensitivity changes rhythmically during the day (47).

4. Therapies for insulin resistance

As the mechanisms underlying the development of insulin resistance are not clear, a therapy that directly targets these mechanisms does not exist. A major goal of therapeutic intervention in diabetes is to reduce circulating glucose levels. Lifestyle changes are the first step towards a reduced risk of developing diabetes or better prognosis for diabetes patients. Lifestyle changes are an ideal method of diabetes prevention because of its beneficial effects on cardiovascular risk factors as well as on other benefits related to weight loss and an improved diet (48). Weight loss in obese patients with diabetes can improve survival. In addition, exercise also improves insulin sensitivity by increasing glucose uptake into skeletal muscle (11). However, these interventions require a strong will as lifestyle modification has been difficult to maintain over a long term. Weight loss is not maintained once exercise or diet has been discontinued, and symptoms of diabetes will recur. Therefore pharmacological strategies are required in addition to exercise or diets.

Oral hypoglycemic drugs, such as (combinations of) metformin, acarbose, sulfonylurea's, thiazolidinediones, and anti-obesity agents (like orlistat) are currently used as pharmacological treatment for diabetes. However, none of these treatments is perfect. Recently, a meta-analysis was performed, in which studies were included that have investigated the effects of several different drug classes on type 2 diabetes incidence (48). Oral hypoglycemic medications and orlistat were the only drugs that had been studied in randomised controlled trials with diabetes incidence as the primary end point. The available evidence suggests that oral hypoglycemic drugs may reduce diabetes incidence compared with placebo. The adequately powered studies showed significant decreases in diabetes incidence with metformin, acarbose,

troglitazone, and orlistat. However, they concluded that the data are not definitive and that no single agent can currently be recommended for diabetes prevention (48).

Interestingly, recent reports show that gastrointestinal hormones appear to have effects both on food intake and glucose metabolism (see next paragraph). Therefore, these hormones might be interesting for therapeutic goals in the battle against type II diabetes mellitus.

4. Gut-brain axis

1. Brain and food intake

Food intake is largely regulated by the central nervous system.

Lesion experiments in the 1950's showed that lesions of the ventromedial nucleus resulted in uncontrollable hyperphagia and obesity, whereas lesions of the lateral hypothalamus resulted in anorexia and weight loss (49). These experiments were the basis of the early concepts of hypothalamic appetite regulation. Although these concepts were a gross oversimplification, the hypothalamus is still regarded as an important feeding center of the brain.

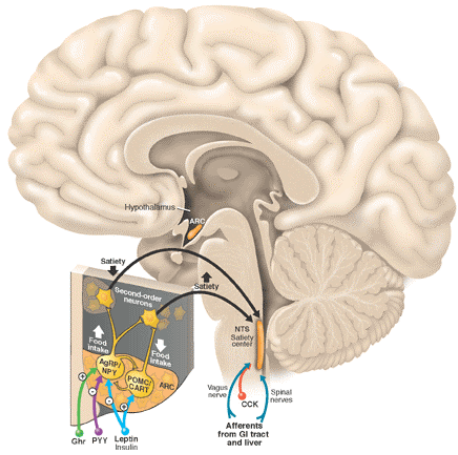

Figure 2. Central command centers. Reprinted with permission from Marx, SCIENCE 299:846 (2003). Illustration: Katharine Cutliff. Copyright 2003 Science.

The hypothalamus consists of several nuclei involved in regulating food intake, including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the ventromedial nucleus (VMH), and the dorsomedial nucleus (DMH).

Located at the bottom of the hypothalamus, around the 3rd ventricle, the ARC can be found (see figure 2). ARC neurons are called 'first-order' neurons, because of their 'direct' contact with peripheral satiety factors. The 'second-order' neurons can be found in the PVN, LHA, VMH and DMH. Within the ARC, at least two populations of 'first-order' neurons controlling appetite are characterized: 1) neurons co-expressing Agouti-related peptides (AgRP) and neuropeptide Y (NPY) and 2) neurons co-expressing pro-opiomelanocortin (POMC), the molecular precursor of

alpha-melanocyte stimulating hormone (α-MSH). The first neuronal circuit (AgRP/NPY) stimulates food intake and the other neuronal circuit (POMC/α-MSH) inhibits food intake (50). There is direct interaction between the NPY/AgRP pathway and the POMC/α-MSH pathway (see figure 3). During fasting conditions, the expression of these neuropeptides is altered; fasting results in an increase in NPY and AgRP mRNA expression and a decrease of POMC mRNA expression levels in the hypothalamus (51). Together, during fasting, food intake is stimulated.

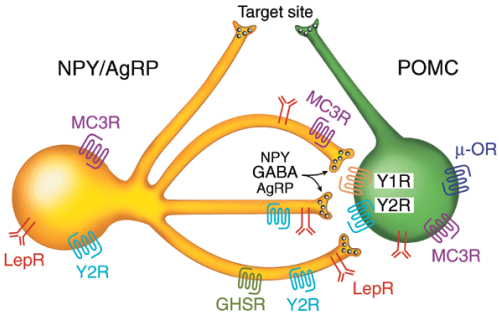

Figure 3. Anatomy and regulation of the NPY and POMC system. Reprinted with permission from Cone, Nat Neuroscience 571-578 (2005). Copyright 2005 Nature Publishing Group

Mutations disrupting these hypothalamic pathways cause obesity in rodents and humans. Examples are obese POMC-/- and MC4R-/- mice (52;53) and humans with POMC, MC4R and CART mutations which are associated with obesity (54-58).

2. Brain and glucose metabolism

The central nervous system is suggested to play a key role in the control of glucose metabolism via brain pathways that overlap with those controlling food intake and body weight (59). The brain is an insulin-sensitive organ. Insulin provides afferent input to the CNS regarding the sufficiency of body fat stores. Receptors for insulin are concentrated in hypothalamic areas. Intracerebroventricular administration of low doses of insulin reduces food intake and body weight (60). Insulin has been shown to increase POMC gene expression, that is normally decreased during fasting, and inhibit the expression of mRNA levels encoding the orexigenic peptide neuropeptide Y (NPY) that are normally increased in the ARC during fasting (61-63).

Brain insulin action nowadays is hypothesised as a requirement for intact glucose homeostasis. Okamoto et al. showed that selective expression of insulin receptors reduces diabetes severity (64). Chronic blockade of hypothalamic insulin receptor signaling was shown to cause hepatic insulin resistance and to increase hepatic glucose production (65;66). In contrast, acute depletion of insulin receptors in the liver impaired downstream insulin signalling, but failed to alter the effect of physiological hyperinsulinemia on the rate of glucose production (67). The

importance of neuronal insulin signalling is further underlined by evidence that mice with neuron-specific insulin receptor deletion are overweight, insulin-resistant, and glucose-intolerant (68).

3. Gastrointestinal hormones and food intake

Hormones secreted from peripheral tissues bind to receptors located in the hypothalamus (see figure 3) (69;70). These hormones are secreted from the pancreas (like insulin), from adipose tissue (like leptin) or from the gastrointestinal tract. In this thesis we focus on the last group of hormones.

Because of permeable blood brain barrier at the bottom of the ARC and the presence of these receptors in the ARC, the AgRP/NPY and POMC/CART neurons can be reached and influenced by these hormones (71). Gastrointestinal hormones may also act indirectly to influence the activity of afferent neuronal pathways and brain stem circuits, which in turn project to the arcuate nucleus (72;73).

Via the hypothalamus these hormones are able to affect food intake (see figure 4). Extensive reviews have been written about gastrointestinal hormones and the regulation of food intake recently (69;74-76). Here, we will give an overview of these hormones and their effects on food intake.

Cholecyctokinin (CCK), which is released from the upper small intestine (duodenal and jejunal mucose) by I cells (77) (78), was the first gastrointestinal hormone shown to decrease food intake. CCK is thought to interact with CCK-1 receptors on vagal sensory fibers, with the signal being relayed to the brainstem. Consistent with this notion, the anorectic effects of CCK can be eliminated by subdiaphragmatic vagotomy or selective damage to vagal afferent nerves. Likewise, lesions of the brainstem area that receives vagal sensory afferents, attenuate CCK-elicited anorexia. Within the brain, recent data suggest that melanocortin-4 receptors (MC4) modulate CCK's action (79-82).

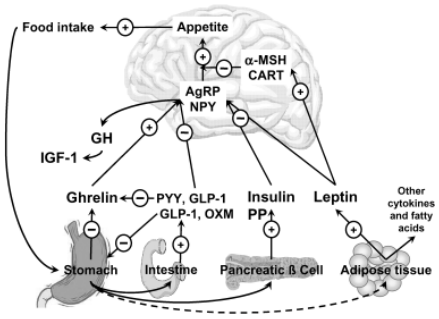

Figure 4. The gut-brain interaction in the regulation of appetite and body weight. Reprinted with permission from Hanusch-Enserer Eur J Clin Invest 35:425-430 (2005). Copyright 2005 Blackwell Publishing Group.

Peptide YY (PYY_{3-36}) is released from L-cells of the distal gut upon feeding. Recently, there has been a lot of discussion about the effects of the gut hormone PYY_{3-36} on food intake (83). However, there is more or less consensus now that PYY_{3-36}

decreases food intake in both rodents (peripheral and central administration) and humans (84-86). To inhibit feeding, PYY_{3-36} may act through the Y2 receptor, a putative inhibitory presynaptic receptor that is highly expressed on NPY neurons in the ARC.

Glucagon Like Peptide 1 (GLP-1) is a proglucagon-derived hormone that is also secreted from the L-cells of the distal gut upon meals and is known to decrease food intake in rodents and humans (87;88). GLP-1 binds to the GLP-1 receptor, that is found in the periphery (gut and endocrine pancreas) and is widespread throughout the central nervous system. The anorectic actions of GLP-1 are probably mediated through both peripheral and central mechanisms. A population of neurons that synthesise GLP-1 is located in the brainstem and projects to hypothalamic and brainstem areas important in the control of energy homeostasis (89;90). GLP-1 is also known to affect glucose metabolism. Numerous studies have shown, that GLP-1 can improve glucose-stimulated insulin secretion and lower fasting and postprandial blood glucose levels in individuals with type 2 diabetes (91;92). Therefore, there is a lot of interest in this peptide for therapeutic goals in type 2 diabetes mellitus. (93;94).

Glucagon Like Peptide 2 (GLP-2) is another product from proglucagon, and is secreted in parallel with GLP-1 from the L-cells of the distal gut. When centrally applied, GLP-2 inhibits food intake (95), which study provides evidence that GLP-2 serves as a neurotransmitter in a distinct ascending pathway linking viscerocepti ve neurons of the brainstem with a hypothalamic target. Recently, a few studies have been performed in which GLP-2 was peripherally administrated in humans. However, these three studies could not find effects of peripheral GLP-2 on appetite, energy intake or satiety (96-98).

Oxyntomodulin (OXM) is a gastrointestinal hormone that is, just like GLP-1 and GLP-2, a product of post-translational processing of preproglucagon and released from the L-cells in response to food ingestion and in proportion to meal calorie content (99;100). OXM inhibits food intake both in rodents and in humans after peripheral administration (101-103). It is currently unclear through which receptor OXM mediates its actions. There is evidence that circulating OXM could mediate its anorectic actions via direct interaction with the hypothalamus, activating POMC neurons within the ARC (103).

Ghrelin, identified in 1999, and released from the X/A-like cells from the stomach, especially just before a meal (104), is, both in rodents and humans, the only peptide hormone found to stimulate appetite when administered peripherally (105-108). To enhance appetite, peripheral produced acylated ghrelin acts in the hypothalamus where it promotes NPY and orexin gene expression and inhibits POMC/α-MSH

expression via activation of the growth hormone secretagogue receptor (GHS-R) (109-115).
Since gastrointestinal hormones influence appetite and food intake in interaction with the brain, especially the ARC, and recent reports point to a central role of the brain in the regulation of insulin sensitivity, we hypothesised that gut-brain interactions might also be involved in the regulation of insulin sensitivity, independently of their effects on food intake and body weight.

5. Outline of the present thesis

The aim of this thesis was to gain more insight in the role of feeding status and gut-brain interaction in the modulation of insulin sensitivity. There is growing evidence that neuropeptides which are situated in the hypothalamus, and gastrointestinal hormones which act on the hypothalamus, and are involved in regulating food intake, seem to be involved in regulating insulin sensitivity as well. Therefore, we first characterized the effects of feeding status itself on insulin sensitivity, and subsequently the effects of some of the signals for feeding status in the gut-brain axis, on insulin sensitivity.

In **chapter 2**, we investigated the effect of fasting on insulin sensitivity in mice. During fasting FFA concentrations and liver TG content are increased. In obesity, increased FFA concentrations and excessive tissue TG storage are associated with tissue insulin resistance. The impact of fasting on tissue insulin sensitivity is unknown. Therefore, we studied the effects of 16 hr of fasting (prolonged fasting) versus 4 hr of fasting (postprandial state) on hepatic and muscle insulin sensitivity in wild-type mice in vivo in relation to tissue TG accumulation and changes in mRNA expression of transcription factors and related proteins involved in glucose and lipid metabolism.

In **chapter 3**, the effects of a 2 week high fat diet on insulin sensitivity in relation to hypothalamic neuropeptides are presented. Studies in rats and dogs on a high fat diet show the induction of hepatic insulin resistance as an early event, followed by muscle insulin resistance later. The question was whether this primacy of hepatic insulin resistance in relation to changes in TG content is also present in mice. Secondly, the aim of this study was to evaluate whether the NPY/POMC circuitry is involved in the induction of insulin resistance during a high fat diet, by measuring

mRNA expression levels of these neuropeptides in the hypothalamus of mice after 2 weeks of high fat diet.

In **chapter 4**, we describe the effect of icv administration of MTII, a synthetic analogue of α-MSH, on insulin sensitivity. It is known that NPY can induce hepatic insulin resistance. However, whether the POMC pathway has effects on insulin sensitivity, independently of changes in food intake and body weight is not investigated. This study was performed to answer that question.

In **chapter 5** we evaluated the effects of acute administration of the gut-hormone PYY_{3-36} on insulin sensitivity. PYY_{3-36} inhibits NPY and activates POMC neuronal activity to inhibit food intake. As both NPY and the POMC pathway affect insulin sensitivity, the aim of this study was to evaluate whether PYY_{3-36} can affect insulin sensitivity independently of its effects on food intake.

In **chapter 6** we focussed on the effects of long-term administration of PYY_{3-36} on insulin sensitivity. A prerequisite for a drug against obesity and insulin resistance is that it has long-term effects. We administered PYY_{3-36} for 7 days, either continuously via subcutaneous mini-pumps or intermittent via daily subcutaneous injections to measure its long-term effects on insulin sensitivity.

In **chapter 7** we investigated whether ghrelin and des-ghrelin, produced by the stomach might affect insulin sensitivity in mice. Ghrelin promotes neuropeptide Y (NPY) gene expression and inhibits pro-opiomelanocortin (POMC)/αMSH expression via activation of the GHS-receptor and thereby stimulates food intake. Our question was whether ghrelin might affect insulin sensitivity. To detect a potential mechanism, we investigated whether GHRP-6, an agonist of the GHS-receptor can also influence insulin sensitivity. Des-ghrelin has not been seen as a bio-active hormone until recently. There are very recent publications that des-ghrelin might affect glucose production in hepatocytes. Therefore, the second aim of this study was to investigate the role of des-ghrelin in the regulation of insulin sensitivity.

In **chapter 8**, the results of the above mentioned studies are summarised and put into perspective.

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Chapter 2

Sixteen hours fasting differentially affects hepatic and muscle insulin sensitivity in mice

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ABSTRACT

Fasting readily induces hepatic steatosis. Hepatic steatosis is associated with hepatic insulin resistance. The purpose of the present study was to document the effects of 16 hours fasting in wildtype mice on insulin sensitivity in liver and skeletal muscle in relation to 1) tissue accumulation of triglycerides (TG's) and 2) changes in mRNA expression of metabolically relevant genes. Sixteen hours of fasting did not show an effect on hepatic insulin sensitivity in terms of glucose production in the presence of increased hepatic triglyceride content. In muscle, however, fasting resulted in increased insulin sensitivity with increased muscle glucose uptake without changes in muscle triglyceride content. In liver, fasting resulted in increased mRNA expression of genes promoting gluconeogenesis and TG synthesis but in decreased mRNA expression of genes involved in glycogenolysis and fatty acid synthesis. In muscle, increased mRNA expression of genes promoting glucose uptake, as well as lipogenesis and β-oxidation was found. In conclusion, 16 hours fasting does not induce hepatic insulin resistance although it causes liver steatosis, whereas muscle insulin sensitivity increases without changes in muscle triglyceride content. Therefore, fasting induces differential changes in tissue-specific insulin sensitivity and liver and muscle TG contents are unlikely to be involved in these changes.

INTRODUCTION

Fasting increases hepatic triglycerides (TGs) in rodents (1). This fasting-induced hepatic steatosis results from repartitioning of FFAs, released from adipose tissue, to the liver. In the liver, FFAs can either be used for β-oxidation in mitochondria, or reesterified into TG. TG can be stored, or secreted as VLDL. In turn, TG-rich VLDL particles are lipolyzed by LPL and deliver FFAs to other tissues, like skeletal muscle (2), where FFAs are used for β-oxidation. If muscle FFA uptake exceeds β-oxidation, excessive TG storage will be the consequence (3).

Evidence is accumulating indicating that accumulation of TG is involved in tissue-specific insulin resistance. For instance, studies in transgenic mice with targeted disturbances in peripheral fatty acid/TG partitioning showed, that there is an inverse relationship between hepatic TG stores and hepatic insulin sensitivity (4;5). In muscle, TG accumulation is also associated with insulin resistance, characterized by a decrease in insulin-stimulated glucose uptake (6). There is a lot of evidence on the action of fatty acid derivatives as agonists and antagonists for nuclear transcription factors, such as peroxisome proliferator-activated receptors (PPARs) and sterol-regulatory element binding proteins (SREBPs) (7;8). These transcription factors profoundly alter the expression of enzymes/proteins involved in glucose and lipid metabolism (8-13) and have interactions with hormones such as insulin (14;15). Therefore, these transcription factors could be molecular links between intracellular fatty acid/TG accumulation and insulin resistance. Because hepatic steatosis is associated with hepatic insulin resistance, we postulated that fasting also induces hepatic insulin resistance. The effects of fasting on muscle TG accumulation and insulin sensitivity have not been studied. Therefore, the aim of the present study was to evaluate the effects of 16 h of fasting on hepatic and muscle insulin sensitivity in wild-type mice in vivo in relation to 1) tissue TG accumulation and 2) changes in mRNA expression of transcription factors and related proteins involved in glucose and lipid metabolism.

MATERIALS AND METHODS

Animals

Male, 12-16 week old, C57Bl/6 mice were used in all experiments. Mice were kept in a temperature- and humidity-controlled environment and had free access to standard mouse chow and water. Control mice were fasted 4 h with food withdrawn at 5:00 AM, and the experimental mice were fasted overnight with food withdrawn at 5:00

PM (16 h fasted). All experiments were performed at 9:00 AM. All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center and Netherlands Organization for Applied Scientific Research (TNO) Prevention and Health (Leiden, The Netherlands).

Hyperinsulimemic euglycemic clamp

Hyperinsulinemic euglycemic clamps of the two experimental groups were performed side by side on the same day. The hyperinsulinemic euglycemic clamp was performed as described previously (4;16). In short, a continuous infusion of D-[^{14}C]glucose (0.3 µCi/kg/min; Amersham, Little Chalfont, UK) was started and blood samples were taken (after 60 and 80 minutes of tracer infusion) to determine basal glucose kinetics. Subsequently, a hyperinsulinemic study started with a bolus of insulin (100 mU/kg Actrapid; Novo Nordisk, Chartres, France) followed by continuous infusion of insulin (6.8 mU/h) and of D-[^{14}C]glucose. A variable infusion of 12.5% D-glucose (in PBS) solution was also started and adjusted to maintain blood glucose levels constant at ~8 mmol/l, measured via tail bleeding (Freestyle, TheraSense; Disetronic Medical Systems BV, Vianen, The Netherlands). During the last hour of the experiment, blood samples (75 µl) were taken every 20 minutes to determine plasma [^{14}C]glucose and insulin concentrations.

To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H]glucose (2-[^{3}H]DG; Amersham) was administered as a bolus (1 µCi) 40 minutes before the end of the clamp procedure.

After the last blood sample was taken, mice were killed and liver and muscle were taken out, immediately frozen using liquid N_2, and stored at -20°C until further analysis.

Analytical procedures

Plasma levels of ketone bodies, glucose and free fatty acids were determined using commercially available kits (#310-A Sigma GPO-Trinder kit and #315-500; Sigma, St.Louis, MO; FFA; Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin concentrations were measured by radio immunoassay using rat insulin standards (Sensitive Rat Insulin Assay; Linco Research, St.Charles, MO). For determination of plasma D-[^{14}C]glucose, plasma was deproteinized with 20% trichloroacetic acid, dried to remove water, resuspended in demiwater and counted with scintillation fluid (Ultima Gold; Packard, Meridien, CT) on dual channels for separation of ^{14}C and ^{3}H, as described earlier (17).

Tissue analysis

Liver and muscle samples were homogenized (~10% w/v) in water. Lipids were extracted according to Bligh and Dyer's method (18). In short, a solution was made of each 200 µg sample of protein in 800 µl of water, then 3 ml methanol-chloroform (2:1) was added and mixed thoroughly, after which 500 µl of chloroform, 100 µl of internal standard, and 1 ml of demiwater were added. After centrifugation, the chloroform layer was collected and dried. The remaining pellet was dissolved in 50 µl chloroform and put on a high-performance TLC plate. With high-performance TLC analysis, TGs, cholesterol, and cholesteryl esters were separated. The amount of TG in the tissues was quantified by scanning the plates with a Hewlett-Packard Scanjet 4c and by integration of the density using Tina® version 2.09 software (Raytest, Staubenhardt, Germany).

For determination of tissue 2-DG uptake, the homogenate of muscle was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-phosphatase from 2-DG, as described previously (16;17;19).

Calculations

Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance [body glucose uptake (BGU)]. The latter was calculated as the ratio of the rate of infusion of D-[^{14}C]glucose (dpm/min) and the steady-state plasma [^{14}C]glucose specific activity (dpm/µmol glucose). Hepatic glucose production (HGP) was calculated as the difference between the rate of glucose disappearance and the infusion rate of exogenous D-glucose.

The hepatic insulin sensitivity index was calculated as the ratio of the relative suppression of HGP during the hyperinsulinemic condition to the change in plasma insulin levels from basal to hyperinsulinemic conditions. The whole body insulin sensitivity index was calculated as the ratio of BGU to plasma insulin levels during hyperinsulinemic conditions.

Muscle-specific tissue glucose uptake was calculated from tissue 2-DG content, which was expressed as percentage of 2-DG of the dosage per gram of tissue, as previously described (19).

Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels in skeletal muscle [glucose transporter 4 (GLUT4), PPARγ

Table 1 Primer and probe sequences of genes used for mRNA quantification

Gene	Forward primer	Reverse primer	TaqMan probe
SREBP1c	5' GGAGCCATGGATTGCACATT 3'	5' CCTGTCTCACCCCCAGCATA 3'	5' CAGCTCATCAACAACCAAGACAGTGACTTCC 3'
FAS	5' GGCATCATTGGGCACTCCTT 3'	5' GCTGCAAGCACAGCCTCTCT 3'	5' CCATCTGCATAGCCACAGGCAACCTC 3'
ACC1	5' GCCATTGGTATTGGGGCTTAC 3'	5' CCCGACCAAGGACTTTGTTG 3'	5' CTCAACCTGGATGGTTCTTTGTCCCAGC 3'
DGAT1	5' CTGGGCATTCACAGCCATG 3'	5' TTCCCTTGGAAGAATCGGC 3'	5' CTCAGGTCCCACTGGCCTGGATTGT 3'
DGAT2	5' TGACTGGAACACGCCCAA 3'	5' ACGGCCCAGTTTCGCA 3'	5' CCACTGCGATCTCCTGCCACCTTT 3'
PPARα	5' CCTCAGGGTACCACTACGGAGT 3'	5' GCCGAATAGTTCGCCGAAA 3'	5' AAGCCCTTACAGCCTTCACATGCGTG 3'
PPARγ	5' TACATAAAGTCCTTCCCGCTGAC 3'	5' GTGATTTGTCCGTTGTCTTTCCT 3'	5' CAAGATCGCCCTCGCCTTGGCTT 3'
PGC1	5' TTTTTGGTGAAATTGAGGAATGC 3'	5' CGGTAGGTGATGAAACCATAGCT 3'	5' GTCTCCATCATCCCGCAGATTTACGG 3'
GLUT4	5' ACCTGTAACTTCATTGTCGGCAT 3'	5' ACGGCAAATAGAAGGAAGACGTA 3'	5' GGACCCATAGCATCCGCAACATACTGG 3'
PEPCK	5' CCATGAGATCTGAGGCCACA 3'	5' GTATTTGCCGAAGTTGTAGCCG 3'	5' CAAGGGCAAGATCATCATGCACGACC 3'
G6P	5' CAGGTCGTGGCTGGAGTCTT 3'	5' GACAATACTTCCGGAGGCTGG 3'	5' TGAAAGTTTCAGCCACAGCAATGCCTG 3'
GP	5' GCGGTGAACGGTGTAGCAA 3'	5' CTTGTCTGGTTCTAGCTCGCTG 3'	5' CCACTCGGACATCGTGAAGACCCAAGTA 3'
GABDH™	Applied Biosystems		
cyclophiline	5' CAAATGCTGGACCAAACACAA 3'	5' GCCATCCAGCCATTCAGTCT 3'	5' CCGGTTCCCAGTTTTTTATCTGCACTGCC 3'

coactivator-1 (PGC1), PPARγ, diacylglycerol acyltransferase 1 (DGAT1), DGAT2, SREBP1c, FAS, acyl-coA carboxylase (ACC) 1, and PPARα] and in liver [phosphoenolpyruvate carboxylase (PEPCK), glucose-6-phosphatase (G6P), glycogen phosphorylase (GP), PGC1, PPARγ, SREBP1c, FAS, ACC1, PPARα, DGAT1, and DGAT2] of mice after 4 and 16 h of starvation. Two other groups of mice, which were not subjected to a hyperinsulinemic clamp, were killed after 4 or 16 h of fasting, and liver and skeletal muscle were taken out for further analysis.

Muscle and liver were homogenized in 1,2 ml RNA-Bee (Tel-Test, Inc.) and total RNA was extracted according to Chomzcinsky and Sacchi (20). The amount of RNA was determined by spectrophotometry at a wavelength of 260nm. The quality was checked by the ratio of absorption at 260nm and absorption at 280nm. cDNA was obtained from total RNA.

For RT-PCR, forward and reverse primers and TaqMan probe (table1) were designed from mouse-specific sequence data (Entrez, National Institutes of Health; and Ensembl, Sanger Institute) using computer software (Primer Express; Applied Biosystems). For each of the genes, a Basic Local Alignment Search Tool search was done to reveal that sequence homology was obtained only for the target gene.

All TaqMan probes were 5'-6-carboxyfluorescein and 3'-BlackHoleQuencher-1 (BHQ1) labeled, except for glyceraldehyde phosphate dehydrogenase (GAPDH) (5'-VIC and 3'-BHQ1; Applied Biosystems) and cyclophiline (5'-TET and 3'-BHQ1).

Each oligonucleotide set was optimized to determine the optimal primer concentrations and probe concentration and verify the efficiency of the amplification. PCR amplification was performed in a total reaction volume of 12.5 μl. The reaction mixture consisted of qPCR™ MasterMix (Eurogentec), the optimal primer and probe concentrations of target gene and the endogenous control, nuclease free water, and cDNA. An identical cycle profile was used for all genes: 50°C for 2 min, 95°C for 10 min, followed by [95°C for 15 sec and 60°C for 1 min for 40 cycles.

Data were analyzed using a comparative critical threshold (Ct) method in which the amount of target normalized to the amount of endogenous control (GAPDH/cyclophiline) and relative to the control sample is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems). For each gene, all samples were run together allowing relative comparisons of the samples of a given gene.

Statistics

The data are presented as means ± SD. The data were analyzed using a non-parametric Mann-Whitney *U* test for independent samples. Differences were considered statistically significant at P≤0.05.

RESULTS

Body weight and plasma parameters

Body weight and basal and hyperinsulinemic plasma concentrations are shown in Table 2. Body weight was significantly lower in 16 h fasted mice compared with control mice (P<0.05). Plasma insulin and FFA concentrations were not significantly different between the groups, whereas basal plasma glucose concentrations were lower and plasma ketone bodies higher in 16 h fasted mice (P<0.01). During the hyperinsulinemic euglycemic clamp procedure, there were no differences in plasma glucose and FFA concentrations between the two groups, whereas insulin concentrations were lower in the 16 h fasted animals (P<0.01).

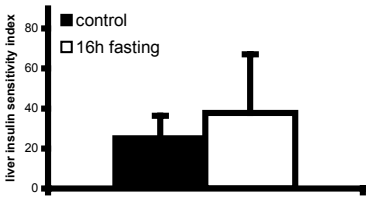

Fig. 1. Liver insulin sensitivity index in 16h fasted and control mice. Data are means ± SD for at least 9 animals per group.

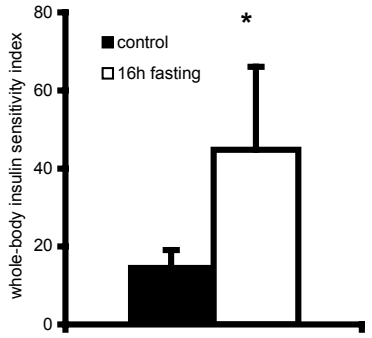

Fig. 2. Whole-body insulin sensitivity index in 16 h fasted and control mice. Data are means ± SD for at least nine animals per group. *P < 0.01 versus control mice.

HGP

Basal HGP was not significantly different between the 16 h fasted mice and the control mice (38±7 *versus* 43±9 µmol/kg/min, respectively). Liver insulin sensitivity index also was not significantly different between 16 h fasted and control mice (38±29 *versus* 25±11; ns), as seen in figure 1.

Glucose uptake

Basal BGU was not significantly different between the 16 h fasted mice and the control mice (38±7 *versus* 43±9 µmol/kg/min, respectively). Interestingly, whole body insulin sensitivity index was higher in 16 h fasted compared with control mice (45±21 *versus* 15±4,

P<0.01), reflecting increased whole body insulin sensitivity after 16 h of fasting (figure 2).

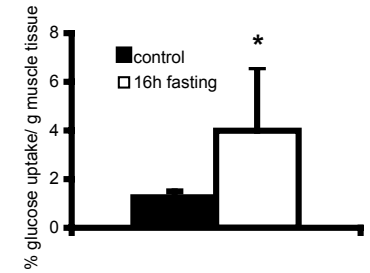

Fig. 3. Muscle-specific glucose uptake under hyperinsulinemic conditions in 16 h fasted and control mice. Data are means ± SD for at least eight animals per group. *P < 0.01 vs control mice.

Muscle-specific glucose uptake was significantly higher under hyperinsulinemic conditions in 16 h fasted compared with control mice (4.0±2.6 % *versus* 1.3 ± 0.2% glucose uptake/ g tissue, P<0.01) (figure 3).

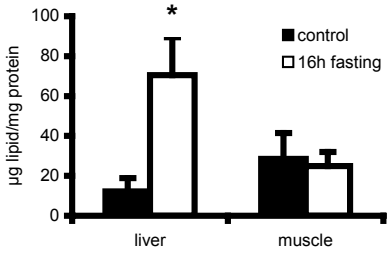

Fig. 4. TG content determined in liver and skeletal muscle of 16h fasted and control mice. Data are means ± SD for at least 6 animals per group. *p < 0.01 vs control mice.

Tissue lipid levels

Hepatic TG content was 6-fold higher in 16 h fasted mice compared with control mice (71±19 *versus* 12±7 µg/mg protein, P<0.01), whereas muscle TG content did not differ between the two groups (25±7 *versus* 28±13 µg/mg protein; ns) (figure 4).

mRNA expression levels

Hepatic mRNA expression levels of transcription factors and related proteins involved in gluconeogenesis and in TG synthesis increased during 16 h of fasting, whereas mRNA expression levels of transcription factors and related proteins involved in glycogenolysis and fatty acid synthesis decreased. The expression levels of G6P and PPARα mRNA were not significantly different (table 3a).

Muscle mRNA expression levels of transcription factors and related proteins involved in glucose uptake, fatty acid synthesis, TG synthesis and β-oxidation increased during 16 h of fasting, whereas SREBP1c (which has a role as a sensor of nutritional status) decreased (table 3b).

DISCUSSION

This study indicates that fasting does not result in changes in hepatic insulin sensitivity with regard to HGP in vivo. However, fasting increases muscle insulin sensitivity in vivo, reflected by an increased ability of insulin to stimulate muscle glucose uptake. In liver, the increased TG accumulation was not associated with

Table 3. mRNA expression levels of different proteins in liver (a) and skeletal muscle (b) of control (n=4) and 16h (n=4) fasted mice.

a

mRNA of	control	16h fasting
Glucose production		
G6P	100 ± 7 %	138 ± 23 %
PEPCK	100 ± 6 %	184 ± 9%**
GP	100 ± 6 %	59 ± 1 % **
PGC1	100 ± 10%	380 ± 32 %**
Fatty acid synthesis		
SREBP1c	100 ± 12 %	34 ± 1%**
FAS	100 ± 11 %	12 ± 1 %**
ACC1	100 ± 14 %	21 ±1 %**
TG synthesis		
PPARgamma	100 ± 9 %	179 ± 4 %**
DGAT1	100 ± 1 %	269 ± 6 %**
DGAT2	100 ± 7%	113 ± 4 %*
ß-oxidation		
PPARalpha	100 ± 15 %	96 ± 4 %

b

mRNA of	control	16h
Glucose uptake		
GLUT4	100 ± 2 %	157 ± 12% **
PGC1	100 ± 17%	166 ± 36 % *
Nutritional status		
SREBP1c	100 ± 8 %	3 ± 0% **
Fatty acid synthesis		
FAS	100 ±10 %	123 ± 8 % *
ACC1	100 ± 26 %	194 ± 35 % **
TG synthesis		
PPARgamma	100 ± 1 %	364 ± 12 % **
DGAT1	100 ± 12 %	193 ± 15 % **
DGAT2	100 ± 4 %	270 ± 20 % **
ß-oxidation		
PPARalpha	100 ± 7 %	278 ± 48% **

*Values are expressed as means ±SD. *p<0.05 compared to control mice, **p<0.01 compared to control mice*

changes in insulin sensitivity.

Moreover, the increase in muscle insulin sensitivity occurred without changes in muscle TG content. Therefore, changes in liver and muscle TG content are unlikely to be involved in changes in insulin sensitivity during conditions of fasting. Studies in transgenic mice with targeted disturbances in peripheral fatty acid/TG distribution showed that there appears to be an inverse dose-effect relationship between hepatic TG stores and hepatic insulin sensitivity (4;5). However, it does not seem possible to expand this theory to cases of fasting and fasting-induced hepatic steatosis.

The increase in muscle insulin sensitivity during fasting is a new and interesting finding.

Table 2. Body weight and plasma glucose, insulin, FFA, and ketone bodies concentrations in 4h fasted (control) and 16h fasted mice.

	Body weight (g)	Glucose (mmol/l)		Insulin (ng/ml)		FFA (mmol/l)		Ketone bodies (mmol/l)
		basal	hyperinsulinemia	basal	hyperinsulinemia	basal	hyperinsulinemia	
4h fasted (control)	27±2	5.9 ± 0.7	8.7±1.2	0.8±0.5	4.0±1.0	1.0±0.2	0.5±0.2	0.50±0.12
16h fasted	24±2*	4.9±0.9*	7.6±1.4	0.5±0.4	2.3±1.3**	1.2±0.5	0.5±0.3	1.21±0.25**

*Plasma glucose, insulin and FFA levels were measured during the hyperinsulinemic euglycemic clamp, during basal as well as hyperinsulinemic conditions. Body weight was measured just before the hyperinsulinemic euglycemic clamp. Values represent the mean ± SD of at least 9 mice per group. Ketone bodies were measured during basal conditions (mean ± SD of 7 mice per group). *p<0.05, **p<0.01 compared to control mice.*

Previous studies showed an inverse relationship between intramuscular TG content and insulin action (21;22). However, because we observed an increase in muscle insulin sensitivity without changes in muscle TG content, it is unlikely that changes in muscle TG play a role in the increased muscle-specific insulin sensitivity during fasting.

During the hyperinsulinemic period of the clamp procedure, we observed significantly lower insulin concentrations in 16 h fasted mice compared with control mice. As both groups received the same amount of insulin during hyperinsulinemic conditions by infusion, this difference suggests an increased insulin clearance during fasting. Because of this difference, we corrected the data on insulin sensitivity for the insulin concentrations.

Another purpose of the present study was to relate the observed in vivo metabolic changes to changes in the transcriptional regulation of genes involved in glucose metabolism, lipogenesis and β-oxidation in liver and muscle. In the liver, there were changes in expression of regulatory transcription factors favouring gluconeogenesis, β-oxidation and TG synthesis, with negative effects on glycogenolysis and fatty acid synthesis. Total HGP is the sum of glycogenolysis and gluconeogenesis. Liver glycogen stores are limited; consequently, during starvation the relative contribution of gluconeogenesis to total glucose production increases, whereas that of glycogenolysis decreases (23). PGC1 promotes gluconeogenesis by stimulation of PEPCK (24;25). Our results document a significant increase in the expression of PGC1 and PEPCK. GP is an enzyme involved in glycogenolysis, and its expression is decreased. G6P is involved in glucose production, with sources from both gluconeogenesis and glycogenolysis. The expression of G6P is not significantly altered by fasting, which seems to reflect the absence of changes in HGP in 16 h fasted compared with control mice.

Our study showed a reduced expression level of SREBP1c. This decrease in SREBP1c mRNA during fasting is in accordance with findings of others in mouse liver (26). Hepatic expression levels of enzymes involved in fatty acid synthesis (FAS and ACC1) decreased during fasting. The expression of these enzymes is stimulated by SREBP1c (27). Although this was not reported in mice before, these findings are in accordance with those of Hillgartner, Charron, and Chesnut (28) in fasted chickens. Because a decrease in expression of SREBP1c, FAS and ACC1 during 16 h of fasting results in decreased suppression of gluconeogenesis and reduced fatty acid synthesis, leaving pyruvate as a substrate for gluconeogenesis, these changes seem to be of physiological importance in the adaptive response of glucose and lipid metabolism to fasting.

The observed increase in liver TG accumulation in our study is in agreement with the observed increase of hepatic mRNA expression levels of PPARγ and of DGAT1 and DGAT2, all three favoring hepatic TG synthesis (29). The increase in hepatic PPARγ is in line with findings of others (26;29;30).

PPARα, which is known as 'the fasting gene', controls the expression of numerous genes related to lipid metabolism in the liver, including genes involved in β-oxidation, fatty acid uptake, and transport. Therefore, it is surprising that PPARα mRNA was not increased after 16 h of fasting, whereas this was found by others in wild-type mice (9). Because these mice had another background (SV129), and others found a decrease in PPARα mRNA after 48 and 72 h of fasting in wild-type mice (1) with the same background as our mice (C57Bl6), it seems likely that these differences in background explain this discrepancy.

In muscle, RT-PCR results demonstrate that fasting-induced expression of genes and enzymes favouring glucose uptake. mRNA expression levels of PGC1 and GLUT4 were increased after 16 h of fasting. PGC1 can control the level of endogenous GLUT4 gene expression in multinucleate myotubes via coactivating MEF2C (31). Moreover, Hammerstedt et al. (32) showed a high correlation (r=0.91) between GLUT4 mRNA and PGC1 mRNA in human skeletal muscle. Therefore, our findings support their hypothesis that PGC1 is associated with increased GLUT4 expression and insulin sensitivity.

The observed decrease in muscle SREBP1c mRNA in our study is in agreement with recently published results (33). These studies found a decrease in SREBP1c mRNA in different rat muscle types that was related to the duration of fasting and consistent with a role for SREBP1c as a sensor of nutritional status in skeletal muscle. Although SREBP1c stimulates the expression of genes involved in lipid metabolism (such as FAS, ACC1) in tissues like the liver, not much is known about the regulatory role of SREBP1c in skeletal muscle. Because our study shows an increase in FAS and ACC1 mRNA during 16 h of fasting but decreased expression of SREBP1c, our data imply that muscle FAS and ACC1 are not stimulated by SREBP1c.

The mRNA expression levels of PPARγ, another lipogenesis-promoting transcription factor in skeletal muscle, strongly increased during 16 h of fasting. This contrasts with a previous study (34), which found no alterations of PPARγ mRNA expression level during fasting in rat skeletal muscle. However, these rats were fasted for 46 h, whereas our mice were fasted for only 16 h. Because mRNA expression levels of DGAT1 and DGAT2, which are involved in muscle TG-synthesis

(35), were significantly increased after fasting in the absence of muscle TG accumulation in this period, it can be speculated that prolonged fasting will be accompanied by muscle TG accumulation.

In the present study, PPARα mRNA, which is involved in β-oxidation, was increased after fasting. This is in agreement with a recent finding (36) showing increased muscle glucose uptake in mice treated with a PPARα agonist (WY14,643).

In conclusion, 16 h of fasting in wild-type mice results in hepatic steatosis without changes in hepatic insulin sensitivity. In muscle, however, 16 h of fasting increased insulin sensitivity without changes in muscle TG content. Therefore, fasting induces differential changes in tissue-specific insulin sensitivity. In addition, changes in liver and muscle TG content are unlikely to be involved in changes in insulin sensitivity during fasting.

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Chapter 3

High fat diet induced hepatic insulin resistance is not related to changes in hypothalamic mRNA expression of NPY, AgRP, POMC and CART in mice

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ABSTRACT

The hypothalamic circuitry, apart from its impact on food intake, modulates insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability. The purpose of the present study was to investigate the effects of 2 weeks high fat feeding in wildtype mice on (1) insulin sensitivity and triglyceride accumulation in liver and muscle in relation to (2) mRNA expression levels of Neuropeptide Y (NPY), Agouti-related protein (AgRP), pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) in the hypothalamus. Two weeks of high fat feeding induced hepatic insulin resistance in the presence of increased hepatic triglyceride accumulation. In muscle, however, 2 weeks of high fat feeding did not result in changes in insulin sensitivity or in triglyceride content. mRNA expression levels of NPY, AgRP, POMC, and CART in the hypothalamus were not different between the groups. This study shows that 2 weeks of high fat feeding in mice does not affect mRNA expression levels of NPY, AgRP, POMC or CART, in the whole hypothalamus, despite induction of hepatic, but not peripheral, insulin resistance. Therefore, a major physiological role of these neuroendocrine factors in the induction of hepatic insulin resistance during a high fat diet seems less likely.

INTRODUCTION

High fat (HF) diet is associated with the development of hepatic and muscle insulin resistance and the induction of liver steatosis and muscle triglyceride accumulation in rodents and humans. The induction of hepatic insulin resistance is an early event, whereas the indication of muscle insulin resistance occurs later in rats and dogs fed a HF diet for 3-12 weeks (11,12). The change in time of hepatic and peripheral insulin sensitivity during high fat feeding in mice has not been reported so far.

The hypothalamic arcuate circuitry, apart from its impact on food intake, modulates insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability. Two types of neurons in the hypothalamus are of major importance for the control of these processes: neurons co-expressing neuropeptide Y (NPY) and Agouti-related protein (AgRP), and neurons co-expressing pro-opiomelanocortin (POMC), the molecular precursor of alpha-melanocyte stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART) (8). These neuropeptides exert opposing effects on food intake and fuel homeostasis. We, and others, recently showed that, independent of its stimulating effect on food intake, NPY is also involved in controlling insulin action in peripheral tissues. Intracerebroventricular (icv) infusion of NPY induces hepatic insulin resistance in mice and rats (15,23). Conversely, subchronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the melanocortin-3/4 receptor (16). These observations suggest that the balance between NPY/AgRP and POMC/CART neuronal activity may be a determinant of peripheral and hepatic insulin sensitivity.

To explore a potential physiological relationship between the arcuate NPY/POMC circuitry and the development of tissue specific insulin resistance during a HF diet, we studied the effects of 2 weeks HF feeding in wildtype mice on (1) insulin sensitivity and TG accumulation in liver and muscle in relation to (2) mRNA expression levels of NPY, AgRP, POMC, and CART in the whole hypothalamus.

MATERIAL AND METHODS

Animals

Male, 12-16 weeks old, C57Bl/6 mice were used in all experiments. Mice were kept in a temperature- and humidity controlled environment and had free access to food and water. Light were on from 07:00 h a.m. till 07:00 h p.m. The control group had

free access to standard chow diet whereas the experimental group had a high fat diet (21.5 wt.% fat or 43 energy% fat; saturated bovine fat) (Hope Farms, Woerden, the Netherlands) ad libitum. Before the experiments mice were fasted 4 h (postprandial) with food withdrawn at 05:00 h a.m., in order to exclude interfering effects of glucose uptake from the gastrointestinal tract. All experiments were performed at 09:00 h a.m. All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center and TNO-Prevention and Health, Leiden, the Netherlands.

Hyperinsulinemic euglycemic clamp study

The hyperinsulinemic euglycemic clamp study was performed as published earlier (7,22,24). In short, a continuous infusion of D-^{14}C glucose, 0.3 µCi/kg.min, (Amersham, Little Chalfont, UK) alone was started and blood samples were taken to determine basal glucose parameters. The hyperinsulinemic study started with a bolus (100 mU/kg, Actrapid, Novo Nordisk, Bagsvaerd, Denmark) followed by continuous infusion of insulin (6.8 mU/h) and of D-^{14}C glucose. A variable infusion of 12.5% D-glucose (in PBS) solution was adjusted to maintain euglycemia, as measured via tailbleeding (Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, the Netherlands). Blood samples (75µl) were taken when glucose levels in the blood were stable for determination of plasma [^{14}C]glucose and insulin concentrations. To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H]glucose (2-[^{3}H]DG; Amersham, Little Chalfont, UK) was administered as a bolus (1µCi), 40 minutes before the end of the clamp. After the last blood sample was taken, mice were sacrificed and liver and muscle were taken out, immediately frozen using liquid N_2 and stored at -20°C until further analysis. For the determination of plasma D-^{14}C glucose, plasma was deproteinized with 20% trichloroacetic acid, dried to remove water, resuspended in demi-water and counted with scintillation fluid (Ultima Gold, Packard, Meridien, CT, USA) on dual channels for separation of ^{14}C and ^{3}H, as described earlier (7,22,24).

Plasma parameters

Plasma glucose, free fatty acid and insulin levels were determined using commercially available kits (Sigma, St.Louis, MO; Wako Pure Chemical Industries, Osaka, Japan; Mercodia, Uppsala, Sweden, respectively).

Tissue analysis

Liver and muscle samples were homogenized (~10% wet w/v) in H_2O. Lipids were extracted according to Bligh and Dyer's method (2). In short, a solution was made of each sample of 200 µg protein in 800 µl H_2O, then 3 ml methanol/chloroform (2:1) was added and mixed thoroughly, after which 500 µl of chloroform, 100 µl internal standard and 1 ml demi-water were added. After centrifugation the chloroform layer was collected and dried. The remaining pellet was dissolved in 50µl chloroform and put on a HPTLC plate. The amount of TG in the tissues was quantified by scanning the plates with a Hewlett Packard Scanjet 4c and by integration of the density using Tina® version 2.09 software (Raytest, Staubenhardt, Germany).

For determination of tissue 2-DG uptake, the homogenate of muscle was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously (7,18,22,24).

Calculations

Under steady-state conditions for plasma glucose concentrations, the rate of glucose disappearance equals the rate of glucose appearance (Ra; i.e. endogenous glucose production plus exogenous D-glucose infusion). The latter was calculated as the ratio of the rate of infusion of D-^{14}C glucose (dpm/min) and the steady-state plasma [^{14}C]glucose specific activity (dpm/µmol glucose). Hepatic glucose production (HPG) was calculated as the difference between Ra and the infusion rate of exogenous D-glucose. Muscle tissue specific glucose uptake was calculated from tissue 2-DG content, which was expressed as percent of 2-DG of the dosage per gram of tissue, as described previously (18).

Extraction of hypothalamus

Two other groups of mice fed on chow or 2 weeks HF diet were, after 4 hours fasting, sacrificed at 9 a.m. by cervical dislocation. After death, the brain was dissected from the skull. A region bordered dorsally by the thalamus, rostrally by the optic chiasm, laterally by the internal capsule, and caudally by the mammillary bodies was excised, as described previously (9). Hypothalami removed were immediately frozen in liquid nitrogen.

Real Time Polymerase Chain Reaction (RT-PCR)

A real time polymerase chain reaction (RT-PCR) was used to measure NPY/AgRP/POMC/CART mRNA expression levels in the hypothalamus of mice after HF feeding and in control mice. Hypothalami were homogenised in 1,2 ml RNA-Bee

(Tel-Test, Inc, Texas, US) and total RNA was extracted according to Chomzcinsky and Sacchi (5). The amount of RNA was determined by spectrophotometry (ND-1000 spectrophotometer, Nanodrop®) at a wavelength of 260 nm. The quality was checked by the ratio of the absorption at 260 nm and the absorption at 280 nm. Complementary DNA (cDNA) was obtained of total RNA.

For RT-PCR, forward and reverse primers were designed from mice specific sequence data (*Entrez*, National Institutes of Health; and *Ensembl*, Sanger Institute) using computer software (Primer Express, Applied Biosystems). For each of the genes a Blast Search was done to reveal that sequence homology was obtained only for the target gene. Forward and reverse primers of NPY (5'CGCTCTGCGACACTACATCAA3'; 5'GGGCTGGATCTCTTGCCAT3'), AgRP (5' GGTGCTAGATCCACAGAACCG 3'; 5' CCAAGCAGGACTCGTGCAG 3'), POMC (5' CGAGGCCTTTCCCCTAGAGT 3'; 5' CCAGGACTTGCTCCAAGCC 3'), and CART (5' TGGATGATGCGTCCCATGA 3'; 5' CGGAATGCGTTTACTCTTGAGC 3') were used. Neuron-specific enolase (NSE) (5' ACGTGGTTCCATTTCAAGATGAC 3'; 5' CGAGCTTCAGTTAGTGCACCAA 3') and hypoxanthine phosphoribosyltransferase (HPRT) (5' GCTCGAGATGTCATGAAGGAGAT 3'; 5' AAAGAACTTATAGCCCCCCTTGA 3') were used as housekeeping genes.

PCR amplification was performed in a total reaction volume of 20 µl. The reaction mixture consisted of SybrGreen mix (Applied Biosystems, United Kingdom), forward and reverse primers, nuclease free water and cDNA. An identical cycle profile was used for all genes: 50°C for 2 min + 95°C for 10 min + [95°C for 15 sec + 60°C for 1 min] * 50 cycles. Specificity of NPY, AgRP, POMC, CART, NSE and HPRT amplifications was confirmed by analyzing the dissociation curves of the target amplicon. Data were analysed using a comparative critical threshold method as described previously (7).

Statistical analysis

Data are presented as means ± standard deviation. Data were analysed using a non-parametric Mann-Whitney *U*-test for independent samples. Differences were considered statistically significant at $P \leq 0.05$.

RESULTS

Body weight and plasma parameters

Two weeks of high fat diet did not lead to differences in body weight, plasma glucose, insulin or FFA levels compared to chow-fed littermates. We also achieved equal plasma glucose, insulin and FFA levels during the hyperinsulinemic euglycemic clamp (table 1).

Table 1. Body weight and plasma glucose, insulin, and FFA concentrations of HF fed and control mice under basal and hyperinsulinemic conditions.

	Body weight (g)	Glucose (mmol/l)		Insulin (ng/ml)		FFA (mmol/l)	
		basal	hyper-insulinemia	basal	hyper-insulinemia	basal	hyper-insulinemia
control	27±2	5.9 ± 0.7	8.7±1.2	0.8±0.5	4.1±1.0	1.0±0.2	0.5±0.2
HF	27±2	6.7±1.2	8.8±0.7	1.9±1.4	3.7±1.7	1.0±0.2	0.6±0.2

Values represent the mean ± SD of at least 8 mice per group

Hepatic glucose production

Basal HGP was not different between the HF fed group and the control group (48±8 µmol/kg.min versus 43±9 µmol/kg.min, ns). Under hyperinsulinemic conditions, HGP was significantly higher in the HF fed group versus the control group (30±9 µmol/kg.min versus 16±10 µmol/kg.min, P<0.05). The relative inhibition of HGP was significantly less after HF feeding compared to the control group (35±20% versus 61±23%, P<0.05), indicating hepatic insulin resistance (figure 1).

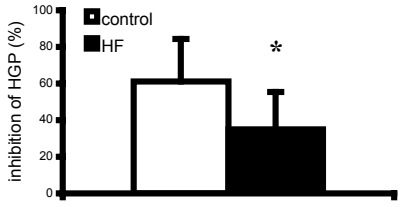

Fig. 1. Inhibition of hepatic glucose production of HF fed and control mice. Data are means ± SD for n=9 in the control group and n=8 in the HF group. *p < 0.05 vs control mice, as assessed by the Mann-Whitney *U* test.

Whole body glucose uptake and muscle specific 2-DG uptake

Basal whole body glucose uptake was not different between the HF fed group and the control group (48±8 µmol/kg.min versus 43±9 µmol/kg.min, ns). Under hyperinsulinemic conditions, whole body glucose uptake was not different between the HF fed group and the control group (66±10 µmol/kg.min versus 59±8 µmol/kg.min, ns).

Under hyperinsulinemic conditions, muscle-specific glucose uptake in the HF fed group was not significantly different from the control group (1.3±0.6 versus 1.3±0.3%/g tissue).

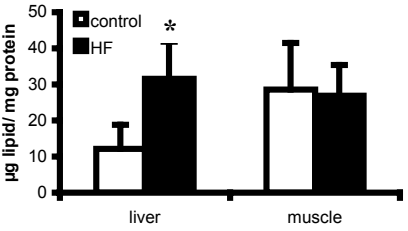

Fig. 2. TG-content determined in liver and skeletal muscle of HF fed and control mice. Data are means ± SD for n=7 in the control group and n=6 in the HF group. *p < 0.05 vs control mice, as assessed by the Mann-Whitney U test.

Tissue lipid levels

Hepatic TG content of HF fed mice was 2.5-fold higher compared to the control group (32±10 versus 12±6 µg/mg protein, P<0.05), whereas muscle TG content was not significantly different between the two groups (27±9 versus 23±7 µg/mg protein, ns) (figure 2).

mRNA expression levels

Hypothalamic mRNA expression levels of NPY, AgRP, POMC, and CART were not different between HF fed and control mice (100±11% versus 111±20%; 100±20% versus 113±20%;100±47% versus 140±54%;100±33% versus 81±12%, respectively, ns).

DISCUSSION

This study shows that 2 weeks of high fat feeding in wildtype mice causes hepatic insulin resistance, reflected by decreased inhibition of hepatic glucose production, without affecting muscle specific insulin sensitivity. However, this was not associated with differences in hypothalamic mRNA expression of NPY, AgRP, POMC, and CART. Therefore, the hypothalamus does not seem to play a major role in the primacy of hepatic insulin resistance during a HF diet in mice.

Although it was concluded from studies in the past in rats (17) and primates (4) that peripheral insulin resistance precedes the development of hepatic insulin resistance, our findings are in line with recent studies in rats (12) and dogs (11) documenting the primacy of hepatic insulin resistance during high fat feeding. Kraegen et al (12) used an extremely high fat diet (59% calorie fat) and found already after 3 weeks muscle specific insulin resistance as well. As this extreme diet is not relevant for the human situation we used a more human like high fat diet. However, even after 6 weeks, we did not observe muscle specific insulin resistance (unpublished data).

In our study, 2 weeks of high fat feeding resulted in hepatic insulin resistance in the presence of hepatic steatosis. These findings are in line with observations of

other groups [18;19] and could imply that hepatic steatosis is either a consequence of metabolic alterations elsewhere in the body and/or an active player in the pathophysiology of the metabolic syndrome.

The mechanisms underlying the impairment of insulin action during a high fat diet are only partly known. Studies performed in vitro and in vivo showed an increase in gluconeogenesis (6,13) and a decrease in the suppressive effect of insulin on glycogenolysis (3). Shulman *et al.* postulated a more direct effect of FFA on hepatic glucose production, by interference of fatty acid derivatives with insulin signalling (10,20).

The past years, data have become available on the potency of the hypothalamus to modulate insulin sensitivity during environmental fluctuations in nutrient availability. Independent of its stimulating effect on food intake and body weight, intracerebroventricular (icv) infusion of NPY causes hepatic insulin resistance in mice and rats (15,23). Conversely, subchronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the melanocortin-3/4 receptor (16). These observations suggest that the balance between NPY/AgRP and POMC/CART neuronal activity is a determinant of peripheral and hepatic insulin sensitivity. In our study, however, we did not find significant changes in NPY, AgRP, POMC and CART mRNA expression levels in the whole hypothalamus during a 2 weeks HF diet, despite tissue-specific differences in insulin sensitivity. Since in our study, mRNA levels were measured in whole hypothalamus without regional dissection of the data, we cannot totally exclude involvement of certain specific hypothalamic areas in the regulation of hepatic insulin resistance during a high fat diet. To our knowledge, differential activation of NPY neurons in the medial and lateral part of the arcuate nucleus is possible (14,21), but no reports of opposite activation have been published. Therefore, a small effect could theoretically be masked by differential expression, but unlikely the absence of an effect, as we report in our study. The same holds true for POMC. In addition, the fasting condition might have a potential masking effect on neuropeptide expression, however, every feeding state could have a masking effect, depending on the neuropeptide of interest.

Our observations are partly in line with those of other groups, who did not find an effect of a 2 weeks HF diet on NPY and AgRP mRNA expression levels in the hypothalamus of C57Bl/6 mice (25) and on NPY mRNA expression level in the hypothalamus of two strains of rats (Osborne-Mendel and S5B/Pl) (19) either. However, in contrast to our findings, Ziotopoulou *et al*. found a significant increase in POMC mRNA expression levels after 2 weeks of HF feeding in mice (25). They

hypothesized that the observed overexpression of POMC, a precursor of melanocyte-stimulating hormone, a potent inhibitor of food intake, is a response to overfeeding and positive energy balance to counteract the effect of high fat diet to promote increased food intake in an effort to maintain energy homeostasis. This is in line with findings of Bergen et al., who found compensatory hypothalamic changes in mRNA expression levels of NPY and POMC in A/J mice (resistant to diet-induced obesity) fed a high fat diet for 14 weeks (1). In line with our study, these studies imply that changes in hypothalamic mRNA expression levels of NPY, AgRP, POMC and CART are rather secondary or compensatory to high fat feeding, in order to maintain energy homeostasis, than primary, i.e. causing increased food intake during high fat feeding and thereby obesity and insulin resistance.

From this study, we conclude that 2 weeks of high fat feeding in mice does not affect mRNA expression levels of NPY, AgRP, POMC or CART in the whole hypothalamus, despite induction of hepatic, but not peripheral, insulin resistance. Therefore, a major physiological role of these neuroendocrine factors in the induction of hepatic insulin resistance during a high fat diet seems less likely.

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Chapter 4

Intracerebroventricular administration of melanotan II increases insulin sensitivity of glucose disposal in mice

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ABSTRACT

***Aims/hypothesis*. The present study was conducted to evaluate the effects of central administration of melanotan II (MTII), a MC3/4 receptor agonist, on hepatic and whole-body insulin sensitivity, independent of food intake and body weight.**

***Methods.* Over a period of 24 h, 225 ng of MTII was injected in three aliquots into the left lateral ventricle in male C57Bl/6 mice. The animals had no access to food. The control group received three injections of distilled water. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic-euglycaemic clamp in combination with [^{3}H]-glucose infusion. *Glut4* mRNA expression was measured in skeletal muscle.**

***Results.* Plasma glucose and insulin concentrations under basal and hyperinsulinaemic conditions were similar in MTII- and placebo-treated mice. Endogenous glucose production (EGP) and glucose disposal in the basal state were significantly higher in MTII-treated mice than in the control group (71±22 *vs.* 43±12 µmol/min/kg, p<0.01). During hyperinsulinaemia, glucose disposal was significantly higher in MTII-treated mice (151±20 *vs*. 108±20 µmol/min/kg, p<0.01). In contrast, the inhibitory effect of insulin on EGP was not affected by MTII (relative decrease of EGP: 45±27 *vs.* 50±20%). *Glut4* mRNA expression in skeletal muscle was significantly increased in MTII-treated mice (307±94 *vs.* 100±56%, p<0.01).**

***Conclusions/interpretation*. Intracerebroventricular administration of MTII acutely increases insulin-mediated glucose disposal, but does not affect capacity of insulin to suppress EGP in C57Bl/6 mice. These data indicate that central stimulation of melanocortin-3/4 receptors modulates insulin sensitivity in a tissue-specific manner, independent of its well-known impact on feeding and body weight.**

INTRODUCTION

The hypothalamus integrates a multitude of behavioural and metabolic adaptations to food intake and starvation, necessary to maintain fuel homeostasis despite profound environmental variations in nutrient availability [1]. Two types of neuron in the arcuate nucleus of the hypothalamus are of major importance for the control of these processes: neurons co-expressing Agouti-related protein (AgRP) and neuropeptide Y (NPY), and neurons expressing pro-opiomelanocortin (POMC), the molecular precursor of alpha-melanocyte-stimulating hormone (α-MSH) [2]. α-MSH binds to and stimulates melanocortin (MC) receptors. AgRP can bind to MC receptors as well, thereby inhibiting the POMC pathway [3]. NPY and POMC neuropeptides exert opposing effects on food intake and fuel homeostasis. NPY acts to promote anabolic pathways, whereas α-MSH counteracts the effects of NPY [4-6]. For instance, during food deprivation, NPY/AgRP neuronal activity is high while POMC/α-MSH expression levels are low [5], and this setting of the arcuate neuronal circuitry strongly stimulates food intake and reduces energy expenditure [7].

Apart from its impact on food intake, intracerebroventricular (icv) administration of NPY acutely hampers capacity of insulin to inhibit hepatic glucose and VLDL production in C57Bl/6 mice, while insulin sensitivity of muscle and adipose tissue remains unaffected [8]. Conversely, chronic (7 days) icv infusion of α-MSH enhances peripheral and hepatic insulin sensitivity in rats through stimulation of the MC3/4 receptor [9] and POMC gene overexpression ameliorates insulin resistance in leptin-deficient mice independently of its effects on food intake and body weight [10]. In the latter studies, the effects on insulin sensitivity occur in the presence of a concomitant reduction in food intake and fat mass, which precludes distinction between putative direct effects of α-MSH or MC4 receptor on insulin sensitivity and indirect effects via feeding and body composition.

In addition to its effect on of MC4 receptor activation on insulin sensitivity, decreased insulin concentration after central activation of the melanocortin neuronal circuitry, and increased levels of insulin in MC4 receptor knock-out mice even before the onset of detectable hyperphagia or obesity [11], have been documented. In humans, MC4 receptor mutations are associated with obesity [12;13].

The aim of the present study was to document the direct effects of the activation of MC3/4 receptors on insulin sensitivity (i.e. via mechanistic routes other than feeding and fat mass). As such, we injected (icv) melanotan II (MTII) [14], an agonist of the MC3/4 receptor [15], and quantified hepatic and peripheral insulin sensitivity of glucose metabolism during a hyperinsulinaemic-euglycaemic clamp in mice with no access to food.

MATERIALS AND METHODS

Animals.

Male 3-month-old C57Bl/6 mice (originating from The Jackson Laboratory [Bar Harbor, ME, USA] and bred in our own animal facility) were housed in a temperature- and humidity-controlled room on a 12-h light-dark cycle with free access to standard mouse chow and water. All animal experiments were performed in accordance with the principles of laboratory animal care and regulations of Dutch law on animal welfare, and the protocol was approved by the institutional ethics committee for animal procedures.

Surgical procedures.

Mice were anaesthetised with 0.5 mg/kg medetomidine (Pfizer, Capelle a/d IJssel, The Netherlands), 5 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.05 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). A 25-gauge guide cannula was stereotactically implanted into the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral and 2.2 mm ventral [16]. The guide cannula was secured to the skull surface using two screws and dental cement (AgnTho's, Lidingö, Sweden). After a recovery period of 1 week, adequate placement of the cannulae was tested by measuring the feeding response to an acute icv injection of NPY (5 µg dissolved in 1 µl sterile water; Bachem, Bubendorf, Germany).

Hyperinsulinaemic-euglycaemic clamp.

Mice fasted for 24 h (with food withdrawn at 09.00 hours on the day before the experiment) were used. At 9.00 and 17.00 hours on the day before the experiment and at 8.45 hours on the day of the experiment mice were given 75 ng (in 1.5 µl distilled water) MTII (PhoenixEurope, Karlsruhe, Germany) or 1.5 µl distilled water (control group) icv. This dose of MTII was based on data from a tudy by Murphy et al [17], which showed inhibition of food intake using this dose. During icv injections, mice were lightly anaesthetised with isoflurane. All experiments were performed at 09.00 hours. Hyperinsulinaemic-euglycaemic clamps were performed as described previously [18;19]. During the experiments, mice were sedated with 6.25 mg/kg acepromazine (Sanofi sante animale, Libourne Cedex, France), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag).

Basal rates of glucose turnover were measured by giving a primed (26 kBq) continuous (44 kBq/h) infusion of [^{3}H]glucose (Amersham, Little Chalfont, UK) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 h to attain steady-state circulating insulin levels of about 4 ng/ml. The [^{3}H]glucose infusion (44

kBq/h) was continued. A variable infusion of 12.5% D-glucose (in PBS) solution was also started and adjusted to maintain blood euglycaemia (measured at 10-min intervals via tail bleeding; Freestyle, TheraSense; Disetronic Medical Systems, Vianen, The Netherlands). Blood samples (60 µl) were taken during the basal period (after 60 and 80 min) and during the clamp period (when glucose levels in the blood were stable, and 20 and 40 min later) for measurement of plasma glucose, NEFAs, insulin and [^{3}H]glucose specific activity.

mRNA expression of Glut4.

Real-time RT-PCR was used to measure mRNA expression levels of *Glut4* (now known as *Slc2a4*) in skeletal muscle. Skeletal muscle was extracted from additional groups of mice directly at 10.30 hours following a 24-h fast and three icv injections with MTII or vehicle (injections at the same time points as in the hyperinsulinaemic-euglycaemic clamp experiment). Muscle was homogenised in 1.2 ml RNA-Bee (Tel-Test, Texas, USA) and total RNA was extracted according to the method described by Chomzcinsky and Sacchi [20]. The amount of RNA was determined by spectrophotometry (ND-1000 spectrophotometer; Nanodrop, Wilmington, DE, USA) at a wavelength of 260 nm. The quality was checked using the ratio of absorption at 260 nm : absorption at 280 nm. Complementary DNA (cDNA) was obtained out of total RNA. For RT-PCR, forward and reverse primers and TaqMan probe were designed from mouse-specific sequence data (Entrez, National Institutes of Health, Bethesda, MD, USA; and Ensembl, Wellcome Trust Sanger Institute, Cambridge, UK) using computer software (Primer Express, Applied Biosystems, Foster City, CA, USA). For each of the genes a Blast Search was done to reveal that sequence homology was obtained only for the target gene. Forward and reverse primers and TaqMan probe (5' CCATGAGATCTGAGGCCACA 3'; 5' GTATTTGCCGAAGTTGTAGCCG 3'; 5' CAAGGGCAAGATCATCATGCACGACC 3') of *Glut4* were used. The TaqMan probe was labelled with 5'-6-carboxyfluorescein and 3'- (BHQ1). The genes encoding GAPDH (5' VIC and 3' BHQ1, Applied Biosystems) and *cyclophilin* (5' TET and 3' BHQ1, 5' CAAATGCTGGACCAAACACAA 3'; 5' GCCATCCAGCCATTCAGTCT 3'; 5' CCGGTTCCCAGTTTTTTATCTGCACTGCC 3') were used as housekeeping genes. PCR amplification was performed in a total reaction volume of 12.5 µl. The reaction mixture consisted of (1) qPCR MasterMix (Eurogentec, Seraing, Belgium); (2) the optimal primer and probe concentrations of *Glut4* and the endogenous control; (3) nuclease-free water; and (4) cDNA. An identical cycle profile was used for all genes: 50°C for 2 min+95°C for 10 min+[95°C for 15 s+60°C for 1 min]*40 cycles. Data were analysed using a comparative critical threshold (Ct) method where the amount of target normalised to the amount of endogenous control (genes encoding GAPDH/cyclophilin) and relative to the control sample

is given by $2^{-\Delta\Delta Ct}$ (Applied Biosystems). All samples were run together allowing relative comparisons of the samples.

Analytical procedures.

Plasma glucose and NEFA levels were determined using a commercially available kit (Instruchemie, Delfzijl, The Netherlands; Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin and corticosterone concentrations were measured by ELISA (both ALPCO Diagnostics, Windham, NH, USA). For the measurement of plasma [^{3}H]glucose, plasma was deproteinised with 20% trichloroacetic acid, dried to remove water and resuspended in distilled water. Samples were counted using scintillation fluid (Ultima Gold; Packard, Meridien, CT, USA).

Calculations.

Glucose turnover rate (μmol/min/kg) was calculated during the basal period and under steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of [^{3}H]glucose (dpm/μmol). The ratio was corrected for body weight. Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Statistical analysis.

Data are presented as mean±SD. Differences between groups were determined by Mann-Whitney *U*-test for two independent samples. A p value of less than 0.05 was considered statistically significant.

Table 1. Body weight, plasma corticosterone, NEFA, glucose and insulin concentration in vehicle (n=10) and MTII (n=8) mice

	Basal		Hyperinsulinemic	
	Vehicle	MTII	Vehicle	MTII
Body weight (g)	17.9 ± 1.7	18.9 ± 1.6	-	-
Corticosterone (mmol/l)	33.3 ± 17.5	37.8 ± 12.4	n.d.	n.d.
Glucose (mmol/l)	5.8 ± 1.0	6.7 ± 1.2	8.4 ± 1.0	8.2 ± 2.4
NEFA (mmol/l)	0.55 ± 0.17	0.62 ± 0.17	0.26 ± 0.14	0.24 ± 0.08
Insulin (ng/ml)	0.31 ± 0.11	0.31 ± 0.07	3.8 ± 2.9	3.7 ± 2.7

Values are expressed as means ± SD. n.d. is not determined.

RESULTS

Plasma parameters.

Body weight, plasma corticosterone, glucose, NEFA and insulin concentrations under basal and hyperinsulinaemic conditions are shown in Table 1. In the basal state, these parameters did not differ between MTII- and vehicle-treated animals. Under steady-state hyperinsulinaemic conditions, plasma NEFA levels decreased approximately two-fold while insulin concentrations increased approximately ten-fold as expected. No differences were observed in plasma glucose, insulin and NEFA levels between MTII- and vehicle-treated mice during hyperinsulinaemia.

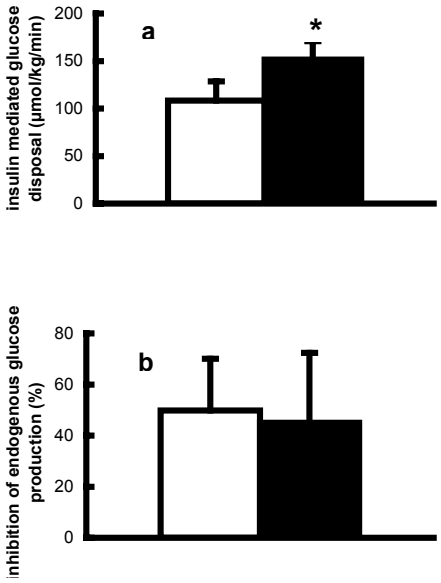

Fig 1. a) insulin mediated glucose disposal in 24 hours fasted mice that received icv injections of MTII (n=10) (black bars) or vehicle (n=8) (white bars); b) inhibition of EGP by insulin in 24 hours fasted mice that received icv injections of MTII (n=10) (black bars) or vehicle (n=8) (white bars). Values represent mean ± SD. *p<0.01 versus vehicle.

Glucose turnover.

Under basal conditions, EGP (and thereby glucose disposal) was significantly higher in MTII-treated animals than in vehicle-treated mice (71 ± 22 *vs.* 43 ± 10 µmol/min/kg, p<0.01). During the hyperinsulinaemic period, the rate of glucose infusion necessary to maintain euglycaemia was significantly higher in MTII- treated animals than in vehicle-treated animals (114 ± 23 *vs.* 85 ± 20 µmol/min/kg, p<0.05). Accordingly, the glucose disposal rate was significantly higher in MTII-treated animals (151 ± 20 *vs.* 108 ± 20 µmol/min/kg, p<0.01, Fig. 1a). In contrast, hyperinsulinaemia suppressed EGP to a similar extent in MTII- treated mice and vehicle-treated mice (45 ± 27% *vs.* 50 ± 20%, NS, Fig. 1b).

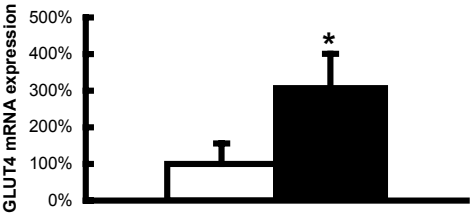

Fig 2. GLUT4 mRNA expression levels in 24 hours fasted mice that received injections of MTII (n=6) (black bars) or vehicle (n=7) (white bars) in basal conditions. *p<0.01 versus vehicle.

mRNA expression of Glut4.

Glut4 mRNA expression in skeletal muscle was higher in the MTII-treated group compared to vehicle-treated mice (307 ± 94 *vs.* 100 ± 56 %, p<0.01, Fig. 2).

DISCUSSION

This study shows that activation of MC3/4 receptors enhances whole-body sensitivity of glucose metabolism for insulin action in mice via mechanisms other than feeding and fat mass. In particular, MTII promotes insulin-mediated glucose disposal, but does not affect the capacity of insulin to suppress EGP. These observations are in line with the emerging notion that neural circuits control insulin action in peripheral tissues.

Interestingly, *Glut4* mRNA was increased in the muscle of MTII-treated animals, which suggests that activation of MC3/4 receptors enhances *Glut4* gene expression to promote glucose uptake. The downstream mechanisms that actuate the effects of hypothalamic neuronal circuits on muscle *Glut4* mRNA expression remain to be fully elucidated. The possibility that MTII increases locomotor activity and subsequently *Glut4* mRNA expression in muscle cannot be ruled out. However, this seems unlikely since other studies have not observed any increase in locomotor activity following central administration of MTII [14;17]. Additional studies are required to elucidate the mechanisms involved in the modulation of insulin sensitivity by central administration of MTII.

Glucose production in the basal state was higher in mice treated with MTII, but MTII did not affect the capacity of insulin to suppress EGP. Thus, central melanocortin pathways appear to directly impact endogenous glucose output. Although Fan et al. [11] reported decreased plasma insulin concentrations after icv administration of MTII, we did not find significant changes in basal plasma insulin concentrations as a potential explanation for the observed increase in basal glucose production. As Fan et al injected more than ten times the amount we injected, this discrepancy may be explained by the difference in dose. Since MTII has been shown to activate the sympathetic nervous system [21;22] and sympathetic outflow promotes glucose production [23], it is conceivable that the autonomic nervous system relays signals from the hypothalamus to peripheral tissues to modulate glucose

metabolism. Unfortunately, our study does not provide data to evaluate this possibility. Alternatively, the brain could interact with the liver via the hypothalamo-pituitary-adrenal axis (HPA). Corticosteroids stimulate glucose production [24] and HPA activity is under strict control of the hypothalamus. In fact, corticotrophin-releasing hormone neurons in the paraventricular nucleus of the hypothalamus express MC4 receptors, and central MTII injection acutely enhances corticotrophin-releasing hormone and corticosterone release in rats [25]. However, MTII did not modify circulating corticosterone concentrations in our experimental setting, which refutes the hypothesis that central melanocortin pathways modulate the HPA axis to enhance glucose output. Interestingly, short-term fasting is accompanied by a decrease in EGP [26;27], and POMC/α-MSH mRNA expressions decreases concomitantly [5]. It is therefore tempting to speculate that blunted melanocortin signalling is involved in the decrease in EGP during fasting. If this was the case, administration of MTII may have prevented the normal decline in EGP associated with fasting in the present study.

Although MTII increased basal EGP, it did not appear to affect capacity of insulin to suppress it. A previous paper [9] reports that chronic (7 days) icv infusion of α-MSH reinforces insulin action on glucose production (as well as on glucose disposal) in rats. However, this effect occurred in the presence of concomitant reductions in food intake, and body adiposity as well as these long-term sequelae of MTII administration can impact on insulin sensitivity. Our data indicate that activation of melanocortin circuits enhances insulin sensitivity independently of food intake and body weight, and that insulin action on glucose disposal is more sensitive to manipulation of MC3/4 receptors than it is capable of suppressing EGP.

We recently showed that icv infusion of NPY in C57Bl/6 mice acutely hampers inhibitory effect of insulin on EGP but does not appear to affect insulin-mediated glucose disposal [8]. We have now shown that activation of melanocortin circuits reinforces insulin action on glucose disposal but does not affect suppression of glucose production. NPY and melanocortin circuits in the arcuate nucleus play critical roles in the control of fuel homeostasis in the face of fluctuations in nutrient availability. NPY neurons, active during fasting, stimulate feeding and inhibit energy expenditure, whereas melanocortin circuits, suppressed in fasting conditions, counteract NPY to exert opposing effects on energy balance. These behavioural and metabolic actions serve to protect the body against the perils of famine. Our data suggest that the brain also modulates glucose metabolism to further reinforce the line of defence. Enhanced activity of NPY neurons promotes glucose production, but reduced melanocortin activity hampers glucose disposal in fasting conditions, keeping glucose available as pivotal fuel for the brain. Conversely, diminished NPY-ergic and increased melanocortin signalling allow insulin to appropriately suppress glucose output and

promote glucose disposal in response to food intake. The current findings may imply that MC-3/4 receptor agonists serve as "insulin sensitisers" in the treatment of the metabolic syndrome and type 2 diabetes mellitus. However, tachyphylaxis to chronic MTII administration has been observed in mice and rats [28;29]. In addition, the present study shows that MTII increases basal EGP. Thus, the impact of chronic MTII administration on glucose metabolism in (insulin resistant) animal models and humans remains to be established.

In conclusion, the present study shows that activation of central melanocortin-3/4 receptors by melanotan II enhances insulin sensitivity of whole body glucose disposal, independently of food intake and fat mass, but does not affect ability of insulin to suppress EGP. These observations are in line with the emerging notion that neural circuits, apart from their effects on feeding, modulate insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability.

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Chapter 5

PYY_{3-36} reinforces insulin action on glucose disposal in mice fed a high fat diet

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ABSTRACT

Peptides YY_{3-36} (PYY_{3-36}) is released by the gut in response to nutrient ingestion. It modulates the activities of orexigenic neuropeptide Y (NPY) neurons and anorexigenic proopiomelanocortin (POMC) neurons in the hypothalamus to inhibit food intake. Because both NPY and POMC have also been shown to impact insulin action, we wondered whether PYY_{3-36} could improve insulin sensitivity. To address this question, we examined the acute effect of intravenous PYY_{3-36} on glucose and free fatty acid (FFA) flux during a hyperinsulinemic-euglycemic clamp in mice maintained on a high-fat diet for 2 weeks before the experiment. We also evaluated the effects of PYY_{3-36} infusion on glucose uptake in muscle and adipose tissue in this experimental context. Under basal conditions, none of the metabolic parameters were affected by PYY_{3-36}. Under hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36}-infused compared with vehicle-infused mice (103.8 ± 10.9 *vs.* 76.1 ± 11.4 µmol/min/kg, respectively; P=0.001). Accordingly, glucose uptake in muscle and adipose tissue was greater in PYY_{3-36}-treated animals, although the difference with controls did not reach statistical significance in adipose tissue (muscle: 2.1 ± 0.5 *vs.* 1.5 ± 0.5 µmol/ g tissue, P=0.049; adipose tissue: 0.8 ± 0.4 *vs.* 0.4 ± 0.3 µmol/ g tissue; P=0.08). In contrast, PYY_{3-36} did not impact insulin action on endogenous glucose production or FFA metabolism. These data indicate that PYY_{3-36} reinforces insulin action on glucose disposal in mice fed a high-fat diet, through a mechanism that is independent of food intake and body weight. In contrast, it leaves glucose production and lipid flux largely unaffected in this experimental context.

INTRODUCTION

Peptide YY (PYY) belongs to a family of peptides that is critically involved in the regulation of appetite. It is synthesized in specialized cells (L-cells) that are found primarily in the distal gastrointestinal tract. Circulating PYY levels rise within 15 minutes after a meal in proportion to the amount of calories ingested and remain elevated for ~6 h (1). The two natural forms of this peptide, PYY_{1-36} and PYY_{3-36}, have opposing effects on food intake (2): PYY_{1-36} stimulates appetite whereas PYY_{3-36} (the major circulating form) inhibits feeding (3-5).

PYY_{3-36} reduces food intake by acting on appetite regulation centers in the hypothalamus (3;6). Specifically, PYY_{3-36} is an agonist of the presynaptic neuropeptide Y (NPY) Y2 receptor, which inhibits NPY neuronal activity in the arcuate nucleus and thereby activates adjacent proopiomelanocortin (POMC) neurons (3). In addition to their critical role in the control of feeding, both NPY and POMC affect insulin action. Intracerebroventricular infusion of NPY induces hyperinsulinemia and insulin resistance in rats (7;8). In contrast, central administration of α-melanocyte stimulating hormone, a splice-product of the POMC peptide, enhances insulin sensitivity (9). In view of the fact that PYY_{3-36} inhibits NPY neuronal activity and activates that of POMC, we wondered whether it could improve insulin sensitivity “directly” (i.e., through a mechanism independent of food intake and body weight). To address this question, we infused PYY_{3-36} intravenously and quantified its acute effects on glucose and fatty acid flux during a hyperinsulinemic-euglycemic clamp in mice without access to food during the experiment.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice were housed in a temperature-controlled room on a 12-h light/dark cycle and were fed a high-fat diet (43 energy% fat derived from bovine lard) with free access to water for 2 weeks to induce insulin resistance. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare, and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinemic-euglycemic clamp

A total of 36 mice were fasted overnight with food withdrawn at 5:00 P.M. the day before the study. The next day, hyperinsulinemic-euglycemic clamps were performed as described earlier (10). PYY or vehicle was administered as a primed (0.15 µg)-continuous (0.25 µg/h) intravenous infusion during the whole experiment (basal and hyperinsulinemic period). In one series of experiments, glucose turnover was quantified, and in another series, free fatty acid (FFA) turnover was determined. First, basal rates of glucose or FFA turnover were measured by giving a primed-continuous infusion of ^{3}H-glucose (prime: 0.7 µCi; continuous: 1.2 µCi/h; Amersham, Little Chalfont, U.K.) or ^{14}C-palmitate (prime: 1.8 µCi; continuous: 3 µCi/h; Amersham), respectively, for 80 min. Subsequently, insulin was administered in a primed (4.1 mU)-continuous (6.8 mU/h) intravenous infusion for 2-3 h to attain steady-state circulating insulin levels of ~6 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycemia, measured at 10 min intervals via tail bleeding (Freestyle; TheraSense, Disetronic Medical Systems, Vianen, The Netherlands). Blood samples (75 µl) were taken during the basal period (after 60 and 80 min) and during the clamp period (when glucose levels were stable and 20 and 40 min later) for determination of plasma glucose, FFA, and insulin concentrations and [^{3}H]glucose and [^{14}C]palmitate specific activities.

To assess insulin-mediated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H] glucose (2-DG; Amersham) was administered as a bolus (1µCi) 40 minutes before the end of the clamp experiments in which FFA turnover was measured. At the end of the clamp, mice were killed, and muscle and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures

Plasma levels of glucose and FFAs were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands, and Wako, Neuss, Germany). Plasma insulin and PYY_{3-36} concentrations were measured by a mouse insulin enzyme-linked immunoabsorbant assay and PYY_{3-36} radioimmunoassay (Alpco, Windham, NH, and Phoenix Pharmaceuticals, Belmont, CA). Total plasma ^{3}H-glucose was determined in 7.5 µl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water. Total plasma ^{14}C-palmitate was determined in 7.5 µl plasma after extraction of lipids by a modification of Bligh and Dyer's (11) method. Briefly, 7.5 µl plasma was dried and resolved in 100 µl water. Then 1.1 ml demi-water and 4.5 ml methanol:chloroform (2:1) were added and mixed thoroughly, after which 1.5 ml chloroform was added and mixed, and, finally, 1.5 ml demi-water was added and mixed. After centrifugation, the chloroform layer was

collected, and the FFA fraction was separated from other lipid components by thin-layer chromatography (TLC) on silica gel plates.

Tissue analysis

For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled, and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-phosphate from 2-DG as described previously (10;12;13).

Calculations

Turnover rates of glucose and FFAs (µmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{3}H-glucose or ^{14}C-palmitate (dpm/µmol). The ratio was corrected for body weight. Endogenous glucose production (EGP) was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adipose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as micromoles per gram of tissue.

Statistical analysis

Differences between groups were determined by Mann-Whitney's non-parametric test for two independent samples. $P < 0.05$ was considered statistically significant. All values shown represent mean ± SD.

RESULTS

Animals

Mice were 16-18 weeks old during the experiments. Body weight was 23.3 ± 1.2 g in the control group and 22.8 ± 1.4 g in the PYY group.

Plasma parameters

Plasma glucose, FFA, insulin, and PYY_{3-36} concentrations in basal and hyperinsulinemic conditions are shown in table 1. In the basal state, plasma parameters did not differ between PYY- and vehicle-infused animals, except for the plasma PYY_{3-36} concentration. Under steady-state clamp conditions, plasma insulin and glucose concentrations were similar in both groups. Hyperinsulinemia suppressed FFA levels to a similar extent in PYY- and vehicle-infused mice.

Table 1. Plasma parameters in overnight fasted mice that received an i.v.-infusion of PYY or vehicle under basal or hyperinsulinemic conditions. Values represent mean ± SD for at least 7 mice per group.

	Basal		Hyperinsulinemic	
	Vehicle	**PYY**	**Vehicle**	**PYY**
Glucose (mmol/l)	**4.9 ± 0.6**	**5.0 ± 0.6**	**9.0 ± 0.9**	**9.2 ± 2.0**
FFA (mmol/l)	**0.9 ± 0.2**	**1.0 ± 0.2**	**0.4 ± 0.1**	**0.5 ± 0.1**
Insulin (ng/ml)	**0.3 ± 0.1**	**0.3 ± 0.1**	**5.2 ± 2.9**	**6.7 ± 2.7**
PYY_{3-36} (pg/µl)	**< 1**	**3.8 ± 0.7**	**< 1**	**3.2 ± 0.7**

Data are the means ± SD for at least seven mice per group

Glucose turnover

Under basal conditions, glucose disposal was similar in PYY- and vehicle-infused mice (39,5 ± 10.9 vs. 45.9 ± 12.6 µmol/min/kg, respectively; P=0.19) (Figure 1a). The rate of glucose infusion necessary to maintain euglycemia during insulin infusion was significantly higher in PYY-infused mice than in vehicle-infused animals (89.1 ± 7.1 vs. 50.9 ± 13.6 µmol/min/kg, P=0.001), indicating that intravenous PYY_{3-36} administration acutely enhances insulin sensitivity. Hyperinsulinemia increased glucose disposal in both groups. However, the disposal rate was significantly higher in PYY-infused animals compared with vehicle-infused controls (103.8 ± 10.9 vs. 76.1 ± 11.4 µmol/min/kg, respectively; P=0.001) (Figure 1a). In contrast, EGP, which was similar in PYY- and vehicle-treated mice under basal conditions, was

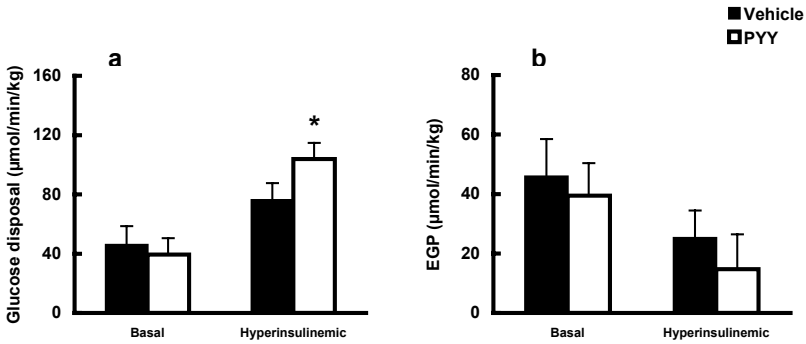

Fig. 1. Glucose disposal (a) and endogenous glucose production (b) in overnight-fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic-euglycemic clamp. Before and during insulin infusion the animals received an intravenous infusion of PYY or vehicle. Values represent mean ± SD for at least seven mice per group. *P<0.01 vs. vehicle.

suppressed by insulin to the same extent in both groups (by 62 ± 29 vs. 42 ± 18% from basal, respectively; P=0.30) (Figure 1b).

Tissue-specific glucose uptake

Insulin-mediated 2-DG uptake in muscle and adipose tissue was greater in PYY-treated animals, but the difference with controls did not reach statistical significance in adipose tissue (muscle: 2.1 ± 0.5 vs. 1.5 ± 0.5 µmol/g tissue, P=0.049; adipose tissue: 0.8 ± 0.4 vs. 0.4 ± 0.3 µmol/g tissue; P=0.08) (Figure 2)

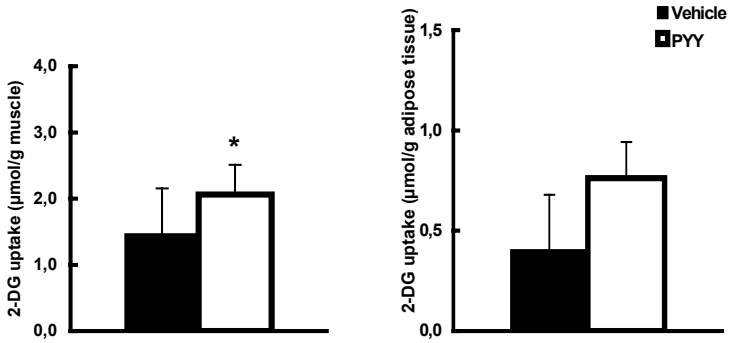

Fig. 2. Muscle-specific (a) and adipose tissue-specific (b) glucose uptake under hyperinsulinemic conditions in overnight-fasted mice that received an intravenous infusion of PYY or vehicle. Values represent mean ± SD for at least seven mice per group. *P<0.05 vs. vehicle.

FFA turnover

Basal FFA turnover (38.0 ± 14.2 vs. 42.3 ± 9.9 µmol/min/kg) was not different between PYY- and vehicle-infused animals and was suppressed to a similar extent by insulin in both groups (22.4 ± 12.3 vs. 21.3 ± 10.9 µmol/min/kg in PYY- and vehicle-infused animals, respectively) (Figure 3).

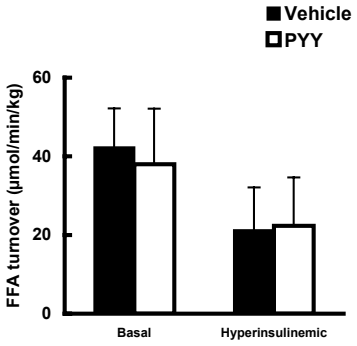

Fig. 3. Free fatty acids (FFA) turnover in overnight fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic-euglycemic clamp. Before and during insulin infusion the animals received an intravenous infusion of PYY or vehicle. Values represent mean ± SD for at least nine mice per group.

DISCUSSION

Here we show that intravenous PYY_{3-36} administration acutely reinforces insulin action on glucose disposal in overnight-fasted mice maintained on a high-fat diet. In contrast, PYY_{3-36} does not appear to impact the effect of insulin on EGP or FFA metabolism in this experimental context.

PYY_{3-36} clearly enhanced insulin-induced glucose disposal as determined by tracer dilution methodology. Accordingly, 2-DG uptake in muscle and adipose tissue under hyperinsulinemic conditions was higher during PYY_{3-36} infusion, albeit the difference with control attained statistical significance only for muscle. In contrast, PYY_{3-36} did not significantly impact the capacity of insulin to inhibit EGP. Insulin action on FFA metabolism was not affected by PYY_{3-36}, either, as indicated by similar fatty acid turnover rates during hyperinsulinemia in PYY_{3-36}- and saline-infused animals. In light of the current experimental context, these data suggest that PYY_{3-36} reinforces the impact of insulin on glucose disposal through a mechanism that is independent of food intake and body weight. In contrast, it appears to leave insulin action on glucose production and FFA turnover largely unaffected.

The plasma PYY_{3-36} concentration rose to 3.2 ± 0.7 pg/µl in response to PYY infusion. Relatively few studies have looked at the physiology of circulating PYY_{3-36} in rodents. According to a recent paper by Batterham et al.(3), postprandial PYY_{3-36} concentrations amount to 112 pmol/l (~0.4 pg/µl) in freely feeding rats. In a study by Lee et al (14), plasma PYY levels in fasting mice were 0.18 pg/µl, which accords with our data (table 1). We are not aware of any study measuring postprandial PYY_{3-36} concentrations in mice. Thus, the dose of PYY_{3-36} we administered may have induced supraphysiological PYY_{3-36} levels. Therefore, our data do not allow a definitive inference as to whether the postprandial rise of circulating PYY_{3-36} can reinforce insulin action.

Postprandial PYY_{3-36} release is decreased in obese compared with lean subjects, and circulating levels correlate negatively with BMI. Conversely, intravenous PYY_{3-36} infusion significantly reduces food intake in humans (15), and repeated administration of PYY_{3-36} attenuates weight gain in rodents (3). These findings suggest that diminished PYY_{3-36} release may contribute to the pathogenesis of obesity in animals and humans. The observations presented here allow us to hypothesize that low circulating PYY_{3-36} levels may also compromise insulin action in obese subjects. Moreover, perhaps even more important, the data suggest that PYY_{3-36} or synthetic analogs of this peptide may be useful tools in the clinical management of obesity and insulin resistance.

It remains to be established whether PYY_{3-36} acts through hypothalamic neural circuits (by analogy with the mechanism guiding its effects on appetite) to enhance insulin-mediated glucose disposal. Because PYY_{3-36} is a high-affinity agonist to the Y2 receptor (2) and the distribution of this receptor subtype is largely confined to the central nervous system (particularly the arcuate nucleus of the hypothalamus) (16), it is most likely that PYY_{3-36} modulates insulin action by activation of Y2 receptors in the brain. As alluded to earlier, Y2 receptor signaling inhibits NPY neuronal activity in the arcuate nucleus of the hypothalamus and thereby activates adjacent POMC neurons (3). Hypothalamic overexpression of NPY induces obesity and insulin resistance in mice (7;8), whereas activation of melanocortin receptor subtypes 3 and 4 in the brain enhances insulin sensitivity (9). Thus, the present data are in keeping with the contention that PYY_{3-36} modulates insulin action via hypothalamic Y2 receptor. The downstream mechanisms that actuate the effects of hypothalamic neuronal circuits on muscle and adipose tissue remain to be fully elucidated. At this point, vagotomy was shown to prevent the hyperinsulinemic effects of NPY, which suggests that the autonomic nervous system may be involved (17). Also, adrenalectomy prevents the obesity syndrome produced by chronic central NPY infusion and reverses the obese phenotype in leptin-deficient *ob/ob* mice (18;19), indicating that the pituitary-adrenal ensemble may also serve as a second messenger. Thus, neural and/or endocrine mechanistic routes may convey signals from hypothalamic nuclei to peripheral tissues to orchestrate energy balance and fuel flux. It is conceivable that similar routes partake in the control of insulin action by PYY_{3-36}.

In conclusion, PYY_{3-36} reinforces insulin action in mice maintained on a high-fat diet through a mechanism that is independent of food intake and body weight. In this context, PYY_{3-36} appears to predominantly impact insulin-mediated glucose disposal, whereas it leaves insulin action on glucose production largely unaffected. These novel data suggest that PYY_{3-36} or synthetic analogues of this peptide may be valuable assets to our armamentarium of drugs designed to battle insulin resistance and type 2 diabetes mellitus.

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Chapter 6

Chronic PYY_{3-36} treatment ameliorates insulin resistance in C57BL6-mice on a high fat diet

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Submitted

ABSTRACT

Aims/hypothesis. **PYY_{3-36} is a gut-derived hormone, that acts on hypothalamic nuclei to modulate energy metabolism. We recently showed, that PYY_{3-36} acutely reinforces insulin action on glucose disposal in insulin resistant mice. However the long-term effects of PYY_{3-36} on insulin sensitivity are still unknown.**

Methods. **To adress this question, we examined the effects of chronic PYY_{3-36} administration (2.5 µg/day s.c. for 7 days) on glucose turnover during a hyperinsulinemic-euglycemic clamp in C57BL6 mice maintained on a high fat diet for 16 weeks before the experiment. In addition, metabolic efficacy of continuous *vs.* intermittent administration of PYY_{3-36} was evaluated.**

Results. **Under hyperinsulinemic conditions, glucose disposal was significantly increased in PYY_{3-36} treated mice *vs.* vehicle-treated mice (78.8 ± 13.3 *vs.* 63.4 ± 15.5 µmol/min/kg, respectively, *P*=0.012). Tissue specific glucose uptake was significantly increased in adipose tissue (0.5 ± 0.2 *vs.* 0.2 ± 0.1 µmol/ g tissue; *P*=0.006), but not in muscle (2.2 ± 1.4 *vs.* 1.6 ± 0.8 µmol/ g tissue for PYY_{3-36} and vehicle-treated animals, respectively, *P*=0.38) of PYY_{3-36} treated animals. In contrast, insulin action on endogenous glucose production was not significantly affected. Furthermore, none of these metabolic parameters were affected by the mode of PYY_{3-36} administration (continuous or intermittent).**

Conclusions/interpretation. **Chronic PYY_{3-36} administration enhances the ability of insulin to promote glucose disposal, whereas it does not significantly affect endogenous glucose production in C57BL6 mice maintained on a high fat diet for 16 weeks. In addition, this study shows that continuous and intermittent administration are equally effective in this respect.**

INTRODUCTION

The metabolic syndrome comprises a cluster of anomalies that increase the risk of cardiovascular disease and type 2 diabetes mellitus: hyperglycemia, abdominal obesity, hypertriglyceridemia, hypertension and low levels of high-density lipoprotein (HDL) cholesterol [1-3]. Insulin resistance may underlie the majority of these pathologies [4] and therapies that effectively reinforce insulin action may therefore ameliorate the risk profile of metabolic syndrome patients [5;6].

Diet-induced obese, insulin resistant C57BL6-mice have increased levels of neuropeptide Y (NPY) and decreased levels of pro-opiomelanocortin (POMC) in hypothalamic nuclei [7-9]. These features of hypothalamic neural circuits may be involved in the pathogenesis of the metabolic syndrome, as intracerebroventricular (icv) administration of NPY or antagonists of POMC induce insulin resistance [10-13]. Therefore, antagonists of NPY and/or agonists of POMC signalling may be useful tools in the clinical management of this syndrome. Peptide YY_{3-36} (PYY_{3-36}) is released in response to food intake by L-cells in the distal gastrointestinal tract. It acts via Y2 receptors on NPY neurons in the arcuate nucleus to inhibit NPY neuronal activity and thereby activates adjacent POMC neurons [14;15]. We recently found that PYY_{3-36} administration acutely reinforces insulin action on glucose disposal through a mechanism that is independent of food intake and body weight [16]. This finding suggests that PYY_{3-36} may be used as a therapeutic tool in the clinical management of insulin resistance and the metabolic syndrome. However, the metabolic effects of long-term PYY_{3-36} administration are currently unknown, and waning of early impact may occur during chronic treatment through down regulation of receptor expression or function [17;18]. Therefore, the aim of this study was to investigate the long-term effects of PYY_{3-36} on insulin action by administering PYY_{3-36} subcutaneously for 7 days in mice fed a high-fat diet, and quantifying the effects on glucose production and disposal during a hyperinsulinemic euglycemic clamp study. As the physiology of PYY_{3-36} entails intermittent release in response to food intake, we also examined whether continuous and intermittent administration of PYY_{3-36} impact glucose metabolism differentially in this experimental context.

MATERIAL AND METHODS

Animals

Male C57BL6 mice were housed in a temperature-controlled room on a 12-hour light-dark cycle and were fed a high fat diet (43 energy% fat derived from bovine lard) with free access to water for 16 weeks to induce insulin resistance. After 15 weeks of high fat diet, osmotic minipumps (Alzet minipump, model 2001, Charles River, Maastricht, The Netherlands) were placed subcutaneously in the back region under light isoflurane anesthesia. All mice received

a saline (n = 15) or PYY_{3-36} (2.5 µg/day, n = 5) infusion via the osmotic minipump at a rate of 0.5 µl/h for 7 days. In addition, daily subcutaneous injections (50 µl at 09.00 am) of saline or PYY_{3-36} (2.5 µg) were given, where mice receiving continuous PYY_{3-36} treatment were additionally injected with saline, and mice receiving saline by minipump were assigned to receive either saline (n = 8) or PYY_{3-36} (n = 7) by injection. Thus, glucose kinetics were determined in 2 experimental groups: 1) receiving saline and 2) receiving PYY_{3-36}, where PYY_{3-36} was administered continuously by minipump or intermittently by daily subcutaneous injection. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinemic euglycemic clamp

Mice were fasted overnight with food withdrawn at 05.00 pm the day prior to the study. The next day, hyperinsulinemic euglycemic clamps were performed as described earlier [19]. First, basal rates of glucose turnover were measured by giving a primed (0.7 µCi) continuous (1.2 µCi/h) infusion of ^{14}C-glucose (Amersham, Little Chalfont, U.K.) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU) continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours to attain steady state circulating insulin levels of ~3.5 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycemia (measured at 10 min intervals via tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (75 µl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels were stable and 20 and 40 minutes later) for determination of plasma glucose, non-esterified fatty acids (NEFA), insulin and PYY_{3-36} concentrations and ^{14}C-glucose specific activities.

To assess insulin-mediated glucose uptake in individual tissues, 2-deoxy-D-[^{3}H] glucose (2-[^{3}H]DG; Amersham, Little Chalfont, UK) was administered as a bolus (1µCi), 40 minutes before the end of the clamp experiments. At the end of the clamp, mice were sacrificed and muscle and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Analytical procedures

Plasma levels of glucose and NEFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands and Wako, Neuss, Germany). Plasma insulin and PYY_{3-36} concentrations were measured by a mouse insulin ELISA and PYY_{3-36} RIA (Mercodia, Uppsala, Sweden; Phoenix pharmaceuticals, Belmont, CA, USA; sensitivity of 1 pg/µl for the PYY_{3-36} RIA). Total plasma ^{14}C-glucose was determined in 7.5 µl plasma and in

supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Tissue analysis

For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled and the supernatant was subjected to an ion-exchange column to separate 2-DG-6-P from 2-DG as described previously [19-21].

Calculations

Turnover rates of glucose (µmol/min/kg) were calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{14}C-glucose (dpm/µmol). The ratio was corrected for body weight. EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adiopose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as µmol per gram of tissue.

Statistical analysis

Differences between groups were determined by Mann-Whitney non-parametric test for 2 independent samples. A P-value < 0.05 was considered statistically significant. All values shown represent means ± SD.

RESULTS

Animals

Body weight did not differ between PYY_{3-36} and vehicle-infused animals (after 7 days of PYY_{3-36}/saline administration: 28.0 ± 3.7 gram in the PYY_{3-36} group and 28.3 ± 1.5 gram in the control group, P=0.68). Overnight food intake was measured at day 2 and day 5 of PYY_{3-36}/saline administration and was similar in both groups (day 2: 2.37 ± 0.68 vs. 2.32 ± 0.33 gram, P=0.96; day 5: 2.76 ± 0.54 vs. 2.75 ± 0.43 gram, P=0.97 in PYY_{3-36} and vehicle-treated animals, respectively). Furthermore, body weight and overnight food intake was not different in groups receiving continuous or intermittent PYY_{3-36} treatment (body weight: 29.5 ± 3.9 vs. 26.9 ± 3.4 gram, P=0.20; food intake day 2: 2.14 ± 0.98 vs. 2.48 ± 0.56 gram, P=0.38; day 5: 2.53 ± 0.69 vs. 2.92 ± 0.39 gram, P=0.27 for continuous and intermittent administration, respectively).

Table 1. Plasma parameters under basal or hyperinsulinemic conditions in overnight fasted mice that received PYY_{3-36} (n=12) or vehicle (n=8) for 7 days.

	Basal		Hyperinsulinemic	
	Vehicle	PYY_{3-36}	Vehicle	PYY_{3-36}
Glucose (mmol/l)	7.7 ± 1.3	8.4 ± 1.5	8.4 ± 1.2	9.4 ± 0.8
NEFA (mmol/l)	1.1 ± 0.2	0.9 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Insulin (ng/ml)	0.7 ± 0.3	0.7 ± 0.4	3.2 ± 0.9	3.6 ± 0.8

Data are the means ± SD. * <0.05 *vs.* vehicle

Plasma parameters

Plasma glucose, NEFA, and insulin concentrations in basal and hyperinsulinemic conditions are shown in table 1. Plasma glucose and insulin concentrations did not differ between vehicle and PYY_{3-36} treated animals under basal and steady state clamp conditions. Furthermore, continuous and intermittent PYY_{3-36} administration had similar impact on these parameters, except for the plasma glucose levels under basal conditions, which were slightly but significantly higher in the group that received continuous PYY_{3-36} administration (basal glucose: 9.3 ± 0.9 vs. 7.7 ± 1.5 mmol/l, P=0.048; hyperinsulinemic glucose: 9.9 ± 0.8 vs. 9.1 ± 0.6 mmol/l, P=0.073; basal insulin: 0.9 ± 0.4 vs. 0.5 ± 0.3 ng/ml, P=0.073; hyperinsulinemic insulin: 3.9 ± 1.0 vs. 3.4 ± 0.6 ng/ml, P=0.43). Plasma NEFA concentrations were slightly, but significantly, lower in PYY_{3-36} treated mice in basal (P=0.025) and steady state clamp (P=0.031) conditions, where continuous and intermittent PYY_{3-36} administration did not have differential effects (basal NEFA: 0.9 ± 0.3 vs. 0.9 ± 0.1 mmol/l, respectively, P=0.76; hyperinsulinemic NEFA: 0.5 ± 0.1 vs. 0.4 ± 0.1 mmol/l, respectively, P=0.073). Plasma PYY_{3-36} concentrations in basal and hyperinsulinemic conditions were below the

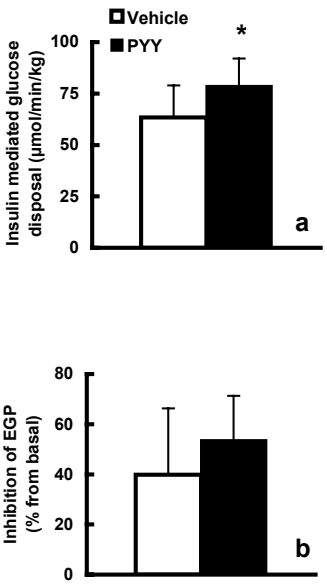

Fig 1. Insulin mediated glucose disposal (a) and inhibition of endogenous glucose production (EGP) by insulin (b) in overnight fasted mice before (basal) and during (hyperinsulinemic) a hyperinsulinemic euglycemic clamp study. Prior to the clamp experiment the animals received PYY_{3-36} (n=12) or vehicle (n=8) for 7 days. Values represent the means ± SD. *P<0.05 *vs.* vehicle

detection level in all groups (<1 pg/µl), except for the basal condition of the mice that received intermittent PYY_{3-36} administration (3.7 ± 0.8 pg/µl).

Glucose turnover

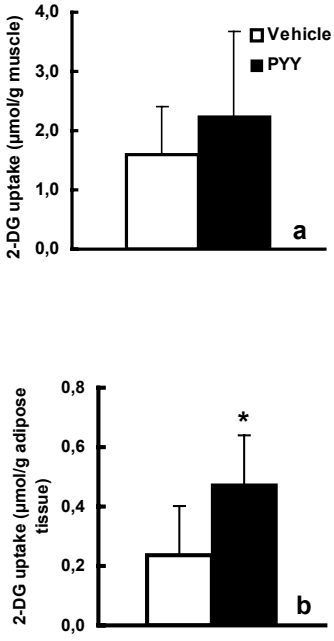

Fig 2. Muscle-specific (a) and adipose tissue-specific (b) glucose uptake under hyperinsulinemic conditions in overnight fasted mice that received PYY_{3-36} (n=11) or vehicle (n=7) for 7 days. Values represent the means ± SD. *P<0.05 vs. vehicle.

In basal conditions, glucose disposal was similar in PYY_{3-36} and vehicle-treated mice (52.0 ± 10.5 *vs.* 50.4 ± 10.4 µmol/min/kg, respectively, P=0.68). The rate of glucose infusion necessary to maintain euglycemia during insulin infusion was significantly higher in PYY_{3-36} treated mice than in vehicle-treated animals (54.0 ± 11.4 *vs.* 33.4 ± 11.6 µmol/min/kg, *P*=0.000), indicating that chronic PYY_{3-36} administration enhances whole body insulin sensitivity. Continuous and intermittent administration of PYY_{3-36} had similar effects on the glucose infusion rate (54.7 ± 9.2 *vs.* 53.6 ± 10.2 µmol/min/kg, respectively, P=0.27). Hyperinsulinemia increased glucose disposal in both groups. However, the disposal rate was significantly higher in PYY_{3-36} treated animals compared with vehicle-treated controls (78.8 ± 13.3 *vs.* 63.4 ± 15.5 µmol/min/kg, respectively, *P*=0.012, Figure 1a) and was similar in animals treated by continuous and intermittent administration (81.2 ± 13.8 *vs.* 77.1 ± 13.7 µmol/min/kg, respectively, P=0.64). Endogenous glucose production was similar in PYY_{3-36} and vehicle-treated mice in basal conditions and was suppressed by insulin to the same extent in both groups (by 54 ± 18 *vs.* 40 ± 26% from basal in PYY_{3-36} *vs.* vehicle treated groups, respectively; *P*=0.27, Figure 1b), where percent inhibition did not differ between animals receiving continuous or intermittent PYY_{3-36} treatment. (52 ± 25 *vs.* 55 ± 12% from basal, respectively, P=0.53).

Tissue-specific glucose uptake

Insulin-mediated 2-deoxy-glucose uptake was measured in muscle and adipose tissue. In muscle, 2-deoxy-glucose was similar in both groups (2.2 ± 1.4 *vs.* 1.6 ± 0.8 µmol/ g tissue for PYY_{3-36} and vehicle-treated animals, respectively, *P*=0.38). In adipose tissue 2-deoxy-glucose uptake was significantly increased in PYY_{3-36} treated animals compared with vehicle treated mice (0.5 ± 0.2 *vs.* 0.2 ± 0.1 µmol/ g tissue; *P*=0.006, Figure 2).

DISCUSSION

Here we show that chronic PYY_{3-36} administration improves whole body insulin sensitivity of glucose metabolism in C57BL6 mice maintained on a high fat diet for 16 weeks. In particular, PYY_{3-36} treatment enhances the ability of insulin to promote glucose disposal via mechanistic routes that are independent of food intake or body weight. In addition, this study documents that continuous and intermittent administration of PYY_{3-36} reinforce insulin action to a similar extent.

These data corroborate our previous findings, which unveil similar acute effects of PYY_{3-36} administration on insulin action [16], and support the emerging concept of neural circuits controlling fuel flux, independent of their impact on food intake and body weight. In addition, our data indicate that the effects of PYY_{3-36} on glucose metabolism do not wane during chronic treatment, which suggests that this peptide may be a novel asset in the battle against insulin resistance and the metabolic syndrome.

Although PYY_{3-36} enhanced insulin-induced glucose disposal, it did not significantly affect the ability of insulin to inhibit endogenous glucose production. Nonetheless, we can not exclude the possibility that the experimental group size may have limited the statistical power necessary to detect a subtle influence of PYY_{3-36} on hepatic glucose metabolism. Alternatively, PYY_{3-36} exerts differential, tissue specific, effects on insulin action.

The mechanism by which PYY_{3-36} affects insulin-mediated glucose metabolism remains to be elucidated. Perhaps, PYY_{3-36} modulates insulin action via the hypothalamic Y2 receptor, in analogy with the mechanism guiding its effects on appetite. Y2-receptor mediated inhibition of NPY and stimulation of POMC neuronal activity by PYY_{3-36} potentially reinforces insulin action on glucose metabolism indeed [10;11;13].

Circulating PYY_{3-36} levels in fasting conditions remained below the level of detection (< 1 pg/μl) during continuous treatment, and rose to 3.7 ± 0.8 pg/μl approximately one hour after i.p injection. During hyperinsulinemia (3-3.5 hours after injection), PYY_{3-36} levels were undetectable by our assay in all animals. Thus, in spite of the fact that continuous PYY_{3-36} treatment did not produce measurable plasma concentrations and intermittent administration induced a merely transitory increase of circulating PYY_{3-36}, both treatments significantly facilitated insulin mediated glucose disposal in high fat fed animals. Relatively few papers report plasma PYY_{3-36} concentrations in rodents. Postprandial levels may be in the range of 112 pmol/L (~ 0.4 pg/μl) and 0.18 pg/μl in freely feeding normal weight rats and mice respectively [14;22], whereas fasting levels are considerably lower, as PYY_{3-36} is primarily released in response to food intake [14;23]. Plasma PYY_{3-36} concentrations in high fat fed mice are unknown, but may be significantly reduced, as obese humans have clearly diminished circulating PYY_{3-36} levels [24]. Taken together, our data suggest, that even a relatively low dose of PYY_{3-36} (in view of the low circulating PYY_{3-36} levels during treatment)

can reinforce insulin action. Further dose-response experiments are warranted to evaluate the potential efficacy of PYY_{3-36} in the treatment of the metabolic syndrome.

Food intake and body weight were not affected by PYY_{3-36} administration in our study. These findings agree with data from Challis *et al.*, indicating that 7 days of PYY_{3-36} administration did not affect food intake and body weight in $POMC^{-/-}$ and wild type mice [25]. In contrast, Batterham *et al.* reported that PYY_{3-36} acutely inhibits food intake [14], an observation that could not be reproduced by Tschöp and coworkers [26;27]. To take this issue further, we compared the acute effects of a single intraperitoneal (2.5 µg) injection of PYY_{3-36} (n = 8) or vehicle (n = 8) at 09.00 am on food intake in our animals, and found that cumulative food intake in 4 hours after injection was significantly inhibited by 21% in overnight fasted mice (P=0.028), whereas subsequent feeding over 24 hours was not affected by PYY_{3-36}. These data suggest that this dose of PYY_{3-36} has a short-term inhibitory impact on food intake in overnight fasted C57BL6 mice, whereas consumption over 24 hours is not affected, probably as a result of a rebound compensatory increase of appetite [14;15].

In conclusion, the present study shows that chronic PYY_{3-36} administration reinforces insulin action on glucose disposal in mice maintained on a high fat diet, whereas it also tends to enhance the ability of insulin to suppress endogenous glucose production. These observations suggest that PYY_{3-36} or potential analogues may be a useful treatment for insulin resistance and the metabolic syndrome.

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Chapter 7

Ghrelin differentially affects hepatic and peripheral insulin sensitivity in mice

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ABSTRACT

Aims/hypothesis. **The present study was conducted to evaluate the effects of ghrelin on insulin's capacity to suppress endogenous glucose production (EGP) and promote glucose disposal (GD) in mice. To establish if the GHS-receptor can mediate the putative impact of ghrelin on insulin action, we also determined the metabolic effects of GHRP-6, a specific GHS-R agonist. In addition, we explored the biological significance of des-ghrelin in this experimental context.**

Methods. **Vehicle, ghrelin, GHRP-6, des-ghrelin or the combination of des-ghrelin and ghrelin were infused i.v. for 3 hours. Simultaneously, EGP and GD were measured by ^{14}C-glucose dilution during a hyperinsulinaemic euglycaemic clamp. Tissue specific glucose uptake in muscle and adipose tissue were measured using ^{3}H-2-deoxy-glucose.**

Results. **During hyperinsulinaemia, GD was 31% higher in ghrelin-treated mice compared with vehicle (77±16 *vs* 59±8 µmol/kg/hour respectively, P<0.05), which was in accordance with enhanced 2-DG uptake in muscle in ghrelin-treated animals. In contrast, EGP was less effectively suppressed by insulin during ghrelin infusion (by 46±22 *vs* 71±11 % in controls, P<0.05). GHRP-6 did not affect insulin action. Des-ghrelin hampered insulin's capacity to inhibit EGP, whereas it did not affect GD. The restraining effects of des-ghrelin and ghrelin on hepatic insulin action were abolished by simultaneous administration of both peptides.**

Conclusions/interpretation. **Ghrelin hampers insulin's capacity to suppress EGP, whereas it reinforces insulin action on GD, independently of food intake and body weight. These metabolic effects are unlikely mediated by the GHS-receptor. Furthermore, simultaneous administration of des-ghrelin abolishes the inhibitory effect of ghrelin on hepatic insulin action.**

INTRODUCTION

Ghrelin is a small 28 amino acid peptide, that is primarily produced by the stomach and binds to the growth hormone secretagogue (GHS) receptor (GHS-R) [1;2]. It circulates in two major forms: n-octanoyl-ghrelin (ghrelin), which contains an n-octanoyl modification at serine-3, and des-octanoyl or unacylated ghrelin (des-ghrelin) [3]. Although des-ghrelin does not bind to the GHS-R [1;4], it may be biologically active [5].

Ghrelin is a component of the gut-brain-axis involved in the control of energy balance. It stimulates food intake in rodents and man [5-8], where peak levels in plasma occur just before each meal and fall rapidly after re-feeding, suggesting that it serves to initiate food consumption [9]. To enhance appetite, ghrelin acts in the hypothalamus where it promotes neuropeptide Y (NPY) and orexin gene expression and inhibits pro-opiomelanocortin (POMC)/α-melanocyte stimulating hormone (αMSH) expression via activation of the GHS-R [10-16]. We recently showed that intracerebroventricular infusion of NPY hampers insulin's capacity to suppress endogenous glucose production (EGP) [17], whereas central injections of melanotan II, a synthetic analog of αMSH, reinforces insulin action on glucose disposal (GD) [18]. In concert, these findings led us to hypothesize that ghrelin might adversely affect insulin sensitivity through activation of the GHS-R. To address this hypothesis, we intravenously administered vehicle, ghrelin, or growth hormone releasing peptide 6 (GHRP-6, a specific agonist of the GHS-R), and measured EGP and GD by ^{14}C-glucose dilution during a hyperinsulinemic clamp in mice.

Until recently, acylation of the serine-3 residue of the ghrelin molecule was believed to be required for bioactivity. However, recent data suggest that des-ghrelin may counteract ghrelin in the control of energy balance [19], and in vitro experiments revealed opposing effects of ghrelin and des-ghrelin on glucose production by primary hepatocytes [20]. To further explore the role of des-ghrelin in the regulation of fuel flux, we studied the effects of this peptide in the same experimental context.

MATERIAL AND METHODS

Animals

Male C57BL/6J mice were housed in a temperature-controlled room on a 12-hour light-dark cycle, with free access to water and chow diet. All animal experiments were performed in accordance with the regulations of Dutch law on animal welfare and the institutional ethics committee for animal procedures approved the protocol.

Hyperinsulinaemic euglycaemic clamp study

Mice were fasted for 4 hours with food withdrawn at 05.00 a.m. Hyperinsulinaemic euglycaemic clamp studies were started at 9.00 a.m. as described earlier [21;22]. During the experiments, mice were sedated with 6.25 mg/kg Acepromazine (Sanofi sante animale, Libourne Cedex, France) 6.25 mg/kg Midazolam (Roche, Mijdrecht, the Netherlands), and 0.3125 mg/kg Fentanly (Janssen-Cilag, Tilburg, the Netherlands).

Vehicle, ghrelin, or GHRP-6 (peptides from PhoenixEurope GmbH, Karlsruhe, Germany) were administered in a primed (0 µg, 0.6 µg, 0.26 µg) continuous (0 µg/h, 1 µg/h, 0.43 µg/h) i.v. infusion during the whole experiment (basal and hyperinsulinemic period). The doses of ghrelin, and GHRP-6 were based on previous reports [6;7;23]. First, basal rates of glucose production/uptake were measured by giving a primed (p) continuous (c) infusion of ^{14}C-glucose (p: 0.7 µCi, c: 1.2 µCi/h) (Amersham, Little Chalfont, U.K.) for 80 min. Subsequently, insulin was administered in a primed (4.1 mU), continuous (6.8 mU/h) i.v. infusion for 2 to 3 hours aimed at steady state circulating insulin levels of approximately 6 ng/ml. A variable infusion of 12.5% D-glucose was used to maintain euglycaemia (measured via tail bleeding, Freestyle, TheraSense, Disetronic Medical Systems BV, Vianen, The Netherlands). Blood samples (75 µl) were taken during the basal period (after 60 and 80 minutes) and during the clamp period (when glucose levels were stable and 20 and 40 minutes later) for determination of plasma glucose, NEFA, insulin and ^{14}C-glucose specific activities.

To assess tissue-specific insulin-mediated glucose uptake, 2-deoxy-D-[^{3}H]glucose (2-DG; Amersham Little Chalfont, U.K.) was administered as a bolus (1 µCi) 40 minutes before the end of the clamp experiment. At the end of the clamp, mice were killed, and skeletal muscle (hindlimb) and adipose tissue were isolated and frozen in liquid nitrogen for subsequent analysis.

Additional studies with des-ghrelin

In addition, a hyperinsulinaemic euglycaemic clamp study was performed while des-ghrelin or a combination of ghrelin en des-ghrelin were administered. Des-ghrelin, or the combination of ghrelin and des-ghrelin were administered in a primed (0.6 µg or 0.6 and 0.6 µg) continuous (1 µg/h or 1 and 1 µg/h) i.v. infusion during the whole experiment (basal and hyperinsulinemic). The doses of des-ghrelin and the combination of ghrelin and des-ghrelin were based on a previous report [23]. Hyperinsulinaemic euglycaemic clamps were performed as stated above and randomised with these other groups.

Analytical procedures

Plasma levels of glucose and NEFA were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands and Wako, Neuss, Germany). Plasma insulin concentration was measured by a mouse insulin ELISA (Mercodia, Uppsala, Sweden). Total

plasma ^{14}C-glucose was determined in 7.5 µl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Tissue analysis

For determination of tissue 2-DG uptake, the homogenate of muscle and adipose tissue was boiled, and the supernatant was subjected to an ion-change column tot separate 2-DG-6-phosphate from 2-DG as described previously [21;24].

Calculations

Turnover rate of glucose (µmol/min/kg) was calculated during the basal period and in steady-state clamp conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of ^{14}C-glucose (dpm/µmol). The ratio was corrected for body weight. During the hyperinsulinemic clamp EGP was calculated as the difference between the tracer-derived rate of glucose appearance and the glucose infusion rate.

Tissue-specific glucose uptake in muscle and adipose tissue was calculated from tissue 2-DG content, corrected for plasma specific activity and expressed as µmol/g tissue.

Statistical analysis

Differences between groups were determined by Kruskal-Wallis non-parametric test for k independent samples. When significant differences were found, Mann-Whitney non parametric test was used as follow-up test to determine differences between experimental groups and the control group. A p-value < 0.05 was considered statistically significant. All values shown represent mean ± SD.

RESULTS

Plasma parameters

Body weight, plasma glucose, NEFA and insulin concentrations in basal and hyperinsulinemic conditions are shown in Table 1. Body weight did not differ between the groups of mice. Basal glucose levels were significantly lower in the group that received GHRP-6 compared to the control group. Basal insulin and NEFA levels were not different between groups. Moreover, in steady state hyperinsulinemic conditions, plasma glucose, NEFA and insulin concentrations were not different between groups.

Glucose turnover

In basal conditions, EGP (and thereby whole body GD) was significantly lower in animals that received GHRP-6 compared to control animals (control: 44±9 µmol/kg/hour, ghrelin: 40±7

Table 1. Body weight and plasma parameters in mice that received ghrelin (n=9), des-ghrelin (n=8), ghrelin and des-ghrelin together (n=7), GHRP-6 (n=9) or vehicle (n=8).

	Body weight (g)	*Glucose (mmol/l)*		*Insulin (ng/ml)*		*NEFA (mmol/l)*	
		Basal	Hyperinsulinemic	Basal	Hyperinsulinemic	Basal	Hyperinsulinemic
Control	27.1 ± 1.8	5.8 ± 0.73	8.8 ± 1.2	0.93 ± 0.63	6.2 ± 1.5	1.0 ± 0.2	0.5 ± 0.2
Ghrelin	26.6 ± 2.2	5.9 ± 0.84	8.8 ± 0.74	0.91 ± 0.73	5.8 ± 2.1	1.0 ± 0.3	0.4 ± 0.3
GHRP-6	26.3 ± 1.5	4.8 ± 0.48**	8.5 ± 1.8	1.0 ± 0.50	6.8 ± 2.6	1.0 ± 0.2	0.3 ± 0.1
Des-ghrelin	26.9 ± 2.2	6.3 ± 1.0	7.8 ± 0.83	1.1 ± 0.38	5.7 ± 1.9	1.0 ± 0.3	0.4 ± 0.1
Des-ghrelin and ghrelin combined	27.6 ± 2.4	4.9 ± 0.90*	7.6 ± 1.1	0.87 ± 0.58	6.6 ± 1.8	1.0 ± 0.2	0.4 ± 0.1

*Values are expressed as means ± sd, * p<0.05 compared to control, ** p<0.01 compared to control*

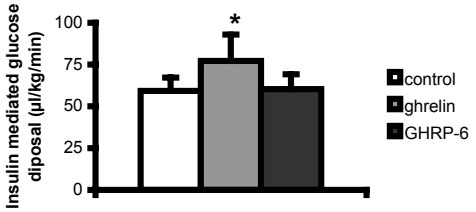

Fig 1. Insulin mediated glucose disposal (µmol/kg/min) in mice that received ghrelin (n=9), GHRP-6 (n=9) or vehicle (n=8). Data are means ± SD. *p<0.05 compared to control mice.

µmol/kg/hour, GHRP-6: 33±4 µmol/kg/hour; p<0.01). During the hyperinsulinemic period, the rate of glucose infusion necessary to maintain euglycemia was significantly higher in animals that received ghrelin than in control mice (control: 91±18 µl/hour, ghrelin: 136±27 µl/hour, GHRP-6: 117±31 µl/hour, p<0.01). Accordingly, GD rate was significantly higher in animals that received ghrelin compared to the control group (control: 59±8 µmol/kg/hour, ghrelin: 77±16 µmol/kg/hour, GHRP-6: 60±9 µmol/kg/hour; p<0.05, see figure 1). In contrast, hyperinsulinaemia suppressed EGP significantly less in animals that received ghrelin compared to controls (control: 71±11 %, ghrelin: 46±22 %, GHRP-6: 70±22 %; p<0.05, see figure 2).

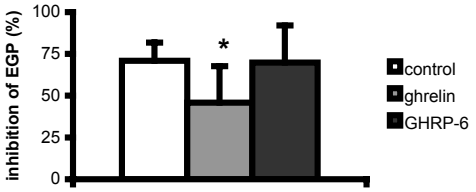

Fig 2. Inhibition of EGP (%) by insulin in mice that received ghrelin (n=9), GHRP-6 (n=9) or vehicle (n=8). Data are means ± SD. *p<0.05 compared to control mice.

Tissue-specific glucose uptake

Insulin-mediated 2-DG uptake in muscle tissue was higher in ghrelin-treated animals, compared to the control group (muscle: control: 8.6 ± 4.4; ghrelin: 26 ± 21; GHRP-6: 7.1 ± 3.4 µmol/g tissue, p<0.05). Insulin-mediated 2-DG uptake in adipose tissue tended to be higher in ghrelin-treated animals compared to the

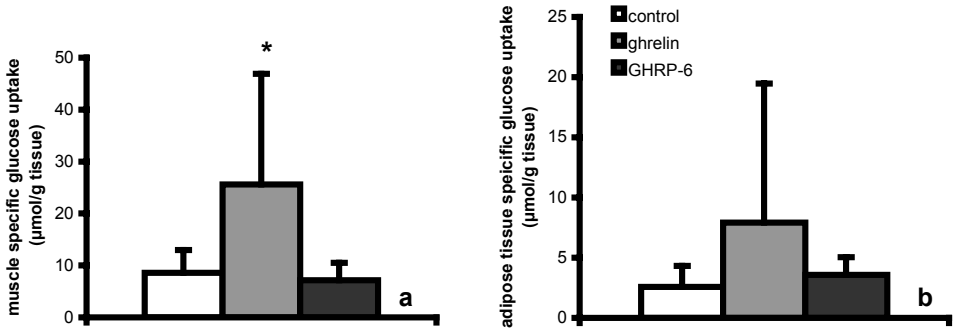

Fig 3 a) Muscle-specific glucose uptake (µmol/g) under hyperinsulinemic conditions in mice that received ghrelin (n=8), GHRP-6 (n=9) or vehicle (n=5). **b)** Adipose tissue-specific glucose uptake (µmol/g) under hyperinsulinemic conditions in mice that received ghrelin (n=8), GHRP-6 (n=9) or vehicle (n=5). Data are means ± SD. *p<0.05 compared to control mice.

control group, although this difference did not reach statistical significance (adipose tissue: control: 2.6 ± 1.7; ghrelin: 7.9 ± 11; GHRP-6: 3.6 ± 1.5 µmol/g tissue, p=0.09) (see figure 3a and b).

Additional studies with des-ghrelin.

Plasma parameters

Body weight, plasma glucose, NEFA and insulin concentrations in basal and hyperinsulinemic conditions are shown in Table 1. Body weight did not differ between the groups of mice. Basal glucose levels were significantly lower in the group that received the combination of ghrelin and des-ghrelin compared to the control group. Basal insulin and NEFA levels were not different between groups. Moreover, in steady state hyperinsulinemic conditions, plasma glucose, NEFA and insulin concentrations were not different between groups.

Table 2. Glucose turnover and tissue specific glucose uptake in mice that received des-ghrelin (n=8) ghrelin and des-ghrelin together (n=7), or vehicle.

	control	*des-ghrelin*	*ghrelin and des-ghrelin combined*
basal EGP (µmol/kg/min)	44 ± 9	48 ± 9	27 ± 7**
glucose infusion rate (µl/hour)	91 ± 18	70 ± 30	104 ± 19
insulin mediated glucose disposal (µmol/kg/min)	59 ± 8	54 ± 15	50 ± 17
inhibition of EGP (%)	71 ± 11	47 ± 13**	75 ± 21
muscle specific glucose uptake (µmol/g tissue)	8.6 ± 4.4	11 ± 6.1	10 ± 9.1
adipose tissue specific glucose uptake (µmol/g tissue)	2.6 ± 1.7	2.6 ± 1.1	2.1 ± 1.3

*Values are expressed as means ± SD, ** p<0.01 compared to control*

Glucose turnover and tissue specific glucose uptake

Basal EGP, Glucose infusion rate, insulin mediated GD, inhibition of EGP, and tissue specific glucose uptake are shown in Table 2. During basal conditions, EGP (and thereby whole body GD) was significantly lower in animals that received the combination of ghrelin and des-ghrelin compared to the control animals. During the hyperinsulinemic period, the rate of glucose infusion necessary to maintain euglycemia, insulin stimulated GD, and tissue specific glucose uptake did not differ between the groups. In contrast, hyperinsulinaemia suppressed EGP significantly less in animals that received des-ghrelin compared to controls.

DISCUSSION

This study shows that intravenous administration of ghrelin reinforces insulin action on GD, whereas it hampers insulin's capacity to inhibit EGP. In contrast, administration of GHRP-6 did not affect insulin action. Des-ghrelin hampered insulin's capacity to inhibit EGP, whereas it did not affect GD. The restraining effects of des-ghrelin and ghrelin on hepatic insulin action were abolished by simultaneous administration of both peptides.

The glucose infusion rate required to maintain euglycemia during insulin infusion was significantly higher in ghrelin treated animals, indicating that this peptide enhances whole body insulin sensitivity of glucose metabolism. In particular, ghrelin clearly stimulated insulin-mediated GD as determined by tracer dilution methodology, an observation that was in line with enhanced 2-DG uptake in muscle and adipose tissue during hyperinsulinaemia in ghrelin treated animals (although statistical significance was reached only for muscle). In contrast, ghrelin hampered inhibition of EGP by insulin. Our results do not fully confirm our a priori hypothesis. As previous papers have reported that ghrelin stimulates NPY release and inhibits POMC/α-MSH neuronal activity in the arcuate nucleus [25], we expected to find ghrelin compromising insulin sensitivity of both liver and muscle/adipose tissue (we recently showed that central NPY hampers insulin's action on EGP, whereas melanocortin pathways promote insulin mediated GD [17;18]. However, the mechanistic routes whereby ghrelin impacts insulin action remain to be established. Intravenously administered peptide may act through central pathways, but also through activation of receptors on peripheral tissues (the GHS-R is present in various tissues like pancreas and liver [15;26]. Indeed, ghrelin blocks insulin's inhibitory effect on gene expression of key gluconeogenic enzymes in a hepatoma cell-line [26]. These data corroborate our own *in vivo* observation of ghrelin hampering insulin action on EGP. In contrast, i.v. administration of ghrelin appears to enhance insulin mediated glucose uptake, where we *a priori* hypothesised that it would block this action (through down-regulation of hypothalamic α-MSH signaling [18]. It is conceivable that ghrelin acts on myocytes and/or adipocytes directly to stimulate GD. We are not aware of any *in vitro* or *in vivo* data documenting ghrelin's effects on insulin action in muscle or adipose tissue. Thus, further studies are required to establish if ghrelin affects insulin action via central or peripheral mechanisms.

Administration of GHRP-6 did not affect insulin action. This observation agrees with a report indicating that, ghrelin stimulates glucose production by primary hepatocytes, but hexarelin does not [20], ghrelin promotes adipogenesis in bone marrow, whereas L163,255, a potent GHS-R agonist does not [27] and ghrelin inhibits preadipocyte cell proliferation via a

novel receptor subtype [28]. In concert, these data strongly suggest that ghrelin impacts insulin action and metabolism via an as yet unidentified receptor.

Des-ghrelin blocked insulin action on EGP as much as ghrelin did. In contrast, it did not affect insulin mediated GD. These data support the emerging view that n-octanoyl modification of the serine (3) residue of ghrelin is not absolutely required for bioactivity [19;20;23]. However, they apparently contradict a report suggesting that ghrelin and des-ghrelin have opposing (stimulatory vs. inhibitory, respectively) effects on glucose production by hepatocytes [20]. The contradiction could be explained by the fact that in vitro and in vivo administration of des-ghrelin may have differential effects, as des-ghrelin, like ghrelin, can act in the brain as well as in peripheral tissues [19;27]. Co-administration of des-ghrelin abolished the impact of ghrelin on hepatic insulin sensitivity, which accords with an *in vitro* study showing similar results in isolated hepatocytes [20]. Our findings corroborate mounting evidence indicating that des-ghrelin has potentially important biological effects. The receptor mediating des-ghrelin bioactivity remains to be identified.

The physiological relevance of the observed effects of ghrelin to decrease hepatic insulin sensitivity and increase peripheral insulin sensitivity remains to be established. Ghrelin secretion by (primarily) the stomach is significantly enhanced during fasting to stimulate appetite and initiate meal consumption [9]. In a previous study, we showed that fasting enhances insulin-mediated glucose uptake in mice [29]. The data we present here allow us to hypothesize that the rise of plasma ghrelin levels during fasting is involved in the physiology of this phenomenon. However, des-ghrelin concentrations clearly dominate the plasma profile of ghrelin-like peptides, at least in the human [30;31]. In view of the current evidence suggesting that des-ghrelin has metabolic effects of its own and indeed appears to interact with ghrelin in the control of metabolism, further studies are required to establish the role of ghrelin-peptides in the regulation of energy balance and fuel flux. Moreover, the receptor(s) mediating the metabolic signals conveyed by (des-)ghrelin need to be identified.

In conclusion, ghrelin differentially affects tissue-specific insulin action, hampering its ability to inhibit EGP while reinforcing its impact on GD. These effects occur acutely and they are not secondary to ghrelin's well-known influence on feeding and body weight. The GHS-R is not likely to mediate ghrelin's metabolic effects. Des-ghrelin also appears to modulate fuel flux and may counteract ghrelin in the control of glucose metabolism.

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Chapter 8

Summary and conclusions

Maintenance of plasma glucose concentration is highly important for normal body physiology, particularly for the central nervous system, which uses glucose as the obligatory fuel and has no endogenous glucose stores. The pancreatic hormone insulin is important in tightly regulating glucose homeostasis. Insulin inhibits endogenous glucose production and stimulates glucose uptake by peripheral tissues, like skeletal muscle and adipose tissue, and thereby lowers glucose levels. In conditions of insulin resistance, these tissues are less sensitive for insulin action, which is reflected in decreased inhibition of endogenous glucose production and decreased tissue glucose uptake at the same concentration of insulin. Initially, pancreatic beta cells compensate for insulin resistance by increasing insulin secretion. However, when this compensatory mechanism fails, hyperglycaemia and type II diabetes will ensue. Insulin resistance is a dominant feature in subjects with obesity and type II diabetes. Without treatment hyperglycaemia progresses in time, making these patients prone for developing secondary complications.

Up till now, only scarce data are available upon physiological regulation of insulin sensitivity by feeding status, in interaction with gastrointestinal hormones and the brain. Feeding status seems to impact insulin sensitivity. Recent studies showed intensive interactions of gut hormones and brain centres in regulating food intake. The hypothalamic arcuate nucleus plays a central role in this interaction. Since fine-tuning of glucose homeostasis is essential to survive times of scarcity, the present thesis is focussed on physiological modulation of insulin sensitivity, with special emphasis on the role of feeding status (fasting, high fat diet), gut hormones (PYY, ghrelin), brain and gut-brain interactions.

Feeding status and insulin sensitivity

During the postabsorptive state and prolonged fasting it is extremely important to keep glucose concentrations high enough for functioning of the brain. During fasting, adipose tissue lipolysis increases, resulting in increased supply of FFA to the liver and muscle. In the liver and muscle, FFA can either be used for β-oxidation or TG storage. By β-oxidation, the liver transforms FFA in ketone bodies, which can be used as energy source by the brain. If tissue FFA uptake exceeds β-oxidation, excessive TG storage will be the consequence. In obesity, increased FFA concentrations and excessive tissue TG storage are associated with tissue insulin resistance. Fatty acid derivatives are ligands for nuclear transciption factors like PPARs and SREBPs, which alter the expression of enzymes/proteins involved in glucose and lipid metabolism and thus interact with insulin effects. The impact of fasting on tissue insulin sensitivity is unknown. Therefore, we studied the effects of 16 hours of fasting (prolonged fasting) versus 4 hr of fasting (postprandial state) on hepatic and muscle insulin sensitivity in wild-type mice in vivo in relation to tissue TG accumulation and changes in mRNA expression of transcription factors and related proteins involved in glucose and lipid

metabolism **(chapter 2).** Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic euglycaemic clamp in combination with D-[^{14}C]glucose infusion. Tissue specific insulin sensitivity was measured by use of 2-deoxy-D[^{3}H]glucose. Sixteen hr of fasting did not impact hepatic insulin sensitivity in terms of glucose production in the presence of increased hepatic triglyceride content (71±19 versus 12±7 µg/mg protein, $p<0.01$). In muscle, however, fasting resulted in increased insulin sensitivity with increased muscle glucose uptake (4.0±2.6 versus 1.3±0.3 % glucose uptake/ g tissue, $p<0.01$) without changes in muscle triglyceride content (25±7 versus 29±13 µg/mg protein, ns). In the liver, fasting resulted in increased mRNA expression of genes promoting gluconeogenesis (PGC1 and PEPCK) and triglyceride synthesis (PPARγ, DGAT1 and DGAT2), but in decreased mRNA expression of genes involved in glycogenolysis (GP) and fatty acid synthesis (SREBP1c, FAS and ACC1). In muscle, increased mRNA expression of genes promoting glucose uptake (PGC1 and GLUT-4), as well as lipogenesis (PPARγ, FAS, ACC1, DGAT-1 and -2) and β-oxidation (PPARα) was found. We conclude from this study that 16 hours of fasting does not induce hepatic insulin resistance, although it causes liver steatosis, whereas muscle insulin sensitivity increases without changes in muscle triglyceride content. Therefore, fasting induces differential changes in tissue-specific insulin sensitivity and liver and muscle TG content are unlikely to be involved in these changes.

With regard to the physiological relevance of the increase in muscle insulin sensitivity during fasting, a state dominated by low insulin levels, it can be speculated that this mechanism might serve as an extremely efficient manner to prepare peripheral tissues metabolically to shift to glucose uptake, when the next meal arrives and insulin levels increase, thereby preventing glucose peaks after meals.

High fat feeding, insulin sensitivity and the brain

At present, the western type of diet contains too much fat. This high fat diet is a risk factor for overweight, insulin resistance, and, thereby, for type II diabetes. Studies in rats and dogs on a high fat diet show the induction of hepatic insulin resistance as an early event, followed by muscle insulin resistance (1;2). The arcuate nucleus integrates a multitude of behavioural and metabolic adaptations to food intake and starvation, necessary to maintain fuel homeostasis despite profound environmental variations in nutrient availability. Two types of neurons in the arcuate nucleus of the hypothalamus are of major importance for the control of these processes: the orexigenic neurons co-expressing AgRP and NPY, and the anorexigenic neurons expressing POMC/ α-MSH. α-MSH binds to and stimulates melanocortin (MC) receptors. Recent data from other groups and our own group showed

involvement of the arcuate NPY/POMC circuitry in the modulation of insulin sensitivity, in addition to its impact on food intake.

The impact of a high fat diet on tissue specific insulin sensitivity in mice and triglyceride accumulation in relation to changes in mRNA expression levels of NPY, POMC, AgRP and CART in the hypothalamus is unknown. Therefore, we studied the effects of a 2 weeks high fat diet in wildtype mice on these parameters **(chapter 3)**. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic-euglycaemic clamp in combination with D-[^{14}C]glucose infusion. Tissue specific insulin sensitivity was measured by use of 2-deoxy-D[^{3}H]glucose. Two weeks of high fat diet did show hepatic insulin resistance with regard to inhibition of hepatic glucose production (35±20% versus 61±23%, $p<0.05$) and in the presence of increased hepatic TG accumulation (32 ±10 versus 12±6 µg/mg protein, $p<0.05$). Under hyperinsulinemic conditions, whole body glucose uptake was not different between the HF fed group and the control group (66±10 µmol/kg.min versus 59±8 µmol/kg.min, ns), also reflected by unaltered muscle-specific glucose uptake in the HF fed group compared to the control group (1.3±0.6 versus 1.3±0.3% per g tissue). Muscle TG content was not significantly different either (27±9 versus 23±7 µg/mg protein, ns). We did not observe changes in hypothalamic mRNA expression levels of NPY, AgRP, POMC and CART after 2 weeks of high fat diet compared to control mice.

From this study, we conclude that 2 weeks of high fat feeding in mice does not affect mRNA expression levels of NPY, AgRP, POMC or CART in the whole hypothalamus, despite induction of hepatic, but not peripheral, insulin resistance. Since in our study mRNA levels were measured in whole hypothalamus without regional assessment within the different nuclei, we cannot exclude involvement of certain specific hypothalamic area's in the regulation of hepatic insulin resistance during a high fat diet. In addition, we did not measure the expression levels of the relevant peptide levels. Additional studies have to be performed to evaluate the potential role of the respective nuclei within the hypothalamus in mediating peripheral insulin sensitivity during high fat feeding.

In addition to the impact of NPY on food intake, icv administration of NPY acutely hampers the capacity of insulin to inhibit hepatic glucose production. The POMC pathway also seems to be involved in regulating insulin sensitivity. Chronic (7 days) icv infusion of α-MSH enhanced peripheral and hepatic insulin sensitivity in rats (3) and POMC gene overexpression ameliorates insulin resistance in leptin-deficient mice (4). In the latter studies, the effects on insulin sensitivity occur in the presence of a concomitant reduction in food intake and fat mass, which precludes distinction of putative direct effects of POMC/α-MSH on

insulin sensitivity from indirect effects via feeding and body composition. To document the direct effects of activation of MC3/4 receptors on insulin sensitivity, we injected melanotan II (MTII), an agonist of these MC3/4 receptors icv, and quantified hepatic and peripheral insulin sensitivity of glucose metabolism in mice without access to food **(chapter 4)**. We performed this study in 24 hour fasted mice, which received 3 times an icv injection with MTII during this period of time. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic euglycaemic clamp in combination with D-[^{3}H]glucose infusion. A real time polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels of GLUT-4 in skeletal muscle. Hepatic insulin sensitivity was unaltered as hyperinsulinaemia suppressed hepatic glucose production to a similar extent in MTII- vs. vehicle-treated animals (45 ± 27% vs. 50 ± 20%, ns). However, glucose disposal rate was significantly higher in MTII treated animals (151 ± 20 vs. 108 ± 20 µmol/min/kg, resp., $p<0.01$), in the presence of increased GLUT-4 mRNA expression in skeletal muscle in the MTII treated group compared to vehicle-treated mice (307 ± 94 vs. 100 ± 56 %, $p<0.01$).

In conclusion, this study shows that activation of central melanocortin-3/4 receptors by MTII enhances insulin sensitivity of whole body glucose disposal, independently of food intake and fat mass, whereas it does not affect insulin's ability to suppress EGP. These observations are in line with the emerging notion, that neural circuits, apart from their effects on feeding, modulate insulin sensitivity to adapt metabolic conditions in the face of environmental fluctuations in nutrient availability.

Gastrointestinal hormones and insulin sensitivity

Gastrointestinal hormones vary according to feeding status and are known to affect food intake. For instance, PYY_{3-36} concentration is low during fasting and increased in the fed state, whereas ghrelin concentration is high after fasting, and peaks just before a meal. Accordingly, PYY_{3-36} inhibits food intake whereas ghrelin stimulates food intake. Gastrointestinal hormones are known to influence appetite by regulating neuropeptides in the hypothalamus. As these neuropeptides can affect insulin sensitivity, gastrointestinal hormones might be involved in regulating insulin sensitivity as well.

PYY_{3-36}

In view of the fact that PYY_{3-36} inhibits NPY- and activates POMC neuronal activity, we wondered whether PYY_{3-36} can improve insulin sensitivity independently of its effects on food intake and body weight. Therefore, we infused PYY_{3-36} intravenously and quantified glucose and lipid metabolism during a hyperinsulinemic euglycemic clamp in mice that were fed a high fat diet for 2 weeks **(chapter 5)**. To measure insulin sensitivity of hepatic and peripheral glucose metabolism, a hyperinsulinaemic euglycaemic clamp with radioactive labelled

glucose and 2-deoxy-glucose was performed. To measure insulin sensitivity of FFA metabolism, a hyperinsulinaemic euglycaemic clamp, in combination with ^{14}C-palmitate was performed. PYY_{3-36} enhanced insulin-induced glucose disposal (103.8 ± 10.9 vs. 76.1 ± 11.4 µmol/min/kg, respectively, p=0.001). Accordingly, 2-DG uptake in muscle and adipose tissue in hyperinsulinaemic conditions was higher during PYY_{3-36} infusion, although the difference with control reached statistical significance only for muscle (2.1 ± 0.5 vs. 1.5 ± 0.5 %/ g tissue, $p<0.05$). In contrast, PYY_{3-36} did not significantly impact insulin's capacity to inhibit endogenous glucose production (62 ± 29 vs. 42 ± 18%, ns). Insulin action on FFA metabolism was not affected by PYY_{3-36} either, as indicated by similar lipolysis rates during hyperinsulinaemia (22.4 ± 12.3 vs. 21.3 ± 10.9 µmol/min/kg, ns) in PYY_{3-36} and saline infused animals. In conclusion, this study shows that PYY_{3-36} reinforces insulin action in mice maintained on a high fat diet, independent of its effects on food intake and body weight. In this context, PYY_{3-36} appears to predominantly impact insulin mediated glucose disposal, whereas it leaves insulin action on glucose production largely unaffected. These data suggest that PYY_{3-36} or synthetic analogues of this peptide may be valuable assets to our armamentarium of drugs designed to battle insulin resistance and type II diabetes mellitus.

The value of PYY_{3-36} as a new drug depends on the duration of its effects. Therefore, we investigated the long-term effects of PYY_{3-36} (administration of 7 days either continuously via subcutaneous mini-pumps or intermittent via subcutaneous injections once a day) on insulin sensitivity **(chapter 6)**. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic euglycaemic clamp in combination with D-[^{14}C]glucose infusion. Tissue specific insulin sensitivity was measured by use of 2-deoxy-D[^{3}H]glucose. Long term PYY_{3-36} administration did not seem to have any adverse or counterregulating effects, as long term administration showed the same effect as acute administration: increased peripheral insulin sensitivity. Notably, there was no difference between continuous and intermittent administration.

Ghrelin

As ghrelin acts in the hypothalamus where it promotes NPY and orexin gene expression and inhibits POMC/αMSH expression via activation of the GHS-receptor, we wondered whether ghrelin might affect insulin sensitivity via the GHS-receptor. To address this hypothesis, we intravenously administered vehicle, ghrelin, or growth hormone releasing peptide 6 (GHRP-6, a specific agonist of the GHS-R), and measured insulin sensitivity in wildtype mice **(chapter 7)**. Until recently, acylation of the serine-3 residue of the ghrelin molecule was believed to be required for bioactivity. However, recent data suggest that des-ghrelin may counteract ghrelin in the control of energy balance (5), and in vitro experiments revealed opposing effects of ghrelin and des-ghrelin on glucose production by primary hepatocytes (6). To further explore

the role of des-ghrelin in the regulation of fuel flux, we studied the effects of this peptide in the same experimental context. Whole-body and hepatic insulin sensitivity were measured by hyperinsulinaemic euglycaemic clamp in combination with D-[^{14}C]glucose infusion. Tissue specific insulin sensitivity was measured by use of 2-deoxy-D[^{3}H]glucose. Hyperinsulinaemia suppressed EGP significantly less in animals that received ghrelin compared to controls (control: 71±11 %, ghrelin: 46±22 %, GHRP-6: 70±22 %; $p<0.05$). In contrast, glucose disposal rate was significantly higher in animals that received ghrelin compared to the control group (control: 59±8 µmol/kg/hour, ghrelin: 77±16 µmol/kg/hour, GHRP-6: 60±9 µmol/kg/hour; $p<0.05$), in accordance with insulin-mediated 2-DG uptake in muscle and adipose tissue, which was higher in ghrelin-treated animals, compared to the control group (muscle: control: 8.6 ± 4.4; ghrelin: 26 ± 21; GHRP-6: 7.1 ± 3.4 µmol/g tissue, $p<0.05$) although this difference did not reach statistical significance in adipose tissue (adipose tissue: control: 2.6 ± 1.7; ghrelin: 7.9 ± 11; GHRP-6: 3.6 ± 1.5 µmol/g tissue, $p=0.09$). With regard to des-ghrelin, during the hyperinsulinaemic period, insulin stimulated glucose disposal and tissue specific glucose uptake did not differ from the control group. In contrast, hyperinsulinaemia suppressed hepatic glucose production significantly less in animals that received des-ghrelin compared to controls (47±13 versus 71±11 %, $p<0.01$). In conclusion, ghrelin differentially affects tissue-specific insulin action, hampering its ability to inhibit endogenous glucose production while reinforcing its impact on glucose disposal. These effects occur acutely and they are not secondary to ghrelin's well-known influence on feeding and body weight. The GHS-R is not likely to mediate ghrelin's metabolic effects. Des-ghrelin also appears to modulate fuel flux and may counteract ghrelin in the control of glucose metabolism.

Conclusions

The hypothalamic arcuate nucleus has a central role in the regulation of appetite and food intake. During fasting, expression of neuropeptides involved in the regulation of food intake in the arcuate nucleus change: expression of the orexigenic neuropeptide NPY increases, whereas expression of the anorexigenic neuropeptide POMC decreases, resulting in stimulation of food intake. Under fed conditions, expression of POMC increases, whereas expression of NPY decreases, resulting in a inhibition of food intake.

Recent studies showed that the brain regulates appetite and food intake in crosstalk with the gut. Secretion of gastrointestinal hormones varies according to feeding status. During fasting, secretion of ghrelin, an activator of NPY neurons and inhibitor of POMC neurons, increases, whereas secretion of PYY_{3-36}, an agonist of the presynaptic NPY Y2 receptor and thereby inhibitor of NPY neurons and activator of POMC neurons, decreases, resulting in increased food intake. During feeding, opposite changes occur.

Until recently, it was thought that regulation of glucose metabolism was the result of crosstalk between the liver, muscle and adipose tissue with the pancreas and adrenals, producing glucoregulatory hormones, with a central role for insulin. Recent studies, however, changed this “peripheral” view to a more “central” concept with a prominent role for the brain regulating glucose homeostasis. Recent studies of Rossetti et al. showed that chronic blockade of hypothalamic insulin receptor signalling caused hepatic insulin resistance of glucose production, whereas acute depletion of insulin receptors in the liver failed to alter the effect of physiological hyperinsulinemia on the rate of glucose production (7-9). These studies show that brain insulin action is required for intact glucose homeostasis.

The studies performed in this thesis show that the brain not only regulates food intake but also insulin sensitivity of glucose metabolism in liver and peripheral tissues, like skeletal muscle and adipose tissue (see figure 1), dependently and independently of feeding status and likely via related changes in gastrointestinal hormones. In this way, at every moment, tissue insulin sensitivity can be fine-tuned, dependently and independently of feeding status. We showed that fasting itself resulted in an increase in muscle insulin sensitivity. Since intravenous administration of ghrelin in mice was shown to increase muscle insulin sensitivity as well, it can be speculated that the effects of fasting on peripheral insulin sensitivity are mediated by increased secretion of ghrelin, probably (partly) in crosstalk with the arcuate nucleus NPY/POMC neuronal circuitry. Interestingly, all intervention studies performed by others and in this thesis showed tissue specific effects of the different interventions on insulin sensitivity. Van den Hoek et al. showed that central infusion of NPY decreased insulin action on hepatic glucose production, without affecting peripheral insulin sensitivity (10). Central injections of MTII (stimulating the POMC pathway) resulted in an increase in peripheral glucose disposal without affecting hepatic insulin sensitivity. Intervenous administration of ghrelin increased muscle insulin sensitivity but decreased hepatic insulin sensitivity. Intravenous infusion of PYY_{3-36} increased peripheral insulin sensitivity as well, but had no impact on hepatic insulin sensitivity. Since, for example, fasting and ghrelin increase peripheral insulin sensitivity whereas NPY, which is also activated by fasting and ghrelin, has no effect on peripheral insulin sensitivity but induces hepatic insulin resistance, the effects of fasting and ghrelin on peripheral insulin sensitivity cannot merely be explained by modulation of NPY neurons. In contrast, the induction of hepatic insulin resistance by ghrelin might be (partly) the result of activation of NPY neurons by ghrelin.

Although it has been shown by others that regulation of food intake by gastrointestinal hormones is mediated by the brain, this remains speculative for the regulation of body insulin sensitivity. Receptors for gastrointestinal hormones are found throughout the body, both centrally and peripherally. From the studies in this thesis no conclusions can be drawn about the site of action of gastrointestinal hormones in the central nervous system. Therefore, more

research, like peripheral and central administration of gastrointestinal hormones in combination with blockers of the NPY/POMC system and denervation studies, is needed to draw conclusions about the contribution of the brain and site of action in the brain with regard to the regulation of insulin sensitivity due to changes in gastrointestinal hormone levels.

As the brain uses glucose as the obligatory fuel, the regulation of feeding and insulin sensitivity is of major importance to the brain. It is therefore not surprising that the brain itself is involved in regulating these processes tightly to secure survival.

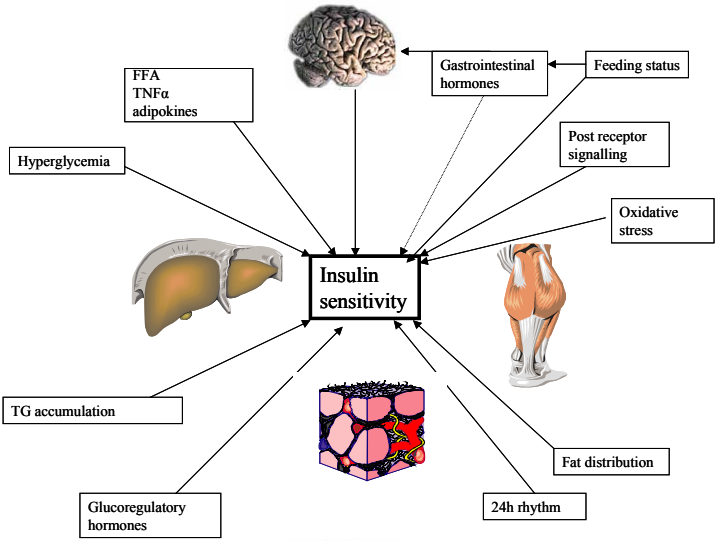

Figure 1. A role for gastrointestinal hormones and the brain in the regulation of insulin sensitivity

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Samenvatting en conclusies

Het handhaven van constante glucose spiegels in het bloed is erg belangrijk voor de normale lichaamsfysiologie en in het bijzonder voor het centrale zenuwstelsel dat glucose als noodzakelijke brandstof gebruikt en geen endogene glucose voorraad heeft. Het hormoon insuline, uitgescheiden door de alvleesklier, is belangrijk bij het nauwkeurig reguleren van de glucose homeostase. Insuline remt de endogene glucose productie en stimuleert de glucose opname door perifere weefsels, zoals spier- en vetweefsel, en verlaagt daardoor glucose spiegels. In het geval van insuline resistentie zijn deze weefsels minder gevoelig voor de werking van insuline, wat gereflecteerd wordt door een verminderde remming van de endogene glucose productie en een verminderde remming van de glucose opname in de periferie bij gelijke insuline concentraties. In het begin zullen de beta cellen van de alvleesklier compenseren voor de insuline resistentie door de insuline secretie te verhogen. Echter, wanneer dit compensatoire mechanisme faalt, zullen hyperglycemie en type II diabetes volgen. Insuline resistentie is een dominant kenmerk van mensen met obesitas en type II diabetes. Zonder behandeling zal de hyperglycemie verergeren in de tijd, wat deze patienten gevoelig maakt voor het ontwikkelen van secundaire complicaties.

Tot nu toe is er slechts een schaarse hoeveelheid data beschikbaar over de fysiologische regulatie van insuline gevoeligheid door voedingsstatus in interactie met gastrointestinale hormonen en de hersenen. De voedingsstatus lijkt effect te hebben op insuline gevoeligheid. Recente studies hebben intensieve interacties tussen darmhormonen en hersenkernen in de regulatie van voedselinname laten zien. De nucleus arcuatus in de hypothalamus lijkt hier een centrale rol bij te spelen. Aangezien nauwkeurige regulatie van de glucose homeostase essentieel is in tijden van voedselschaarste, hebben we ons in dit proefschrift geconcentreerd op de fysiologische modulatie van insuline gevoeligheid, met speciale aandacht voor de rol van voedingsstatus (vasten, hoog vet dieet), darmhormonen (PYY, ghrelin), de hersenen en darm-brein interacties.

Voedsel status en insuline gevoeligheid

Tijdens de nuchtere staat en langdurig vasten is het extreem belangrijk om glucose spiegels hoog genoeg te houden om de hersenen te laten functioneren. Tijdens vasten stijgt de lipolyse in vetweefsel, wat resulteert in een verhoogde toevoer van vrije vetzuren naar de lever en spierweefsel. In lever en spierweefsel kunnen de vetzuren of gebruikt worden voor β-oxidatie of worden opgeslagen als triglyceriden. In het geval van β-oxidatie transformeert de lever de vetzuren in ketonen, die als energiebron kunnen worden gebruikt door de hersenen. Als de vetzuuropname groter is dan de β-oxidatie, zal een buitensporige triglyceriden-opslag de consequentie zijn. Bij obesitas zijn verhoogde vetzuurconcentraties en overmatige triglyceridenopslag geassocieerd met insuline resistentie. Vetzuurderivaten zijn liganden voor transcriptiefactoren zoals PPARs en SREBPs, die bekend staan om hun

vermogen de expressie van enzymen/eiwitten betrokken bij het glucose en lipiden metabolisme te veranderen. Deze transcriptiefactoren hebben effecten op de insuline gevoeligheid. Het effect van vasten op weefsel-specifieke insuline gevoeligheid was onbekend. Daarom hebben we de effecten bestudeerd van 16 uur vasten (langdurig vasten) versus 4 uur vasten (postprandiaal) op insuline gevoeligheid van lever en spier in wildtype muizen in vivo, in relatie met weefsel-specifieke triglyceridenstapeling en met veranderingen in mRNA expressie van transcriptie factoren en gerelateerde eiwitten betrokken bij glucose en lipiden metabolisme **(hoofdstuk 2)**. Totale en lever-specifieke insuline gevoeligheid werden gemeten met behulp van een hyperinsulinemische euglycemische clamp, in combinatie met D-[^{14}C]glucose infusie. Voor het meten van weefsel-specifieke insuline gevoeligheid werd gebruik gemaakt van 2-deoxy-D-[^{3}H]glucose. Zestien uur vasten had geen effect op de hepatische insuline gevoeligheid met betrekking tot de glucose productie in de aanwezigheid van een verhoogde triglyceridenstapeling in de lever (71$\pm$19 versus 12$\pm$7 µg/mg eiwit, $p<0.01$). In spier echter, resulteerde vasten in een verhoogde insuline gevoeligheid met verhoogde glucose opname door de spier (4.0$\pm$2.6 versus 1.3$\pm$0.3 % glucose opname/g weefsel, $p<0.01$), zonder veranderingen van triglyceride inhoud in spierweefsel (25$\pm$7 versus 29$\pm$13 µg/mg eiwit, ns). In de lever resulteerde vasten in verhoogde mRNA expressies van genen die de gluconeogenese (PGC1 en PEPCK) en triglyceride synthese (PPARγ, DGAT1 and DGAT2) promoten, maar in verlaagde mRNA expressies van genen die betrokken zijn bij glycogenolyse (GP) en vetzuursynthese (SREBP1c, FAS en ACC1). In spier resulteerde vasten in verhoogde mRNA expressies van genen die glucose opname (PGC1 en GLUT-4), als ook lipogenese (PPARγ, FAS, ACC1, DGAT-1 and -2) en β-oxidatie (PPARα) promoten. We concluderen uit deze studie dat 16 uur vasten geen hepatische insuline resistentie induceert, ondanks het veroorzaken van lever steatose, terwijl spier-specifieke insuline gevoeligheid verbetert zonder veranderingen in triglyceridenstapeling in de spier. Vasten induceert dus differentiële veranderingen in weefsel-specifieke insuline gevoeligheid en het is onwaarschijnlijk dat triglyceriden stapeling in lever en spier betrokken zijn bij deze veranderingen. Met betrekking tot de fysiologische relevantie van de verbetering van de spier-specifieke insuline gevoeligheid tijdens vasten, kunnen we speculeren dat dit mechanisme zou kunnen dienen als een extreem efficiënte manier om de perifere weefsels metabool gezien voor te bereiden op glucose opname zodra de volgende maaltijd komt en insuline spiegels gaan stijgen, om op deze manier glucose pieken na een maaltijd te voorkomen.

Hoog vet dieet, insuline gevoeligheid en de hersenen

Het westerse type dieet in onze huidige tijd bevat te veel vet. Dit hoog vet dieet is een risicofactor voor het krijgen van overgewicht, insuline resistentie en dus voor type II diabetes. Studies in ratten en honden op een hoog vet dieet laten de inductie van hepatische insuline resistentie als een vroege consequentie zien, wat later gevolgd wordt door insuline resistentie in de spier (1;2). De nucleus arcuatus integreert een groot aantal gedrags- en metabole aanpassingen aan voedselinname en gebrek aan voeding, wat nodig is om de energie homeostase te handhaven ondanks variaties in de beschikbaarheid van voedsel. Twee types neuronen in de nucleus arcuatus in hypothalamus zijn hoogst belangrijk voor de controle van deze processen: de eetluststimulerende neuronen die zowel AgRP als NPY tot uiting brengen, en de eetlustremmende neuronen die POMC/α-MSH tot uiting brengen. α-MSH bind aan en stimuleert melanocortine (MC) receptoren. Recente data van andere groepen en ook onze groep, laat zien dat het NPY/POMC circuit in de nucleus arcuatus betrokken is bij de modulatie van insuline gevoeligheid, onafhankelijk van de effecten van NPY/POMC op voedselinname.

Het effect van een hoog vet dieet op weefsel-specifieke insuline gevoeligheid in muizen en triglyceridenstapeling, in relatie tot veranderingen in mRNA expressie van NPY, POMC, AgRP en CART in de hypothalamus is onbekend. Wij bestudeerden daarom de effecten hierop van 2 weken hoog vet dieet in wildtype muizen **(hoofdstuk 3)**. Totale en lever-specifieke insuline gevoeligheid werden gemeten met behulp van een hyperinsulinemische euglycemische clamp, in combinatie met D-[^{14}C]glucose infusie. Voor het meten van weefsel-specifieke insuline gevoeligheid werd gebruik gemaakt van 2-deoxy-D-[^{3}H]glucose. Twee weken hoog vet dieet liet hepatische insuline resistentie met betrekking tot de remming van de hepatische glucose productie zien (35$\pm$20% versus 61$\pm$23%, $p<0.05$), in de aanwezigheid van verhoogde triglyceridenstapeling in de lever (32 ±10 versus 12$\pm$6 µg/mg eiwit, $p<0.05$). Tijdens hyperinsulinemische omstandigheden was de totale glucose opname niet verschillend tussen de hoog vet gevoede groep en de controle groep (66$\pm$10 µmol/kg.min versus 59$\pm$8 µmol/kg.min, ns), wat ook gereflecteerd werd door een onverandere spier-specifieke glucose opname in de hoog vet gevoede groep vergeleken met de controle groep (1.3$\pm$0.6 versus 1.3$\pm$0.3% per g tissue). We zagen geen veranderingen in hypothalame mRNA expressies van NPY, AgRP, POMC en CART in muizen na 2 weken hoog vet dieet vergeleken met controle muizen.

Uit deze studie kunnen we concluderen dat 2 weken hoog vet dieet in muizen mRNA expressies van NPY, AgRP, POMC of CART in de hele hypothalamus niet beinvloedt, ondanks inductie van hepatische, maar niet perifere, insuline resistentie. Omdat in onze

studie mRNA expressies gemeten zijn in de gehele hypothalamus zonder regionale dissectie van de verschillende kernen, kunnen we de betrokkenheid van specifieke hypothalame kernen in de regulatie van hepatische insuline resistentie tijdens een hoog vet dieet niet totaal excluderen. Bovendien hebben we geen expressie niveaus van relevante eiwitten gemeten. Meer onderzoek is nodig om de mogelijke rol van de verschillende specifieke hypothalame kernen bij de regulatie van insuline gevoeligheid tijdens hoog vet diet te evalueren.

Naast effecten op voedselinname, belemmert icv toediening van NPY ook acuut de capaciteit van insuline om de hepatische glucose productie te remmen. POMC lijkt ook betrokken te zijn bij de regulatie van insuline gevoeligheid. Chronische (7 dagen) icv infusie van α-MSH verbetert perifere en hepatische insuline gevoeligheid in ratten (3) en POMC overexpressie verbetert insuline resistentie in leptine deficiente muizen(4). In deze laatste studies doen de effecten op insuline gevoeligheid zich voor in de aanwezigheid van een begeleidende reductie in voedselinname en vetmassa, wat het maken van een onderscheid tussen vermeende *directe* effecten van POMC/α-MSH op insuline gevoeligheid en *indirecte* effecten via voedselinname en lichaamscompositie belet. Om de *directe* effecten van activatie van MC3/4 receptoren op de insuline gevoeligheid te documenteren, injecteerden we melanotan II (MTII), een agonist van deze MC3/4 receptoren, icv en kwantificeerden we hepatische en perifere insuline gevoeligheid van het glucose metabolisme in muizen zonder toegang tot voedsel **(hoofdstuk 4)**. We hebben deze studie uitgevoerd in 24 uur gevaste muizen, die gedurende deze periode 3 keer een icv injectie kregen met MTII. Totale en lever-specifieke insuline gevoeligheid werden gemeten met behulp van een hyperinsulinemische euglycemische clamp, in combinatie met D-[^{3}H]glucose infusie. Een real-time PCR werd gebruikt om mRNA expressie van GLUT4 in spierweefsel te meten. Hepatische insuline gevoeligheid was onveranderd, aangezien hyperinsulinemie de hepatische glucose productie van MTII- en vehicle behandelde muizen met gelijke hoeveelheid remde (45 $\pm$ 27% *vs.* 50 $\pm$ 20%, ns). De glucose opname snelheid was echter significant hoger in MTII behandelde muizen (151 $\pm$ 20 *vs.* 108 $\pm$ 20 µmol/min/kg, resp., $p<0.01$), in aanwezigheid van een verhoogde GLUT4 mRNA expressie in spier in de MTII behandelde groep vergeleken met de controle groep (307 ± 94 *vs.* 100 ± 56 %, $p<0.01$).

Uit deze studie kunnen we concluderen dat activatie van centrale MC3/4 receptoren door MTII zorgt voor een verbetering van de insuline gevoeligheid met betrekking tot de totale glucose opname, onafhankelijk van voedselinname en lichaamsgewicht, terwijl er geen effect is op het vermogen van insuline om de endogene glucose productie te remmen. Deze observaties komen overeen met het concept dat steeds meer naar voren komt, dat neurale circuits, naast effecten op voedselinname, insuline gevoeligheid moduleren om metabole

condities aan te passen in het aangezicht van fluctuaties in beschikbaarheid van voedingmiddelen.

Gastrointestinale hormonen en insuline gevoeligheid

Gastrointestinale hormonen variëren afhankelijk van voedingsstatus en staan bekend om hun effect op voedselinname. Ter illustratie, PYY_{3-36} concentraties zijn laag tijdens vasten en verhoogd in de gevoede staat, terwijl ghrelin concentraties hoog zijn na vasten en net voor de maaltijd pieken. Dienovereenkomstig remt PYY_{3-36} voedselinname, terwijl ghrelin voedselinname stimuleert. Van gastrointestinale hormonen is bekend dat ze de eetlust kunnen beinvloeden door de regulatie van neuropeptides in de hypothalamus. Aangezien deze neuropeptides insuline gevoeligheid kunnen beïnvloeden, zouden gastrointestinale hormonen ook betrokken kunnen zijn bij de regulatie van insuline gevoeligheid.

PYY_{3-36}

Aangezien PYY_{3-36} de neuronale activiteit van NPY remt en die van POMC stimuleert, vroegen we ons af of PYY_{3-36} insuline gevoeligheid kan verbeteren, onafhankelijk van de effecten op voedselinname en lichaamsgewicht. Daarom hebben we PYY_{3-36} intraveneus geinfuseerd en het glucose en lipidenmetabolisme gekwanitficeerd tijdens een hyperinsulinemische euglycemische clamp in muizen die 2 weken een hoog vet dieet hadden gegeten **(hoofdstuk 5)**. Om insuline gevoeligheid met betrekking tot het glucose metabolisme van de lever en de periferie te meten werd een hyperinsulinemische euglycemische clamp, in combinatie met radioactief gelabeld glucose en 2-deoxy-glucose, uitgevoerd. Om de insuline gevoeligheid met betrekking tot het vetzuurmetabolisme te meten werd ook een hyperinsulinemische euglycemische clamp, in combinatie met ^{14}C palmitaat, uitgevoerd. PYY_{3-36} verbeterde de door insuline geinduceerde glucose opname (103.8 ± 10.9 *vs.* 76.1 ± 11.4 µmol/min/kg, respectievelijk, p=0.001). Dienovereenkomstig was glucose opname in spier en vetweefsel tijdens hyperinsulinemische condities hoger gedurende PYY_{3-36} infusie, hoewel het verschil met de controle groep alleen statistische significantie bereikte voor spierweefsel (2.1 ± 0.5 *vs.* 1.5 ± 0.5 %/ g tissue, $p<0.05$). Daartegenover had PYY_{3-36} geen effect op de capaciteit van insuline om de endogene glucose productie te remmen (62 ± 29 *vs.* 42 ± 18%, ns). PYY_{3-36} had ook geen effect op actie van insuline met betrekking tot het vetzuur metabolisme, wat aangeduid werd door gelijke lipolyse snelheden gedurende hyperinsulinemie (22.4 ± 12.3 *vs.* 21.3 ± 10.9 µmol/min/kg, ns) in dieren die met PYY_{3-36} of vehicle zijn geinfuseerd. Uit deze studie kunnen we concluderen dat PYY_{3-36} de actie van insuline versterkt in muizen die op hoog vet dieet gehouden zijn, onafhankelijk van zijn effecten op voedselinname en lichaamsgewicht. In deze context blijkt PYY_{3-36} voornamelijk effect te hebben op insuline gemedieerde glucose opname, terwijl het de actie van insuline

op de glucose productie grotendeels onbeinvloed laat. Deze data suggereren dat PYY_{3-36} of synthetische analogen van dit eiwit waardevolle middelen zijn in de strijd tegen insuline resistentie en type II diabetes mellitus.

De waarde van PYY_{3-36} als een nieuw geneesmiddel hangt af van de duur van zijn effecten. Daarom hebben we onderzoek gedaan naar de lange termijn effecten van PYY_{3-36} (toediening gedurende 7 dagen, of continu via subcutane minipompjes of intermitterend via subcutane injectie eenmaal per dag) op insuline gevoeligheid **(hoofdstuk 6)**. Totale en lever-specifieke insuline gevoeligheid werden gemeten met behulp van een hyperinsulinemische euglycemische clamp, in combinatie met D-[^{14}C]glucose infusie. Weefsel specifieke insuline gevoeligheid werd gemeten met behulp van 2-deoxy-D[^{3}H] glucose infusie. Lange termijn toediening van PYY_{3-36} leek geen nadelige of counterregulatoire effecten te hebben, aangezien lange termijn toediending dezelfde effecten liet zien als acute toediening, namelijk verhoogde perifere insuline gevoeligheid. Opmerkelijk genoeg was er geen verschil tussen continue en intermitterende toediening.

Ghrelin

Aangezien ghrelin zijn werking heeft in de hypothalamus, waar het NPY en orexine expressie stimuleert en POMC/α-MSH expressie remt via activatie van de growth hormone secretagogue (GHS) receptor, vroegen we ons af of ghrelin effect zou kunnen hebben op insuline gevoeligheid via deze GHS receptor. Om onze hypothese te staven, hebben we vehicle, ghrelin en growth hormone releasing peptide 6 (GHRP-6, een specifieke agonist van de GHS receptor) intraveneus toegediend in wildtype muizen en insuline gevoeligheid gemeten **(hoofstuk 7)**. Tot voor kort werd geloofd dat acylatie van serine-3 van het ghrelin molecuul noodzakelijk zou zijn voor bioactiviteit. Recente data suggereren echter dat des-ghrelin ghrelin kan tegenwerken in de controle van de energie balans (5), en in vitro experimenten laten tegengestelde effecten van ghrelin en des-ghrelin op glucose productie in hepatocyten zien (6). Om de rol van des-ghrelin in de regulatie van energie fluxen verder te onderzoeken, hebben we de effecten van dit peptide in dezelfde context onderzocht. Totale en lever-specifieke insuline gevoeligheid werden gemeten met behulp van een hyperinsulinemische euglycemische clamp, in combinatie met D-[^{14}C]glucose infusie. Weefsel specifieke insuline gevoeligheid werd gemeten met behulp van 2-deoxy-D[^{3}H] glucose infusie. Hyperinsulinemie onderdrukte de endogene glucose productie significant minder in muizen die ghrelin kregen dan in de controles (controle: 71±11 %, ghrelin: 46±22 %, GHRP-6: 70±22 %; $p<0.05$). In tegenstelling, de glucose opname snelheid was significant hoger in de dieren die ghrelin ontvingen ten opzichte van de controle groep (controle: 59±8 µmol/kg/hour, ghrelin: 77±16 µmol/kg/hour, GHRP-6: 60±9 µmol/kg/hour; $p<0.05$), wat in overeenstemming was met de insuline gemedieerde 2-DG opname in spier en vetweefsel,

welke hoger was in de met ghrelin behandelde dieren vergeleken met de controle groep (spier: control: 8.6 ± 4.4; ghrelin: 26 ± 21; GHRP-6: 7.1 ± 3.4 µmol/g weefsel, $p<0.05$), hoewel dit niet statistisch significant was in vetweefsel (vetweefsel: controle: 2.6 ± 1.7; ghrelin: 7.9 ± 11; GHRP-6: 3.6 ± 1.5 µmol/g weefsel, $p=0.09$). Met betrekking tot des-ghrelin was, gedurende de hyperinsulinemisch periode, de insuline gestimuleerde glucose opname en de weefsel specifieke glucose opname niet verschillend van de controle groep. Hyperinsulinemie onderdrukte, in tegenstelling, de hepatische glucose productie significant minder in dieren die des-ghrelin kregen dan in de controle dieren (47±13 versus 71±11 %, $p<0.01$). Uit deze studie kunnen we concluderen dat ghrelin differentiele effecten heeft op weefsel-specifieke insuline actie, namelijk het verminderen van het vermogen om de endogene glucose productie te remmen, terwijl het de impact op de glucose opname versterkt. Deze effecten komen acuut voor en zijn niet secundair aan ghrelin's bekende effecten op voedselinname en lichaamsgewicht. De GHS receptor is waarschijnlijk niet de mediair van de metabole effecten van ghrelin. Des-ghrelin lijkt ook energie fluxen te moduleren en kan ghrelin tegenwerken tijdens de controle van het glucose metabolisme.

Conclusies

De nucleus arcuatus in de hypothalamus speelt een centrale rol in de regulatie van eetlust en voedselinname. Tijdens vasten veranderen de expressies van neuropeptiden in de nucleus arcuatus die betrokken zijn bij de regulatie van voedselinname: expressie van het eetluststimulerende neuropeptide NPY stijgt, terwijl de expressie van het eetlustremmende neuropeptide POMC daalt, wat resulteert in de stimulatie van voedselinname. In gevoede toestand stijgt de expressie van POMC en daalt de expressie van NPY, wat resulteert in remming van voedselinname.

Recente studies laten zien dat de hersenen de eetlust en voedselinname regulerene in samenspraak met het maagdarmstelsel. De uitscheiding van gastrointestinale hormonen in het bloed varieert afhankelijk van voedingsstatus. Tijdens vasten stijgt de uitscheiding van ghrelin, een activator van NPY neuronen en een remmer van POMC neuronen, terwijl de uitscheiding van PYY_{3-36}, een agonist van de presynaptische NPY Y2 receptor en daardoor een remmer van NPY neuronen en activator van POMC neuronen, vermindert, wat resulteert in een verhoogde voedselinname. In gevoede toestand worden tegenovergestelde effecten gezien.

Tot voor kort werd gedacht dat de regulatie van het glucose metabolisme het resultaat was van de samenspraak tussen lever, spier en vetweefsel met de alvleesklier en bijnieren, die glucoregulatoire hormonen produceren, met een hoofdrol voor insuline. Recente studies echter veranderden deze 'perifere' blik naar een meer 'centraal' concept met een prominente rol voor de hersenen die de glucose homeostase reguleren. Recente studies van Rossetti et

al laten zien, dat chronische blokkade van insuline receptor signalen in de hypothalamus resulteert in hepatische insuline resistentie van de glucose productie, terwijl bij acute verwijdering van insuline receptoren in de lever het effect van fysiologische hyperinsulinemie op de snelheid van de glucose productie niet verandert (7-9). Deze studies laten zien dat insuline actie in de hersenen noodzakelijk is voor een intacte glucose homeostase.

De studies die uitgevoerd zijn binnen dit proefschrift laten zien dat de hersenen niet alleen voedselinname reguleren, maar ook insuline gevoeligheid van het glucose metabolisme in de lever en perifere weefsels, zoals spier en vetweefsel (zie figuur 1), afhankelijk en onafhankelijk van voedingsstatus en waarschijnlijk via gerelateerde veranderingen in gastrointestinale hormonen. Op deze manier kan op ieder moment de weefsel-specifieke insuline gevoeligheid nauwkeurig worden gereguleerd, afhankelijk en onafhankelijk van voedingsstatus.

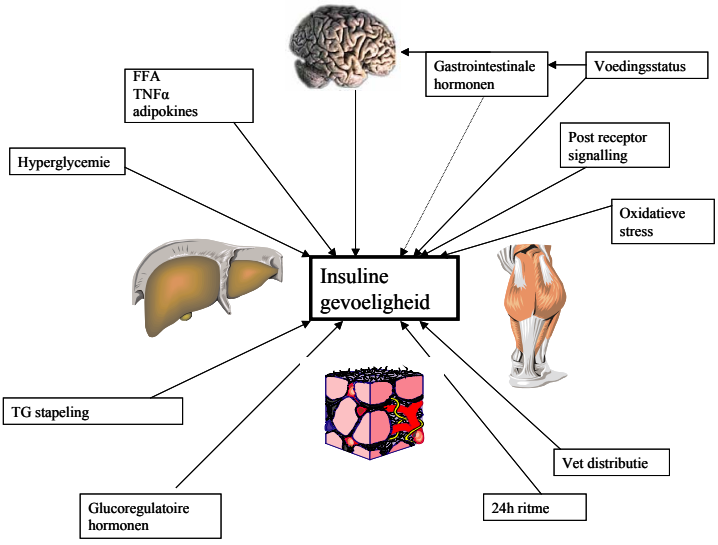
We hebben laten zien, dat vasten zelf resulteert in een verhoging van de spier-specifieke insuline gevoeligheid. Aangezien intraveneuze toediening van ghrelin in muizen ook een verhoogde spier-specifieke insuline gevoeligheid liet zien, kan worden gespeculeerd dat de effecten van vasten op de perifere insuline gevoeligheid gemedieerd worden door een verhoogde secretie van ghrelin, waarschijnlijk (gedeeltelijk) in samenspraak met de NPY/POMC neuronale circuits in de nucleus arcuatus. Interessant genoeg laten alle interventie studies, uitgevoerd door anderen en in dit proefschrift, weefsel-specifieke effecten van de verschillende interventies op insuline gevoeligheid zien. Van den Hoek et al lieten zien dat centrale infusie van NPY de insuline actie op hepatische glucose productie verminderde, zonder effecten op perifere insuline gevoeligheid (10). Centrale injecties van MTII (stimuleert het POMC pad), resulteerden in een verhoging van de perifere glucose opname zonder effect op hepatische insuline gevoeligheid. Intraveneuze toediening van ghrelin verhoogde de perifere insuline gevoeligheid, maar verlaagde de lever-specifieke insuline gevoeligheid. Intraveneuze infusie van PYY_{3-36} verhoogde de perifere insuline gevoeligheid maar had geen effect op hepatische insuline gevoeligheid.

Aangezien, bijvoorbeeld, vasten en ghrelin de perifere insuline gevoeligheid verhogen, terwijl NPY, dat ook geactiveerd wordt door vasten, geen effect heeft op perifere insuline gevoeligheid, maar hepatische insuline resistentie veroorzaakt, kunnen de effecten van vasten en ghrelin op de perifere gevoeligheid niet slechts verklaard worden door de modulatie van NPY neuronen. Echter, de inductie van hepatische insuline resistentie door ghrelin zou (gedeeltelijk) het resultaat kunnen zijn van de activatie van NPY neuronen door ghrelin.

Hoewel anderen hebben laten zien dat de regulatie van voedselinname door gastrointestinale hormonen wordt gemedieerd door de hersenen, blijft dit speculatief voor de regulatie van insuline gevoeligheid in het lichaam. Receptoren van gastrointestinale

hormonen zijn overal in het lichaam te vinden, zowel centraal als perifeer. Uit de studies in dit proefschrift kunnen bovendien geen conclusies getrokken worden over de plaats van actie van gastrointestinale hormonen in het centrale zenuwstelsel. Er is meer onderzoek nodig, zoals perifere en centrale toediening van gastrointestinale hormonen in combinatie met blokkers van het NPY/POMC systeem en denervatie studies, om conclusies te trekken over de bijdrage van de hersenen en de plaats van actie in de hersenen met betrekking tot de regulatie van insuline gevoeligheid door veranderingen in concentraties gastrointestinale hormonen.

Aangezien glucose onontbeerlijk is als energiebron voor de hersenen, is de regulatie van voedselinname en insuline gevoeligheid van het grootst mogelijk belang voor de hersenen. Het is daarom niet verwonderlijk dat de hersenen zelf betrokken zijn bij de uiterst nauwkeurige regulatie van deze processen, om overleving veilig te stellen.



Figuur 1. Een rol voor gastrointestinale hormonen en de hersenen in de regulatie van insuline gevoeligheid

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CURRICULUM VITAE

Annemieke Heijboer werd geboren op 27 maart 1978 te Noordwijk (Zuid-Holland). In 1996 behaalde zij haar atheneum diploma aan het Teylingen College te Noordwijkerhout. Datzelfde jaar werd de studie Bewegingswetenschappen aangevangen aan de Vrije Universiteit te Amsterdam. Tijdens haar studie behaalde zij in 1999 een European Master Degree in Adapted Physical Activity aan de Katholieke Universiteit Leuven in België.

In 2000 volgde een afstudeerstage aan het Sunnaas Sykehus te Nesodden in Noorwegen, onder begeleiding van dr. P.C. de Groot en dr. N. Hjeltnes. Deze wetenschappelijke stage richtte zich op de effecten van trainingsintensiteit op fysieke capaciteit, lipiden profielen en insuline gevoeligheid tijdens de vroege revalidatie van dwarslaesie patiënten. Daarna schreef zij een wetenschappelijke scriptie over fysieke activiteit en glucose transport, onder begeleiding van drs. H. Westra van de faculteit Bewegingswetenschappen aan de Vrije Universiteit van Amsterdam.

Na haar afstuderen in 2001, werkte zij bij Dr. M.W. Thompson aan de University of Sydney in Australië aan de effecten van verhoogde lichaamstemperatuur op vermoeidheid.

In september 2002 begon zij haar promotieonderzoek onder begeleiding van prof. dr. J.A. Romijn, dr. E.P.M. van der Kleij-Corssmit en dr. H. Pijl. De resultaten van dit onderzoek naar de rol van de gut-brain axis bij de regulatie van insuline gevoeligheid zijn in dit proefschrift beschreven.