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Gut hormones - Novel tools in the treatment of insulin resistance

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Gut hormones

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Novel tools in the treatment of insulin resistance

Gut hormones

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Novel tools in the treatment of insulin resistance

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Chapter 1

General introduction

Glucose homeostasis

Blood glucose levels are tightly regulated as a part of metabolic homeostasis. Mean normal blood glucose levels in humans are about 90 mg/dl, equivalent to 5 mmol/l. Glucose levels rise after meals for an hour or two by a few mmol and are usually lowest in the morning, before the first meal of the day. Transported via the bloodstream from the intestines or liver to body cells, glucose is the primary source of energy for body cells, fats and lipids being primarily a compact energy store. Failure to maintain blood glucose in the normal range leads to conditions of persistently low (hypoglycemia) or high (hyperglycemia) blood sugar. Since the brain functions on glucose, hypoglycemia is dangerous and can cause seizures, loss of consciousness, and death. Hyperglycemia, on the other hand, can lead to dehydration and hyper-osmolality, which can eventually result in a coma. Chronic hyperglycemia at levels more than slightly above normal can produce a very wide variety of serious complications over a period of years, including kidney damage, neurological damage, cardiovascular damage, and loss of vision.

Normal glucose homeostasis is regulated by endogenous glucose production and the uptake and utilization of glucose by peripheral tissues. In the fasted state glucose is mainly derived from the liver by 2 pathways, glycogenolysis and gluconeogenesis. Glycogenolysis is the biochemical breakdown of stored glycogen to glucose, while gluconeogenesis is the process of generating new glucose molecules from precursor molecules like amino acids, lactate, and glycerol. Glucose disposal takes place in peripheral tissues like skeletal muscle, adipose tissue, and heart tissue. The initial fate of glucose in tissues is essentially limited to glycolysis (catabolism of glucose) and direct storage either as glycogen or as the glycerol moiety of triglycerides in adipose tissue. The latter, however, accounts for only about 5% of postprandial glucose disposal, whereas liver and muscle are the sites of glycogen formation.

The homeostatic mechanism which keeps the blood value of glucose in a narrow range is composed of several interacting systems, of which hormone regulation is the most important. Two types of mutually antagonistic metabolic hormones affect blood glucose levels; they include the catabolic hormones (such as glucagon), and the anabolic hormone insulin. Insulin secretion by the pancreatic β -cells is stimulated after a meal. Then, insulin inhibits glycogenolysis and gluconeogenesis, and as a result inhibits endogenous glucose production. In addition, insulin stimulates glucose disposal in peripheral tissues. The net result of these actions of insulin is a decrease in plasma glucose levels. In response, glucagon is secreted by the pancreatic α -cells, which stimulates glycogenolysis and glucose production by the liver. Collectively with the interplay of other catabolic hormones (like growth hormone, cortisol, and catecholamine), endogenous glucose production is kept at a relatively constant level.

Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) has become a global epidemic rapidly. Now the disease affects 171 million individuals globally with an estimated mortality of about 4 million deaths each year¹. Having T2DM increases the risk for complications including heart disease, retinopathy, neuropathy, and nephropathy. T2DM has become one of the major causes of premature illness and death, mainly through the increased risk for cardiovascular disease. The World Health Organization (WHO) predicts that diabetes deaths will increase by more than 50% worldwide in the next 10 years. T2DM is a chronic, multifactorial disease characterized by a combination of impaired insulin secretion by the pancreatic β -cells and insulin resistance of target organs, leading to hyperglycemia. The diagnosis of having diabetes mellitus is based on WHO recommendations from 2006. Criteria include fasting plasma glucose levels ≥ 7.0 mmol/l or 2-h plasma glucose levels ≥ 11.1 mmol/l after a glucose load¹.

The development of T2DM is the result of both a deficient insulin secretion and insulin resistance. Insulin resistance is a state of reduced sensitivity in peripheral tissues of the body to the action of insulin². Muscle and adipose tissue in particular display a decreased responsiveness to insulin mediated glucose disposal. Resistance of the liver to the effects of insulin will increase endogenous glucose production. In the early stage of the development of T2DM, insulin resistance leads to an increase in insulin secretion by the β -cells to overcome the impairment of glucose disposal and endogenous glucose production and thereby maintaining normal glucose levels. Later in the development β -cells fail to compensate, leading to progressive hyperglycemia and ultimately T2DM^{3,4}.

Consequences of insulin resistance can be viewed in two ways. First, during fasting, the liver represents a major site of insulin resistance, which is reflected by overproduction of glucose despite presence of both fasting hyperinsulinemia and hyperglycemia. This accelerated rate of hepatic glucose output is the primary determinant of elevated fasting plasma glucose concentration in T2DM. Second, with eating, failure of adequate insulin-mediated nutrient disposal into skeletal and adipose tissue combined with an attenuated inhibition of hepatic glucose production cause the raised postprandial glycemia.

T2DM and lipid metabolism

Besides glucose metabolism, lipid metabolism is also disturbed in T2DM patients. Increased plasma levels of triglycerides (TG) in very low density lipoproteins (VLDL) are characteristic of the dyslipidemia associated with insulin resistance and T2DM. Overproduction of VLDL leads to increased plasma levels of TG which, via an exchange

process, results in low levels of high density lipoprotein cholesterol and the generation of small, dense, cholesterol ester depleted low density lipoproteins. Increased assembly and secretion of VLDL by the liver results from the complex, post-transcriptional regulation of apolipoprotein B (apoB) metabolism in the liver. In the presence of low levels of hepatic TG and cholesterol, much of the constitutively synthesized apoB is degraded by both proteosomal and non-proteosomal pathways. When excess TG, and to a lesser extent, cholesterol, are present, apoB is targeted for secretion. The major sources of TG in the liver: uptake of fatty acids (FA) released by lipolysis of adipose tissue TG, uptake of TG in VLDL and chylomicrons remnants, and hepatic de novo lipogenesis (the synthesis of FA from glucose) are all abnormally increased in insulin resistance.

Obesity

Body Mass Index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in meters (kg/m^2). Overweight is defined when BMI is $\geq 25 \text{ kg/m}^2$ and obese when $\geq 30 \text{ kg/m}^2$. The prevalence of obesity has reached epidemic proportions for a number of years now. Recent numbers from the WHO show that more than 1 billion adults are overweight worldwide¹. In the Netherlands, 46.9% of the total population is overweight of which 11.1% is obese. A dramatic increase has been found in the percentages of obese youngsters (age 15-25)⁵. Overweight and obesity lead to serious health consequences. They are a major risk factor for developing chronic diseases like cardiovascular disease, some type of cancers, musculoskeletal disorders, and T2DM.

Obesity, and in particular visceral adiposity, is negatively correlated with insulin sensitivity. With respect to visceral adiposity, a great deal of evidence suggests two strong links with insulin resistance. First, visceral adipose cells produce significant amounts of proinflammatory cytokines such as tumor necrosis factor-alpha, and interleukins-1 and -6, resistin, and leptin. In numerous experimental models, these proinflammatory cytokines profoundly disrupt normal insulin action in fat and muscle cells, and may be a major factor in causing the whole-body insulin resistance observed in patients with visceral adiposity⁶.⁷ Second, visceral adiposity is related to an accumulation of hepatic fat stores, a condition known as nonalcoholic fatty liver disease (NAFLD). The result of NAFLD is an excessive release of FFA into the bloodstream (due to increased lipolysis), and an increase in hepatic glucose production, both of which have the effect of exacerbating peripheral insulin resistance and increasing the likelihood of T2DM⁸.

Therapies for T2DM

T2DM and obesity associated insulin resistance impose a major health risk, due to increased morbidity and mortality. The major goal of therapeutic intervention is to ameliorate hyperglycemia and insulin resistance, but also increased risk factors such as dyslipidemia and hypertension should be taken into account. Without medication, the outcome of the disease can significantly be improved by lifestyle changes. Restricted caloric intake, weight loss, and exercise will significantly improve whole body insulin sensitivity^{9, 10}. Sometimes, these adaptations are not enough to reduce blood glucose levels near the normal range. In addition, lifestyle changes are not easy and require a strong will. Moreover, it has to be continued for a long term, since weight gain and symptoms of T2DM will come back once discontinued. Therefore, the need for pharmacological strategies is required. Sulfonylureas, meglitinides, biguanides, thiazolidinediones, and alpha-glucosidase inhibitors comprise classes of drugs that lower blood glucose levels. Because these drugs work in different ways to accomplish this, they may be used together to get the desired effects. However, taking more than one drug is more costly and can increase the risk of side effects. Recently, the gut hormones have become interesting therapeutic targets in the battle against T2DM, because of their effects on both food intake and glucose metabolism.

Gut-brain axis

The gastrointestinal tract is the largest endocrine organ in the body and releases more than 20 different regulatory peptide hormones that influence a number of physiological processes and act on tissues including exocrine glands, smooth muscle, and the central nervous system. Most of the gut hormones are sensitive to gut nutrient content, and short-term feelings of hunger and satiety are believed to be mediated, in part, by coordinated changes in circulating gut hormone levels. The interaction between the gut hormones and the central nervous system, the gut-brain axis, is regulated via both hormonal and neuronal pathways. At the base of the third ventricle these hormones can cross the semi-permeable blood-brain-barrier to reach the hypothalamus. Alternatively, they act indirectly by altering the activity of afferent neuronal pathways and brain stem circuits, which in turn project to the hypothalamus.

The hypothalamus is regarded as the main feeding center of the brain and consists of several nuclei involved in food intake, including the arcuate nucleus (ARC), the paraventricular nucleus, the lateral hypothalamic area, the ventromedial nucleus, and the dorsomedial nucleus. The ARC located in the hypothalamus is the most important center that regulates food intake. It contains at least two distinct groups of neurons controlling

energy balance: the orexigenic pathway, containing neurons co-expressing neuropeptide Y and agouti-related protein, stimulating food intake, and the anorexigenic pathway, containing neurons co-expressing pro-opiomelanocortin and cocaine- and amphetamine-regulated transcript, inhibiting food intake¹¹.

Over the past decades the pathways of food intake by the gut-brain axis have become increasingly well delineated¹². Recently, compelling evidence indicates that the gut-brain axis is also a key partner of glucose homeostasis. This is accomplished via brain pathways that overlap with those controlling food intake and body weight. The gut hormones have emerged as key factors able to convey metabolic information to the brain and also to modulate endogenous glucose production and disposal^{13, 14}. The present thesis deals with a variety of gut hormones, of which their effects on food intake and glucose metabolism will be summarized.

Glucagon-like peptide-1 (GLP-1) is a cleavage product of the proglucagon molecule which is secreted by the intestinal L-cells and in the brain^{15, 16}. It is released in response to food intake in proportion to caloric content to stimulate glucose-dependent insulin secretion^{15, 17}. In addition, GLP-1 exerts multiple other effects, including inhibition of food intake, slowing of gastric emptying, and inhibition of glucagon secretion^{18, 19}. GLP-1 is also known to beneficially affect glucose metabolism in T2DM patients^{19, 20}. However, GLP-1 is easily degraded by the enzyme dipeptidyl-peptidase IV (DPP-IV), limiting its therapeutic efficacy²¹. Therefore, pharmaceutical companies are developing GLP-1 analogues resistant to inactivation by DPP-IV. Clinical studies have shown that these analogues are very effective in reducing body weight and improving glucose metabolism in patients with T2DM. Exenatide is a synthetic version of exendin-4, a naturally-occurring GLP-1 analogue, which was first isolated from the saliva of the lizard known as the Gila monster. Exenatide, currently on the market (brand name Byetta), works to lower blood glucose levels primarily by increasing insulin secretion. Because it exerts this effect only in the presence of elevated blood glucose levels, it does not tend to increase the risk of hypoglycemia on its own, although hypoglycemia can occur if taken in conjunction with a sulfonylurea. The primary side effect is nausea, which tends to improve over time. Another class of medications called DPP-IV inhibitors work by preventing the breakdown of GLP-1. By interfering in the process that breaks down GLP-1, DPP-IV inhibitors allow it to remain active in the body for a longer duration, lowering blood glucose levels only when they are elevated. DPP-IV inhibitors do not tend to cause weight gain and tend to have a neutral or positive effect on cholesterol levels. Sitagliptin (Januvia) is currently the only DPP-IV inhibitor on the market.

The anorexic effect of GLP-1 is mediated via the GLP-1 receptor in the brain¹⁸. Recent findings provide evidence linking GLP-1 action in the brain with the peripheral control of glucose flux, insulin sensitivity and insulin secretion²². Complementary studies reveal that the arcuate GLP-1 receptors regulate glucose homeostasis but not food intake²³. In addition, central administration of GLP-1 reduces lipid storage in peripheral adipose tissue²⁴.

Oxyntomodulin (OXM) is, just like GLP-1, a cleavage product of the proglucagon molecule secreted from the intestinal L-cells in response to food intake in proportion to caloric content^{25, 26}. It potentially inhibits meal-stimulated gastric acid and enzyme secretion in rodents and man²⁷. OXM is insulinotropic, however, as a circulating hormone in humans, its concentration is probably too low to influence insulin secretion under physiological circumstances^{28, 29}. OXM is a potential regulator of appetite and body weight, when administered both intracerebroventricular and intraperitoneal in fasted rats^{30, 31}. It is suggested that OXM causes these anorexigenic effects by affecting the arcuate nucleus in the brain.

Peptide YY (PYY) is produced and released from the L-cells postprandially³². Most of the circulating PYY is the truncated form of the full length peptide, PYY₃₋₃₆. PYY₃₋₃₆ is transported to the brain via the blood-brain-barrier. There, it binds the Y2 receptor, which is expressed on NPY neurons of the arcuate nucleus, resulting in decreased food intake³³. Peripheral administration of PYY₃₋₃₆ has been shown to regulate long-term body weight reduction^{34, 35}. In addition, PYY₃₋₃₆ administration to mice shows an improvement of insulin action to stimulate glucose disposal independently of its effects on food intake and body weight³⁶.

Pancreatic polypeptide (PP) is synthesized in the so-called F-cells, located in the pancreatic islet of Langerhans. PP is the endogenous ligand for the Y4 receptor in the brain. Peripheral administration of PP has been shown to reduce food intake in both rodents and humans^{37, 38}. Chronic treatment with PP ameliorates insulin resistance as indicated by decreased plasma glucose accompanied by a moderate decrease in insulin levels³⁹.

Outline of the thesis

The aim of the present thesis was to gain more insight into the potential of gut hormones or newly developed analogues to impact on insulin sensitivity.

Chapter 2 describes the metabolic effects of peripheral administration of OXM in insulin resistant mice. OXM appears to be an effective regulator of food intake and appetite. OXM probably impacts on food intake via neural routes in the ARC, which are similarly engaged by other gut-derived peptides modulating appetite, like GLP-1, ghrelin, and PYY₃₋₃₆. These peptides affect insulin sensitivity via mechanistic routes that are independent of food intake and body weight. Therefore, we speculated that OXM could also impact on insulin action in insulin resistant mice.

In **chapter 3**, we evaluated the effects of a novel GLP-1 analogue, CNTO736, on insulin sensitivity of glucose and VLDL metabolism in high-fat-fed mice. GLP-1 appears to be a promising drug target for the treatment of T2DM, however its therapeutic potency is limited by its short half-life. CNTO736 has an increased half-life over GLP-1 and has been shown to retain GLP-1-like biological activity. The aim of our study was to evaluate the potential of this analogue in terms of its metabolic effects.

In **chapter 4**, we further investigated the potential of the GLP-1 analogue described in **chapter 3** to modulate lipid metabolism. Lipid metabolism is also disturbed in patients with T2DM. Increased VLDL-TG concentrations and decreased HDL cholesterol in plasma significantly contribute to the long term risk of macrovascular disease in these patients. We explored the chronic effects of the analogue on lipid metabolism in APOE*3-Leiden transgenic mice, a “human-like” animal model for studies evaluating the impact of drugs on hyperlipidemia.

GLP-1 improves insulin sensitivity in humans and rodents. It is currently unknown to what extent the (metabolic) effects of circulating GLP-1 are mediated by central GLP-1 receptors. Therefore, in **chapter 5** we aimed to evaluate the impact of blocking central GLP-1 receptors on the metabolic effects of peripherally administered GLP-1.

Chapter 6 describes the effects of chronic treatment with the novel compounds Obinipitide, a synthetic analogue of PYY₃₋₃₆ and PP, and TM30339, a synthetic analogue of PP. Both compounds, designed as anti-obesity drugs, show reduced food intake and long-term body weight reduction. We administered the compounds for 4 weeks subcutaneously to measure its long-term effects on insulin sensitivity of glucose and VLDL metabolism.

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Chapter 2

Oxyntomodulin ameliorates glucose intolerance in mice fed a high-fat diet

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Abstract

We evaluated the acute effects of oxyntomodulin (OXM), a proglucagon derived peptide, on glucose metabolism in diet-induced insulin resistant male C57Bl/6 mice. To determine the effects on glucose tolerance, mice were intraperitoneally injected with OXM (0.75, 2.5, or 7.5 nmol) or vehicle prior to an intraperitoneal glucose tolerance test. 0.75 nmol/h OXM or vehicle was infused during a hyperinsulinemic euglycemic clamp to quantify insulin action on glucose production and disposal. OXM dose-dependently improved glucose tolerance as estimated by the area under the curve (AUC) for glucose (OXM, 7.5 nmol: 1564 ± 460 , $p < 0.01$; 2.5 nmol: 1828 ± 684 , $p < 0.01$; 0.75 nmol: 2322 ± 303 , $p < 0.05$; control: 2790 ± 222 mmol/l/120 min). Insulin levels in response to glucose administration were higher in 7.5 nmol OXM-treated animals compared to controls (AUC_{insulin} : 343 ± 113 vs. 231 ± 39 ng/ml/120 min, $p < 0.05$). In basal clamp conditions, OXM increased endogenous glucose production (82.2 ± 14.7 vs. 39.9 ± 5.7 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.001$). During insulin infusion, insulin levels were twice as high in OXM-treated mice compared to controls (10.6 ± 2.8 vs. 4.4 ± 2.2 ng/ml, $p < 0.01$). Consequently, glucose infusion rate (118.6 ± 30.8 vs. 38.8 ± 26.4 $\mu\text{l}/\text{h}$, $p < 0.001$) and glucose disposal (88.1 ± 13.0 vs. 45.2 ± 6.9 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.001$) were enhanced in mice that received OXM. In addition, glucose production was more suppressed during OXM infusion (35.7 ± 15.5 vs. $15.8 \pm 11.4\%$ inhibition, $p < 0.05$). However, if these data were expressed per unit concentration of circulating insulin, OXM did not affect insulin action on glucose disposal and production. These results indicate that OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. It ameliorates glucose intolerance, most likely because it elevates glucose-induced plasma insulin concentrations. OXM does not appear to impact on insulin action.

Introduction

Posttranslational processing of proglucagon in the intestine and the central nervous system (CNS) yields glucagon-like peptide-1 (GLP-1), glucagon-like peptide-2 (GLP-2), glicentin, and oxyntomodulin (OXM)¹⁻³. OXM contains 37-amino acids, including the complete glucagon sequence with a basic octapeptide carboxyterminal extension⁴. OXM is released from intestinal L-cells into the blood in response to food ingestion in amounts proportional to caloric content^{5, 6}. It potentially inhibits meal-stimulated gastric acid and enzyme secretion in rodents and man⁷.

OXM appears to be an effective regulator of appetite and body weight. Intravenous (i.v.) infusion of OXM suppresses appetite and reduces food intake in humans during a buffet meal⁸. Furthermore, both intracerebroventricular and intraperitoneal (i.p.) administration of OXM reduces food intake and body weight gain during refeeding in fasted rats^{9, 10}. OXM most likely causes these anorexigenic effects by modulating neuronal activity in the arcuate nucleus (ARC) of the hypothalamus¹⁰. OXM-like immunoreactivity has been found in the CNS, particularly in the hypothalamus^{11, 12}, and i.p. administration of OXM inhibits neuronal activity in the ARC as detected by magnetic resonance imaging¹³. A distinct receptor for OXM has not been identified to date. However, OXM can bind to both the glucagon and GLP-1 receptor, which is to be expected on the basis of its molecular structure^{4, 14}. Its anorexigenic effect requires the (central) GLP-1 receptor only¹⁵. Thus, OXM probably impacts on food intake via neural routes in the ARC, which are similarly engaged by other gut-derived peptides modulating appetite, like GLP-1, ghrelin, and peptide YY₃₋₃₆ (PYY₃₋₃₆)¹⁶⁻²⁰.

Gut-hormones are important for the regulation of glucose metabolism. We recently showed that ghrelin and PYY₃₋₃₆ affect insulin sensitivity via mechanistic routes that are independent of food intake and body weight^{21, 22}. Also, recent evidence suggests that GLP-1 ameliorates insulin resistance and hyperglycemia in diabetic ob/ob mice²³. Therefore, we speculated that OXM could impact on insulin sensitivity in insulin resistant mice. The *aim* of this study was to explore the effects of systemic OXM administration on glucose tolerance and insulin sensitivity in diet-induced insulin resistant C57Bl/6 mice. To this end, we injected OXM intraperitoneally (i.p.) prior to an i.p. glucose tolerance test (IPGTT), and administered OXM intravenously (i.v.) during a hyperinsulinemic euglycemic clamp in high-fat-fed, insulin resistant C57Bl/6 mice.

Methods

Animals and diet. Male C57Bl/6 mice (12 weeks old)(Charles River, Maastricht, the Netherlands) were housed in a temperature and humidity-controlled environment and

were fed a high-fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) with free access to water for 16-20 weeks to induce insulin resistance²⁴. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Drugs. OXM (human, mouse, rat; molecular weight = 4449, 90 g/mol) was purchased from Phoenix Pharmaceuticals (Belmont, USA).

Intraperitoneal glucose tolerance test. After 16 weeks on diet, mice were fasted for 10 hours with food withdrawn at 23:00 p.m. the day prior to the study. At 9:00 a.m. the experimental day, mice were given an i.p. injection of OXM dissolved in PBS (0.75, 2.5, or 7.5 nmol) or PBS alone in a volume of 100 μ l. The doses were based on a previous report in which OXM 0.75 nmol reduced food intake in rats^{9, 10}. Fifteen minutes after injection blood was taken, immediately followed by i.p. injection of a 2 g/kg 20% D-glucose solution (t=0). Additional blood samples were taken via tail bleeding at 5, 15, 30, 45, 60, and 120 minutes after glucose injection for measurement of plasma glucose and insulin levels.

Hyperinsulinemic euglycemic clamp study. The same cohort of mice was used for the clamp study as for the IPGTT with 4 weeks of intermission. Mice were fasted for 16 hours with food withdrawn at 17:00 p.m. the day prior to the study. Hyperinsulinemic euglycemic clamp studies started at 9:00 a.m. and were performed as described earlier²⁵. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Vehicle or OXM was administered in a primed (0 and 0.45 nmol respectively) continuous (0 and 0.75 nmol/h respectively) i.v. infusion during the whole experiment. First, basal rate of glucose turnover was determined by giving a primed (p) continuous (c) i.v. infusion of ¹⁴C-glucose (p: 0.7 μ Ci; c: 1.2 μ Ci/h, GE Healthcare, Little Chalfont, U.K.) for 60 minutes. Subsequently, insulin was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 minutes to attain steady state circulating insulin levels of ~4 ng/ml. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding (< 3 μ l) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 μ l) were taken during the basal period (after 50 and 60 min) and during the clamp period (after 70, 80, and 90 min) to determine plasma concentrations of glucose, non-esterified fatty acids (NEFA), insulin, and plasma ¹⁴C-glucose specific activities. To estimate insulin-stimulated glucose uptake in individual tissues, 2-deoxy-D-[³H] glucose (2-[³H]DG; Amersham, Little Chalfont, UK) was

administered as a bolus (2 μ Ci) 40 minutes before the end of the clamp. At the end of the clamp, mice were sacrificed and skeletal muscle (hind limb) and white adipose tissue (abdomen) were isolated and immediately 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). Plasma insulin concentrations were measured by a mouse insulin ELISA (Mercodia AB, Uppsala, Sweden). Total plasma ^{14} C-glucose was determined in 7.5 μ l plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water as described earlier²⁶.

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 previously²⁷.

Calculations. Areas under the curve (AUC) for glucose (AUC_{glucose}) and insulin (AUC_{insulin}) were calculated using the trapezoidal rule. The turnover rate of glucose (μ mol/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 ^{14} C-glucose (dpm/ μ mol). 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. The whole body insulin sensitivity index was calculated as the ratio of glucose disposal to plasma insulin levels during hyperinsulinemic conditions. The hepatic insulin sensitivity index was calculated as the ratio of the relative suppression of EGP during the hyperinsulinemic condition to the change in plasma insulin levels from basal to hyperinsulinemic conditions. Tissue-specific 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 described²⁷.

Statistical analysis. Differences between groups were determined with the Kruskal–Wallis non-parametric test for k independent samples. When significant differences were found, the Mann–Whitney non-parametric test was used as a post hoc test to determine differences between two independent groups. A P-value of less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD.

Results

Glucose tolerance test

OXM significantly improved glucose tolerance in a dose-dependent way, as evidenced by a reduction of the area under the curve of glucose concentrations (AUC_{glucose} : OXM, 7.5 nmol: 1564 ± 460 , $p < 0.01$; 2.5 nmol: 1828 ± 684 , $p < 0.01$; 0.75 nmol: 2322 ± 303 , $p < 0.05$; control: 2790 ± 222 mmol/l/120 min)(figure 1A and B). Insulin levels in response to glucose administration were significantly increased in animals receiving 7.5 nmol OXM compared to control animals (AUC_{insulin} : 343 ± 113 vs. 231 ± 39 ng/ml/120 min, $p < 0.05$)(figure 2A and B), but not in animals treated with 2.5 or 0.75 nmol of OXM.

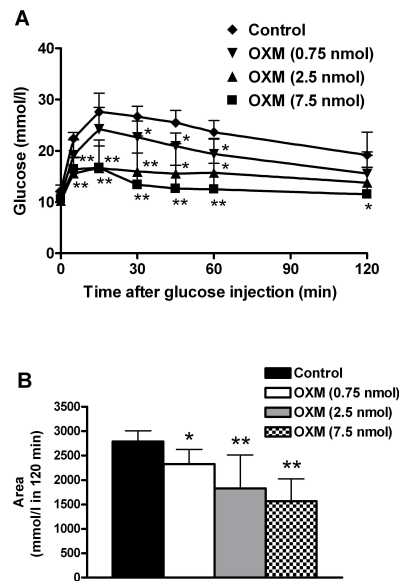


Figure 1. Plasma glucose (A) immediately before and 5, 15, 30, 45, 60, and 120 min after an intraperitoneal (i.p.) injection of glucose (2g/kg) in mice that received a single i.p. injection of vehicle or OXM at $t = -15$ (n=6/group). (B) Area under 120-min glucose curve. Values represent mean $\pm$ SD. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

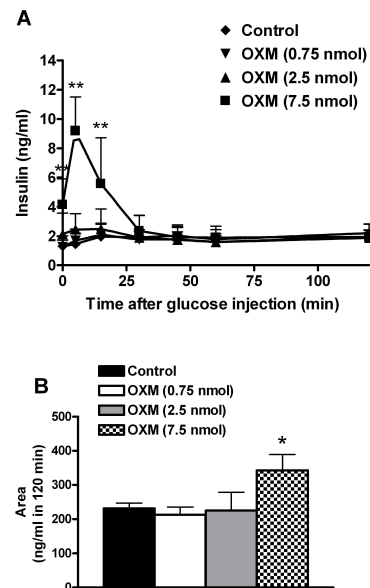


Figure 2. Plasma insulin (A) immediately before and 5, 15, 30, 45, 60, and 120 min after an intraperitoneal (i.p.) injection of glucose (2g/kg) in mice that received a single i.p. injection of vehicle or OXM at $t = -15$ (n=6/group). (B) Area under 120-min insulin curve. Values represent mean $\pm$ SD. * $P < 0.05$ vs. control. ** $P < 0.01$ vs. control.

Hyperinsulinemic euglycemic clamp

Plasma parameters and body weight

Body weight, plasma glucose, insulin, and NEFA concentrations in basal and hyperinsulinemic conditions are shown in table 1. Basal glucose and insulin levels were significantly higher and basal NEFA levels significantly lower in mice that received OXM

compared to control mice. In hyperinsulinemic steady state clamp conditions, insulin levels remained significantly higher and plasma NEFA levels significantly lower in OXM treated mice compared to control mice.

Table 1. Body weight and plasma parameters under basal and hyperinsulinemic conditions in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7).

	Basal		Hyperinsulinemic	
	Control	OXM	Control	OXM
Body weight, g	37.1 ± 3.2	38.4 ± 1.8		
Glucose, mmol/l	5.9 ± 0.8	13.4 ± 2.8**	6.5 ± 1.0	7.1 ± 1.1
Insulin, ng/ml	2.0 ± 1.4	14.1 ± 2.6***	4.4 ± 2.2	10.6 ± 2.8**
NEFA, mmol/l	0.6 ± 0.2	0.2 ± 0.1**	0.3 ± 0.2	0.1 ± 0.1*

Values are ± means SD. OXM, oxyntomodulin; NEFA, nonesterified fatty acids. *P<0.05 vs. control. **P<0.01 vs. control.

***P<0.001 vs. control.

Glucose turnover and tissue-specific glucose uptake

In basal conditions, EGP was significantly higher in the OXM-treated animals compared to control animals (82.2 ± 14.7 vs. 39.9 ± 5.7 $\mu\text{mol}/\text{min}/\text{kg}$, $p<0.001$). The rate of glucose infusion required to maintain euglycemia during insulin infusion was significantly higher in OXM-treated mice than in control mice (118.6 ± 30.8 vs. 38.8 ± 26.4 $\mu\text{l}/\text{h}$, respectively, $p<0.001$). This was because glucose disposal was significantly enhanced (88.1 ± 13.0 vs. 45.2 ± 6.9 $\mu\text{mol}/\text{min}/\text{kg}$ in controls, $p<0.001$, figure 3A) and glucose production more suppressed (35.7 ± 15.5 vs. $15.8 \pm 11.4\%$ inhibition in controls, $P<0.05$, figure 4A) during insulin infusion in OXM-treated animals. Accordingly, insulin-mediated 2-deoxy-glucose uptake in adipose and muscle tissue were significantly higher in OXM-treated mice (adipose tissue: 1.3 ± 0.8 vs. 0.5 ± 0.2 $\mu\text{mol}/\text{g}$ tissue in controls, $p<0.01$; muscle: 5.2 ± 2.9 vs. 2.2 ± 1.1 $\mu\text{mol}/\text{g}$ tissue in controls, $p<0.05$). However, the plasma insulin concentrations attained by insulin infusion were significantly higher in OXM-treated mice compared to control (10.6 ± 2.8 vs. 4.4 ± 2.2 ng/ml , $p<0.01$). Therefore, we also present the data per unit of circulating insulin concentration. The whole body and hepatic insulin sensitivity indices were not different between groups (whole body insulin sensitivity index: 8.9 ± 2.6 vs. 12.2 ± 5.2 $\mu\text{mol}/\text{min}/\text{kg}/\text{ng}$ insulin/ml, NS; hepatic insulin sensitivity index: 3.5 ± 1.5 vs. $3.3 \pm 1.8\%$ inhibition from basal per ng insulin/ml, NS, figure 3B and 4B respectively).

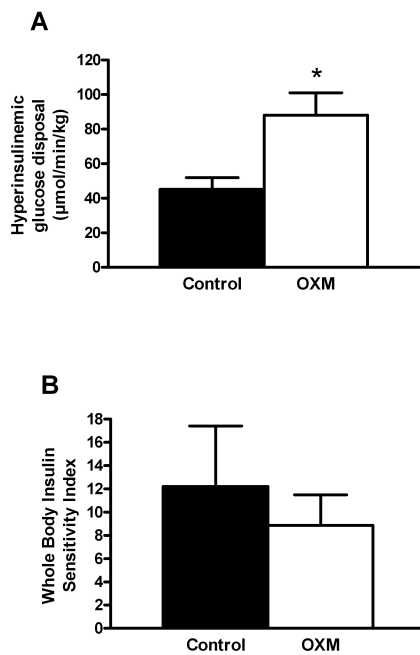


Figure 3. Hyperinsulinemic glucose disposal (A) and whole body insulin sensitivity index (B) in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7) during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD. *P<0.001 vs. control.

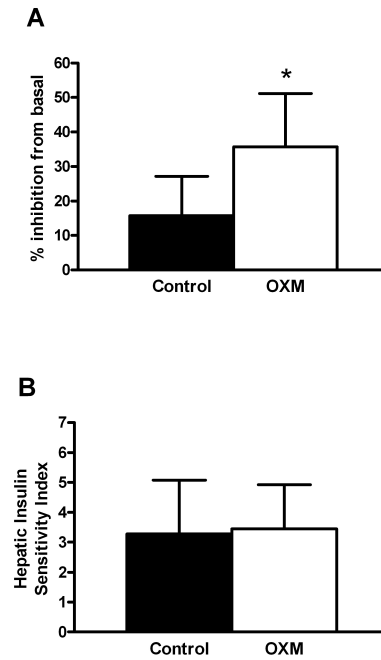


Figure 4. Inhibition of endogenous glucose production (EGP) (A) and hepatic insulin sensitivity index (B) in mice that received continuous intravenous infusion of vehicle (n=8) or OXM (n=7) during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD. *P<0.05 vs. control.

Discussion

This is the first study to show that systemic administration of OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. A single i.p. injection of OXM dose-dependently ameliorates glucose intolerance in these animals. The plasma insulin concentrations in response to glucose administration were higher in OXM treated animals, particularly after the highest dose. In apparent contrast, i.v. OXM administration clearly enhanced EGP in fasted animals. During the euglycemic clamp, i.v. OXM stimulated glucose uptake and curtailed EGP, suggesting that it improved insulin action. However, circulating insulin levels were more than double during insulin infusion in OXM treated mice, complicating the interpretation of the data. Indeed, per unit of ambient insulin concentration, glucose uptake and EGP were not different in OXM and placebo treated animals. In aggregate, our data indicate that systemic OXM administration improves glucose tolerance in insulin resistant C57Bl/6 mice, primarily because it elevates plasma

insulin levels. Minor effects on insulin action cannot be excluded. In fasting condition, systemic OXM injection stimulates EGP.

Oxyntomodulin dose-dependently improved glucose tolerance in mice maintained on a high fat diet. Post-load plasma glucose concentrations are the result of the concerted effects of insulin secretion, clearance and action on glucose metabolism. Insulin levels in response to glucose administration were significantly higher in OXM treated animals, suggesting that OXM stimulates insulin secretion and/or inhibits insulin clearance from the circulation. Indeed, OXM has been shown to stimulate glucose-induced insulin release in perfused rat pancreas²⁸, which may be mediated by binding of OXM to the GLP-1 and/or glucagon receptor in β -cells. A unique receptor for OXM has not been identified to date. However, OXM was shown to bind and activate GLP-1 and glucagon receptors, albeit with lower affinity than the cognate ligands^{4, 14}. β -cells express GLP-1 as well as glucagon receptors^{29, 30} and both ligands reinforce glucose-induced insulin secretion³¹⁻³⁴. Thus, OXM most likely triggered insulin secretion by simultaneous activation of GLP-1 and glucagon receptors on β -cells³⁵. However, interaction with (an)other receptor(s) cannot be excluded as OXM was shown to induce contraction of antral smooth muscle cells in an *in vitro* model in which GLP-1 and glucagon had no effect at all³⁶.

GLP-1 retards insulin clearance from the circulation in mice³⁷, which suggests that OXM may also slow insulin clearance to elevate glucose-induced plasma insulin levels. This inference is strongly supported by our finding that OXM administration more than doubled plasma insulin concentrations in the face of insulin infusion rates similar to those in placebo treated animals during the hyperinsulinemic clamp. In any case, our data clearly suggest that the beneficial effect of OXM on glucose tolerance is entirely due to elevation of post-load plasma insulin concentrations, because OXM did not significantly impact on insulin action as quantified by hyperinsulinemic euglycemic clamp.

OXM inhibits food intake in rats, mice and humans⁸⁻¹⁰. Other gut peptides, that similarly decrease appetite, also modulate insulin action and glucose metabolism²¹⁻²³. In fact, many peptides involved in the control of feeding simultaneously regulate metabolism, illustrating the intuitively obvious notion that food intake and metabolism share common regulatory pathways³⁸⁻⁴⁰. Therefore, we speculated that oxyntomodulin could also impact on insulin action. However, although OXM dose-dependently improved glucose tolerance, it did not appear to affect insulin action, as glucose disposal and EGP on a per-unit-of-circulating-insulin-concentration basis were not different in OXM versus placebo infused animals during the euglycemic clamp. Correction of glucose production and uptake rates for plasma insulin levels is rather complex, as insulin action does not linearly correlate with plasma insulin concentrations⁴¹. Thus, glucose production and disposal expressed per unit of circulating insulin concentration may not be an optimal

measure of insulin's capacity to impact on these metabolic processes. However, we believe it's the best measure we have, given the fact that circulating insulin levels were almost twofold higher in OXM treated animals during the clamp. Therefore, we cannot exclude minor effects of OXM on insulin sensitivity, also in light of the data from the IPGTT, showing that the two lowest doses of OXM did improve glucose tolerance without increased insulin levels.

The failure of OXM to deeply impact on insulin action may have to do with its affinities for both the GLP-1 and the glucagon receptors. Although GLP-1 and glucagon similarly stimulate insulin secretion, these peptides generally relay opposing metabolic messages. GLP-1 inhibits food intake¹⁹ and simultaneously improves insulin action in diabetic mice²³, whereas glucagon antagonizes insulin action (it stimulates EGP and indirectly inhibits glucose disposal by stimulating lipolysis^{42, 43}). Thus, simultaneous activation of both GLP-1 and glucagon receptors by systemic administration of OXM is unlikely to significantly impact on insulin action.

Our findings suggest that OXM may be the base of new pharmacological tools to ameliorate glucose intolerance in insulin resistant subjects. The mice examined in our studies were maintained on a high fat diet, which invariably induces obesity and insulin resistance in the C57Bl/6 strain²⁴. Thus, the fact that OXM can improve glucose tolerance in these animals indicates that this peptide (or any novel peptidomimetic) may be useful in the treatment of obesity and type 2 diabetes, like other proglucagon derived peptides⁴⁴. However, OXM enhanced glucose production in basal condition, probably by virtue of its capacity to activate the glucagon receptor in the liver^{4, 45}. Obviously, stimulation of glucose production is an undesirable quality of drugs used to treat insulin resistance, which requires careful evaluation. At the end of the day, if it turns out to be a consistent metabolic effect of treatment with OXM (-mimetics), excessive glucose production clearly limits the clinical applicability of this peptide.

In conclusion, OXM beneficially affects glucose metabolism in diet-induced insulin resistant C57Bl/6 mice. It improves glucose tolerance, enhances glucose disposal by peripheral tissues, and reduces glucose production, primarily by elevating glucose induced plasma insulin levels.

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Chapter 3

CNT0736, a novel GLP-1 receptor agonist, ameliorates insulin resistance and inhibits very low density lipoprotein production in high-fat-fed mice

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Abstract

CNTO736 is a glucagon-like peptide-1 (GLP-1) receptor agonist that incorporates a GLP-1 peptide analogue linked to the Mimetibody™ platform. We evaluate the potential of acute and chronic CNTO736 treatment on insulin sensitivity and very low density lipoprotein (VLDL) metabolism. For acute studies, diet-induced insulin resistant C57Bl/6 mice received a single i.p. injection of CNTO736 or vehicle. Chronic effects were studied following 4 weeks daily i.p. administration. A hyperinsulinemic-euglycemic clamp monitored insulin sensitivity. A single dose of CNTO736 reduced fasting plasma glucose levels (CNTO736: 4.4 ± 1.0 ; control: 6.3 ± 2.4 mM) and endogenous glucose production (EGP) (CNTO736: 39 ± 11 ; control: 53 ± 13 $\mu\text{mol}/\text{min}/\text{kg}$) and increased insulin mediated glucose uptake (CNTO736: 76 ± 25 ; control: 54 ± 13 $\mu\text{mol}/\text{min}/\text{kg}$). Chronic administration of CNTO736 reduced fasting glucose levels (CNTO736: 4.1 ± 0.8 ; control 6.0 ± 1.0 mM), improved insulin dependent glucose uptake (CNTO736: 84 ± 19 ; control: 61 ± 15 $\mu\text{mol}/\text{min}/\text{kg}$), and enhanced inhibition of EGP (CNTO736: 91 ± 18 ; control: 80 ± 10 % inhibition). In addition, chronic dosing with CNTO736 reduced fasting EGP (CNTO736: 39 ± 9 ; control: 50 ± 8 $\mu\text{mol}/\text{min}/\text{kg}$) and VLDL production (CNTO736: 157 ± 23 ; control: 216 ± 36 $\mu\text{mol}/\text{h}/\text{kg}$). These results indicate that CNTO736 reinforces insulin's action on glucose disposal and production in diet-induced insulin resistant mice. In addition, CNTO736 reduces basal hepatic glucose and VLDL output in these animals. The data suggest that CNTO736 may be a useful tool in the treatment of type 2 diabetes.

Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone synthesized in enteroendocrine L-cells and brain tissue^{1, 2}. It is released by the gut in response to food intake to stimulate glucose-induced insulin production^{1, 3}. In addition, GLP-1 exerts multiple other effects, including inhibition of food intake, slowing of gastric emptying and inhibition of glucagon secretion^{4, 5}. Some, but certainly not all, studies suggest that GLP-1 secretion in response to meals is reduced in patients with type 2 diabetes mellitus (T2DM)⁶⁻⁸, which attenuates postprandial insulin release and potentially blunts satiety. Continuous infusion of GLP-1 lowers circulating glucose levels after ingestion of a meal and normalizes fasting hyperglycemia in these patients^{5, 9}. Native GLP-1 is not an appropriate candidate molecule for therapeutic intervention, because of its rapid degradation by dipeptidyl-peptidase-4 (DPP-4)¹⁰. However, GLP-1 analogues with extended *in vivo* stability are currently under development by many pharmaceutical companies. Byetta™ (exendin-4 (Ex-4)) was approved in 2005 to treat T2DM¹¹.

We have developed the Mimetibody™ platform for display and delivery of bioactive peptides¹². Mimetibodies™ have longer terminal half-lives relative to peptides due to their increased size and antibody Fc properties. CNTO736 incorporates a DPP-4 resistant GLP-1 peptide analogue into the Mimetibody™ platform. Despite these physical adaptations, it retains GLP-1-like biological activity: it stimulates glucose-dependent insulin secretion, inhibits food intake and gastric emptying, and improves glucose tolerance in diet-induced obese mice in a GLP-1 receptor dependent manner¹³.

Here, we further explore the pharmacological characteristics of CNTO736 in terms of its metabolic effects. In particular we evaluate the acute and chronic impact of the compound on insulin sensitivity of glucose and lipid metabolism. Overproduction of very low density lipoprotein (VLDL), which is a prominent feature of diabetic dyslipidemia (as the proximate cause of hypertriglyceridemia), contributes significantly to cardiovascular risk in T2DM patients¹⁴. A few studies show that GLP-1 treatment and DPP-4 inhibition reduce circulating triglyceride (TG) levels in healthy humans and T2DM patients, which suggest that these drugs can inhibit VLDL production and/or clearance¹⁵⁻¹⁷.

Methods

Animals and diet. Male C57Bl/6 mice (12 weeks old) (Charles River, Maastricht, the Netherlands) were housed in a temperature and humidity-controlled environment and were fed a high-fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) with free access to water for 10 weeks to induce insulin resistance¹⁸. All

animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Expression and Purification of CNTO736. CNTO736 was constructed by fusing a GLP-1 peptide analogue to a flexible Gly/Ser linker and a fragment of a VH domain linked directly to the CH2 and CH3 domains of an Fc¹³. A gene encoding CNTO736 was cloned into a vector for mammalian expression under control of the CMV promoter. For transient expression, HEK 293E cells were expanded (DMEM (Invitrogen, Carlsbad, CA, USA) + 10% FBS) and used to seed a 10-tier cell factory (5 x 10⁷ cells in growth medium (1200 ml)). 24 h after seeding, the cells were transfected. 24 h later, the growth medium/transfection mix was removed and replaced with 293-SFMII medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5 mmol/l sodium butyrate (1200 ml). Four days later, the conditioned medium was harvested, filtered, and stored at 4°C until purification. CNTO736 was purified using a Protein A MabSelect column (GE Healthcare, Piscataway, NJ, USA) and Immunopure Gentle Ag/Ab binding and elution buffers (Pierce, Rockland, IL, USA). The purified product was dialyzed into 20 mmol/l Tris, pH 7.4 prior to concentration. The final column was a Superdex 200 column (GE Healthcare, Piscataway, NJ, USA) in phosphate buffered saline (PBS). Selected fractions were pooled and concentrated.

Treatments. Mice enrolled in the *acute* study of CNTO736 and Ex-4 (generic name: exenatide) were divided into 4 groups. Each group received a single i.p. injection of CNTO736 (0.1 or 1.0 mg/kg, dissolved in PBS), Ex-4 (7.1 µg/kg, dissolved in PBS. This dose of Ex-4 is on a molar base equivalent to 0.1 mg/kg CNTO736) (Sigma-Aldrich, Zwijndrecht, The Netherlands), or PBS in a volume of 100 µl at 08.00 a.m. at the end of the 10 week high-fat diet period. The clamp studies began exactly 1 h later. Mice enrolled in the *chronic* study of CNTO736 and Ex-4 were matched for body weight and fasting plasma glucose concentration after 6 weeks of high-fat-feeding, where after they were divided into 4 groups. Each group received daily i.p. doses of CNTO736 (0.1 or 1.0 mg/kg), Ex-4 (7.1 µg/kg), or PBS in a volume of 100 µl at 08.00 a.m. during the remaining 4 weeks on diet. At 08.00 a.m. on the last day of the 10 week high-fat diet period, animals were given a last i.p. dose and the clamp studies were initiated 1 h later.

Hyperinsulinemic-euglycemic clamp study. Mice were fasted for 10 h with food withdrawn at 23:00 p.m. the day prior to the start of the study. Hyperinsulinemic-euglycemic clamps started 1 h after the last dose. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-

Cilag, Tilburg, The Netherlands). First, basal rates of glucose and glycerol turnover were determined by giving a primed (p) continuous (c) i.v. infusion of D-[U- ^{14} C]Glucose (p: 0.2 μ Ci; c: 0.3 μ Ci/h, GE Healthcare, Little Chalfont, U.K.) and [1-(3)- ^{3} H]Glycerol (p: 0.6 μ Ci; c: 0.9 μ Ci/h, GE Healthcare) for 60 min. Subsequently, insulin was administered in a primed (4.5 mU) continuous (6.8 mU/h) i.v. infusion for 90 min to attain steady state circulating insulin levels of $\sim$ 4 ng/ml. A variable i.v. infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding ($< 3 \mu$ l) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 μ l) were taken via tail bleeding during the basal period (after 50 and 60 min) and during the clamp period (after 70, 80, and 90 min) to determine plasma concentrations of glucose, NEFA, insulin, glycerol, and plasma D-[U- ^{14} C]Glucose and [1-(3)- ^{3} H]Glycerol specific activities. At the end of the clamp, mice were used to determine VLDL-production.

VLDL production. VLDL production was determined in basal conditions (1h after the last treatment) and in hyperinsulinemic conditions (after the clamp experiment) in parallel experiments. Mice were fasted for 10h with food withdrawn at 23:00 p.m. the day prior to the start of the study. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag). At t=0 min blood was taken via tail bleeding and mice were i.v. injected with 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline, which completely blocks VLDL clearance from serum¹⁹. Additional blood samples were taken at t=10, 20, 40, and 60 min after tyloxapol injection and used for determination of plasma TG concentration. Plasma TG concentrations were related to body weight and hepatic VLDL-TG production rates were calculated from the linear increase in TG in time and expressed as μ mol/h/kg. After the last sampling mice were sacrificed by cervical dislocation and livers were immediately removed from the mice and snap-frozen in liquid nitrogen for determination of hepatic TG content.

Analytical procedures. Plasma levels of glucose, NEFA, TG, and glycerol were determined using commercially available kits (Instruchemie, Delfzijl, The Netherlands). Plasma insulin (Mercodia AB, Uppsala, Sweden) and glucagon (Alpco, Salem, NH, USA) concentrations were measured by ELISA. Due to limited plasma, samples were pooled for glucagon measurements. Total plasma ^{14} C-glucose and ^{3} H-glycerol were determined in 7.5 μ l plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Hepatic TG content. A small piece of liver was homogenated in 400 μ l PBS and 1.5 ml $\text{CH}_3\text{OH}:\text{CHCl}_3$ (2:1, v/v) was added. After centrifugation, TG was extracted from the supernatant with CHCl_3 and H_2O (1:1, v/v) and the CHCl_3 phase was dried. TG was dissolved in H_2O with 2% Triton-X100 (Sigma). TG levels were assayed as described above.

Calculations. The turnover rates of glucose and glycerol ($\mu\text{mol}/\text{min}/\text{kg}$) were 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 D-[U- ^{14}C]Glucose or [1-(3)- ^3H]Glycerol (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. All metabolic parameters were expressed per kg of body weight.

Statistical analysis. Differences between groups were determined with the Kruskal–Wallis non-parametric test for k independent samples. When significant differences were found, the

Mann–Whitney non-parametric test was used as a follow-up test to determine differences between two independent groups. A p -value of less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD.

Results

Plasma metabolites and body weight. Body weight, plasma glucose, NEFA, insulin, glucagon, and glycerol before chronic treatment and in basal and hyperinsulinemic conditions after acute and chronic treatment are shown in table 1.

Acute effects of a single injection

The fasting plasma glucose concentration was significantly reduced by a single dose of CNT0736 (1.0 mg/kg). Similarly, Ex-4 and a lower dose of CNT0736 (0.1 mg/kg) decreased fasting blood glucose, although the difference did not reach statistical significance. Other fasting metabolite concentrations (NEFA, insulin, glucagon, and glycerol) were not affected by any single injection.

In the hyperinsulinemic state, insulin levels were slightly, but significantly elevated after a single dose of Ex-4 compared to control mice, although insulin infusion rates were identical and glucose concentrations were clamped at a similar level in all groups. Perhaps as a result, plasma NEFA concentrations were slightly more suppressed in

response to insulin infusion in mice treated with Ex-4 compared to control, whereas CNTO736 did not affect circulating NEFA levels during hyperinsulinemia.

Effects of 4 weeks of daily injections

Chronic administration of both drugs significantly reduced the fasting plasma glucose concentration compared to baseline values, while glucose levels remained high in the control group. The other plasma parameters did not differ between groups.

In the steady state clamp condition, insulin and glucose concentrations were similar in all groups and hyperinsulinemia suppressed NEFA levels to a similar extent in all groups. Body weight was significantly reduced by chronic administration of the highest dose of CNTO736 only.

Table 1. Plasma parameters in mice before chronic treatment and in basal or hyperinsulinemic conditions after a single or chronic i.p. injection of CNTO736, exendin-4 (Ex-4), or vehicle.

		Acute Study				Chronic Study			
		Control	Ex-4	CNTO736		Control	Ex-4	CNTO736	
				0.1 mg/kg	1.0 mg/kg			0.1 mg/kg	1.0 mg/kg
Body weight (g)	Start treatment	-	-	-	-	26.7 ± 1.2	27.0 ± 1.9	26.6 ± 1.7	26.8 ± 1.4
	10 wks diet/ after treatment	30.9 ± 2.4	31.4 ± 0.9	31.3 ± 1.6	31.2 ± 2.2	27.3 ± 1.8	26.3 ± 2.0	26.1 ± 1.9	25.1 ± 1.3 ^{††}
Glucose (mM)	Start treatment	-	-	-	-	6.5 ± 0.8	6.8 ± 0.8	6.7 ± 1.1	6.6 ± 1.4
	Basal	6.3 ± 2.4	4.6 ± 1.1	5.5 ± 1.2	4.4 ± 1.0*	6.0 ± 1.0	4.9 ± 1.1 ^{††}	4.3 ± 1.0 ^{††}	4.1 ± 0.8 ^{††}
	Hyperinsulinemic	7.3 ± 1.0	7.7 ± 0.9	7.3 ± 0.5	8.0 ± 0.8	6.9 ± 0.9	7.3 ± 0.9	7.6 ± 1.1	7.6 ± 0.9
NEFA (mM)	Start treatment	-	-	-	-	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
	Basal	1.3 ± 0.6	1.0 ± 0.2	1.1 ± 0.5	1.1 ± 0.5	1.1 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.2
	Hyperinsulinemic	0.7 ± 0.3	0.5 ± 0.2*	0.7 ± 0.3	0.6 ± 0.4	0.5 ± 0.2	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Insulin (ng/ml)	Start treatment	-	-	-	-	1.1 ± 0.4	1.0 ± 0.5	1.0 ± 0.5	1.1 ± 0.4
	Basal	1.1 ± 0.8	0.7 ± 0.5	1.1 ± 0.8	0.9 ± 0.6	1.0 ± 0.7	1.4 ± 0.7	1.1 ± 0.6	1.4 ± 1.3
	Hyperinsulinemic	3.1 ± 1.1	4.4 ± 0.8*	3.5 ± 1.3	4.5 ± 1.7	5.4 ± 2.4	4.4 ± 0.9	4.6 ± 1.0	5.0 ± 1.9
Glucagon (pg/ml)	Start treatment	-	-	-	-	491	502	551	580
	Basal	556	513	455	592	565	787	699	675
	Hyperinsulinemic	569	509	476	670	575	631	580	632
Glycerol (mM)	Start treatment	-	-	-	-	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0
	Basal	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
	Hyperinsulinemic	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

Values represent mean ± SD for at least 8 mice per group. * $p < 0.05$ vs control. ^{††} $p < 0.01$ vs start treatment.

Glucose turnover*Acute effects of a single injection*

A single dose of CNTO736 (1.0 mg/kg) inhibited endogenous glucose production (EGP) in the basal condition. Ex-4 reduced EGP to a similar extent, but the difference with control did not reach statistical significance. The lowest dose of CNTO736 (0.1 mg/kg) did not affect glucose metabolism in basal conditions (control: 53 ± 13 ; Ex-4: 40 ± 11 ; 0.1 mg/kg CNTO736: 47 ± 10 ; 1.0 mg/kg CNTO736: 39 ± 11 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.05$; figure 1).

The high dose of CNTO736 (1.0 mg/kg) and Ex-4 significantly increased the rate of glucose infusion necessary to maintain euglycemia during the insulin infusion relative to the control (control: 52 ± 14 ; Ex-4: 75 ± 13 , $p < 0.05$; 0.1 mg/kg CNTO736: 63 ± 24 ; 1.0 mg/kg CNTO736: 75 ± 16 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.05$; figure 2). This was largely because both drugs enhanced the ability of insulin to stimulate glucose uptake (control: 54 ± 13 ; Ex-4: 72 ± 17 , $p < 0.05$; 0.1 mg/kg CNTO736: 69 ± 20 ; 1.0 mg/kg CNTO736: 76 ± 25 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.05$). In contrast, neither drug acutely affected insulin's inhibitory action on EGP (control: 89 ± 12 ; Ex-4: 90 ± 14 ; 0.1 mg/kg CNTO736: 84 ± 14 ; 1.0 mg/kg CNTO736: 79 ± 19 % from basal; figure 4). The lowest dose of CNTO736 did not affect insulin action in this experimental context.

Effects of 4 weeks of daily injections

Chronic dosing of CNTO736 (1.0 and 0.1 mg/kg) significantly inhibited EGP in basal condition. In contrast, Ex-4 did not affect EGP in the fasting condition (control: 50 ± 8 ; Ex-4: 51 ± 18 ; 0.1 mg/kg CNTO736: 35 ± 9 , $p < 0.05$; 1.0 mg/kg CNTO736: 39 ± 9 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.05$; figure 1).

Chronic administration of CNTO736 and Ex-4 increased the rate of glucose infusion necessary to maintain euglycemia during insulin infusion ((control: 47 ± 18 ; Ex-4: 88 ± 16 , $p < 0.05$; 0.1 mg/kg CNTO736: 80 ± 12 , $p < 0.05$; 1.0 mg/kg CNTO736: 89 ± 16 $\mu\text{mol}/\text{min}/\text{kg}$, $p < 0.05$; figure 2). Chronic administration of both 1.0 mg/kg CNTO736 and Ex-4 enhanced insulin stimulated glucose disposal relative to control (CNTO736: 84 ± 19 ; Ex-4: 83 ± 19 ; control: 61 ± 15 $\mu\text{mol}/\text{min}/\text{kg}$). The lowest dose of CNTO736 (0.1 mg/kg) did not significantly affect glucose uptake during hyperinsulinemia relative to the control (CNTO736: 74 ± 15 ; control: 61 ± 15 $\mu\text{mol}/\text{min}/\text{kg}$). However, if expressed as percentage increase of baseline values, 0.1 mg/kg CNTO736 significantly increased glucose disposal during hyperinsulinemia (control: 30 ± 36 ; Ex-4: 76 ± 49 , $p < 0.05$; 0.1 mg/kg CNTO736: 125 ± 69 , $p < 0.01$; 1.0 mg/kg CNTO736: 132 ± 77 % from basal, $p < 0.01$; figure 3). All chronic interventions significantly reinforced insulin's capacity to inhibit EGP (control: 80 ± 10 ; Ex-4: 98 ± 4 , $p < 0.05$; 0.1 mg/kg CNTO736: 95 ± 10 , $p < 0.05$; 1.0 mg/kg CNTO736: 91 ± 18 % from basal, $p < 0.05$; figure 4).

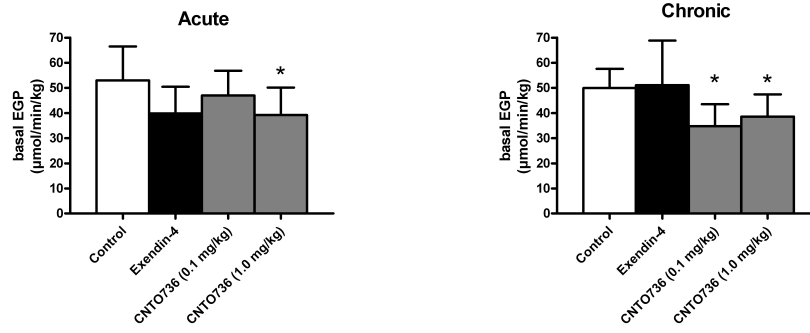


Figure 1. Basal endogenous glucose production (EGP) in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD for at least 8 mice per group. * p < 0.05 vs control. ** p < 0.01 vs control

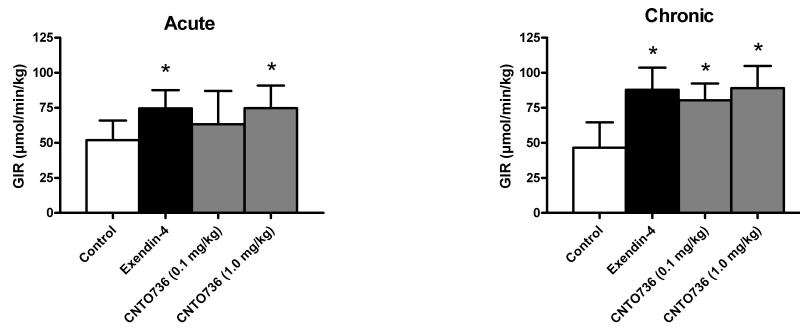


Figure 2. Glucose infusion rate (GIR) in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD for at least 8 mice per group. * p < 0.05 vs control.

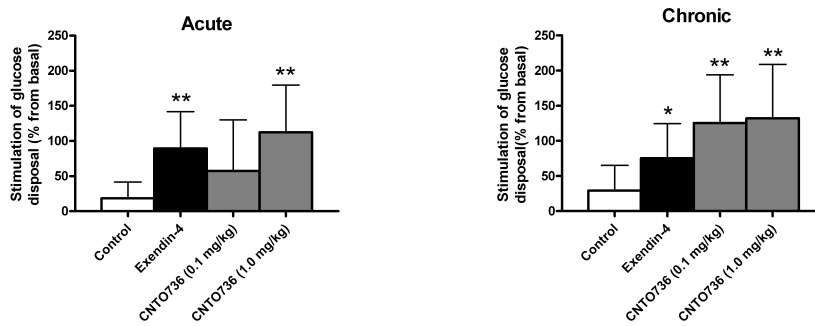


Figure 3. Stimulation of glucose disposal by insulin in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD for at least 8 mice per group. * p < 0.05 vs control. ** p < 0.01 vs control

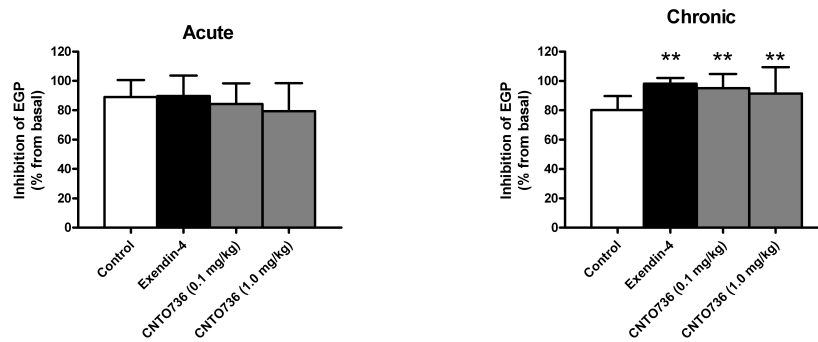


Figure 4. Inhibition of endogenous glucose production (EGP) by insulin in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle during a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD for at least 8 mice per group. ** $p < 0.01$ vs control.

Glycerol turnover

Acute effects of a single injection

Basal rates of glycerol turnover were not different after a single dose of CNTO736, Ex-4, or vehicle. None of the treatments affected insulin's ability to inhibit glycerol turnover (figure 5).

Effects of 4 weeks of daily injections

Chronic administration of CNTO736 and Ex-4 did not affect glycerol turnover in basal conditions. Hyperinsulinemia suppressed glycerol turnover to a similar extent in all groups (figure 5).

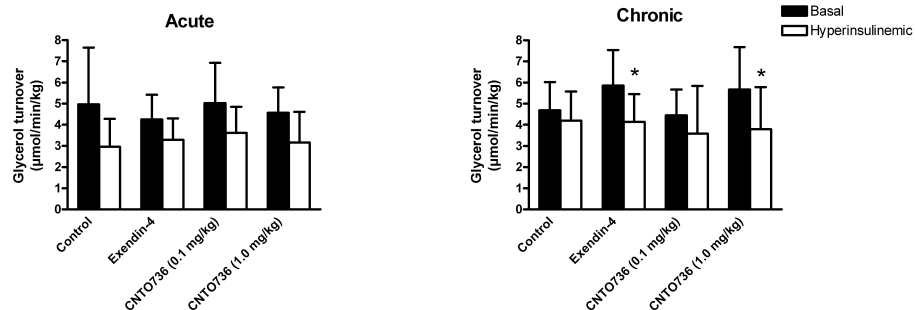


Figure 5. Glycerol turnover in mice that received acute or chronic i.p. injection of CNTO736, exendin-4, or vehicle before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic euglycemic clamp. Values represent mean $\pm$ SD for at least 8 mice per group. * $p < 0.05$ vs basal.

VLDL production

Acute effects of a single injection

VLDL production after one single injection was similar in all groups both in basal and hyperinsulinemic conditions (table 2).

Table 2. Body weight, plasma TG levels and VLDL production rate under basal or hyperinsulinemic conditions in mice that received a single or chronic i.p. injection of CNTO736 (0.1 or 1.0 mg/kg), exendin-4 (Ex-4), or vehicle.

		Acute Study				Chronic Study			
		Control	Ex-4	CNTO736		Control	Ex-4	CNTO736	
				0.1 mg/kg	1.0 mg/kg			0.1 mg/kg	1.0 mg/kg
Body weight (g)	Start treatment	-	-	-	-	27.0 ± 1.1	27.2 ± 1.7	27.3 ± 1.8	26.8 ± 1.3
	10 wks diet/ end treatment	31.6 ± 2.5	31.4 ± 1.9	31.5 ± 1.8	31.5 ± 2.5	27.5 ± 1.6	26.6 ± 1.8	26.6 ± 1.8	25.2 ± 1.2 ^{††}
TG (mM)	Start treatment	-	-	-	-	0.79 ± 0.3	0.77 ± 0.2	0.78 ± 0.2	0.81 ± 0.3
	10 wks diet/ end treatment	0.39 ± 0.1	0.35 ± 0.1	0.34 ± 0.1	0.39 ± 0.1	0.56 ± 0.2 ^{††}	0.52 ± 0.1 ^{††}	0.53 ± 0.1 ^{††}	0.52 ± 0.1 ^{††}
VLDL production rate (μmol/h/kg)	Basal	229 ± 61	237 ± 16	205 ± 34	220 ± 48	216 ± 36	212 ± 55	160 ± 30*	157 ± 23*
	Hyperinsulinemic	190 ± 45	202 ± 47	196 ± 45	186 ± 32	176 ± 51	179 ± 40	117 ± 37*	111 ± 32*

Values represent mean ± SD for at least 8 mice per group. * $p < 0.05$ vs control and exendin-4. ^{††} $p < 0.01$ vs start treatment.

Effects of 4 weeks of daily injections

Chronic treatment decreased plasma TG levels to a similar extent in all groups compared to baseline values (table 2). Chronic administration of CNTO736 (0.1 and 1.0 mg/kg) led to a significant decrease in VLDL production both in basal and in hyperinsulinemic conditions, whereas chronic Ex-4 treatment did not affect VLDL production (table 2 and figure 5).

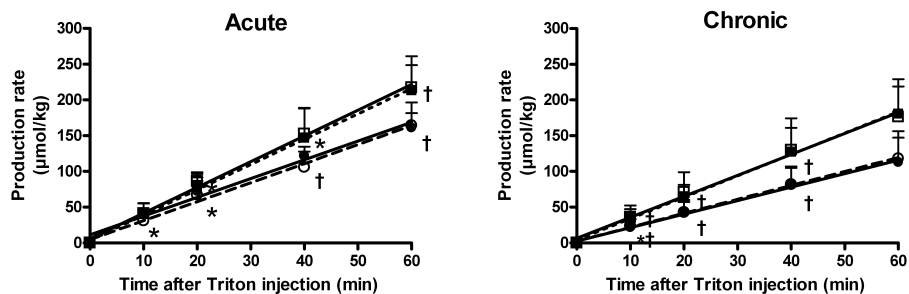


Figure 6. VLDL production rate in mice that received chronic i.p. administration of CNTO736, exendin-4, or vehicle in basal (a) or hyperinsulinemic (b) conditions. white squares = control; black squares = exendin-4; white circles = CNTO736 (0.1 mg/kg); black circles = CNTO736 (1.0 mg/kg). Values represent mean ± SD for at least 9 mice per group. * $p < 0.05$ vs control and vs exendin-4. ** $p < 0.05$ vs control. *[†] $p < 0.05$ vs exendin-4.

Hepatic TG content

Hepatic TG content did not differ between groups (control: 118 ± 10 ; Ex-4: 112 ± 14 ; 0.1 mg/kg CNTO736: 113 ± 27 ; 1.0 mg/kg CNTO736: 120 ± 29 $\mu\text{g TG}/\text{mg protein}$).

Discussion

Here we show that CNTO736, a novel GLP-1 receptor agonist, beneficially affects glucose and VLDL metabolism in diet-induced insulin resistant C57Bl/6 mice. In particular, a single injection of the highest dose of CNTO736 (1.0 mg/kg) acutely reduced fasting plasma glucose concentration and inhibited basal endogenous glucose production (EGP). Moreover, the capacity of insulin to stimulate glucose disposal was acutely reinforced by CNTO736. Chronic administration also clearly reduced plasma glucose levels and EGP during the fasting state and reinforced insulin's action on glucose disposal and production. Moreover, chronic treatment with CNTO736 significantly inhibited VLDL production. Ex-4 had a similar impact on glucose disposal and production in the hyperinsulinemic state, indicating that these effects are likely attributable to common GLP-1-like actions of the compounds. However, chronic Ex-4 treatment did not affect basal EGP or VLDL production, suggesting that CNTO736 and Ex-4 differentially act to modulate metabolism. The half-life of CNTO736 (15-20 h) in mice is considerably longer than that of Ex-4 (~ 2 h)¹³. At this time, we do not know whether the difference between Ex-4 and CNTO736 on fasting EGP and VLDL production is due to sustained exposure of CNTO736 because of its longer plasma half-life or due to some other unique characteristic that is specific to CNTO736. However, Ex-4 and CNTO736 stimulated glucose disposal and inhibited glucose production in hyperinsulinemic conditions to a similar degree, which suggests that compound kinetics cannot fully explain the CNTO736-specific effect on fasting EGP and VLDL production.

The data corroborate earlier reports indicating that GLP-1 and its analogues in the long run ameliorate whole body insulin resistance in obese animal models²⁰⁻²² and in T2DM patients²³. They further extend our knowledge of the precise actions of GLP-1 analogues on distinct components of glucose flux in insulin resistant animals, inasmuch as they show that these compounds acutely reinforce insulin's ability to promote glucose disposal and boost insulin action on both glucose uptake and production in the long term.

Interestingly, the data also clearly show that CNTO736 inhibits VLDL production both in the fasting state and during hyperinsulinemia, whereas Ex-4 does not. To our knowledge, this is the first report of a GLP-1 analogue affecting VLDL synthesis. Native GLP-1 reduces postprandial plasma triglyceride levels in healthy, normal-weight humans and T2DM patients, and vildagliptin, a DPP-4 inhibitor that increases plasma GLP-1 levels,

lowers the postprandial concentration of triglyceride-rich chylomicron particles in type 2 diabetic patients¹⁵⁻¹⁷. T2DM is a complex metabolic disorder, frequently marked by more anomalies than hyperglycemia alone. Overproduction of VLDL by the liver is the proximate cause of hypertriglyceridemia in patients with T2DM¹⁴, and hypertriglyceridemia contributes significantly to cardiovascular risk²⁴. Thus, the capacity to inhibit VLDL production is a favorable quality of drugs designed to treat T2DM.

The mechanisms underlying the beneficial impact of GLP-1 analogues on glucose metabolism remain to be established. They facilitate glucose induced insulin release by beta cells of course, which dampens postprandial hyperglycemia and thereby ameliorates glucose toxicity. The effects of chronic administration of both CNTO736 and Ex-4 on glucose disposal and production are conceivably at least in part attributable to this feature of the drugs. However, the impact of a single drug dose on glucose metabolism clearly indicates that other, direct mechanisms are involved. CNTO736 and Ex-4 may act on peripheral tissues directly via the GLP-1 receptor (GLP-1R). However, it is unclear if the liver expresses GLP-1R, since there is conflicting data^{25, 26}. CNTO736 and Ex-4 may reinforce insulin's action via neural routes that have recently emerged as key players in the control of glucose and lipid metabolism²⁷. GLP-1 receptors that mediate its anorexigenic action are expressed in multiple hypothalamic nuclei and peripheral dosing of CNTO736 could have allowed the molecule to reach the hypothalamus¹³. One possibility is that CNTO736 and Ex-4 in the hypothalamus modulate the NPY pathway. It has been shown previously that intracerebroventricular (i.c.v.) administration of GLP-1 completely prevents the orexigenic effects of neuropeptide Y (NPY), which suggests that GLP-1 acts by blocking NPY transmission to inhibit food consumption²⁸. I.c.v. administration of NPY acutely impairs insulin's ability to suppress EGP²⁹. Also, animal models of obesity and type 2 diabetes (including diet-induced obesity) are marked by elevated NPY expression in hypothalamic nuclei^{30, 31}. For these reasons, it is conceivable that these exceedingly active NPY neurons are involved in the pathophysiology of enhanced EGP in the face of hyperinsulinemia in these models (and obese humans). Peptide YY₃₋₃₆, which inhibits NPY release in the arcuate nucleus, enhances insulin sensitivity in the same experimental context³². Therefore, chronic administration of CNTO736 may have antagonized NPY-induced insulin resistance to explain the findings presented here.

Alternatively, CNTO736 and Ex-4 may activate neurons in the nucleus of the solitary tract (NTS) in the brain stem via afferent vagal inputs to modulate glucose metabolism. GLP-1 evokes vagal afferent nerve activity to initiate a hepato-pancreatic reflex that is critically involved in the control of insulin release³³. It is conceivable, that vagal afferent output in this context is not limited to the pancreas, but affects liver and other visceral

organs as well to modulate glucose production. Moreover, in addition to its direct effects on hypothalamic neurons, GLP-1 conveys its message to the hypothalamus via the vagus nerve and the NTS³⁴. Indeed, vagal ablation attenuates the anorexigenic effects of peripheral GLP-1 administration³⁴. In analogy, activation of vagal afferents by GLP-1 may impact on glucose metabolism via the hypothalamus. Finally, it remains possible that GLP-1 acts on the liver and other peripheral tissues via a structurally and functionally distinct GLP-1R. Indeed, GLP-1 was reported to have insulin-like actions in liver and skeletal muscle which are not mediated by the classical GLP-1R^{35, 36}.

Chronic administration of CNTO736 clearly inhibited VLDL production in basal and hyperinsulinemic conditions. The assembly of VLDL particles in the endoplasmatic reticulum of hepatocytes is dependent on the intracellular presence of triglycerides, other lipids, and apolipoprotein B (apoB) as its major components. Correct apoB lipidation and translocation probably limit the rate of VLDL secretion³⁷. Thus, the supply of NEFA, released by lipolysis of circulating and stored triglycerides, to the liver is a major determinant of VLDL production³⁸. However, CNTO736 did not affect lipolysis or insulin's capacity to suppress this process and, accordingly, it did not change plasma NEFA concentrations. Moreover, chronic CNTO736 treatment did not impact on liver TG content. Therefore, CNTO736 does not appear to inhibit VLDL production via reduction of triglyceride lipolysis and diminution of NEFA supply to the liver.

CNTO736 may affect liver function via one of the mechanisms discussed above. ICV administration of NPY acutely impairs the capacity of insulin to inhibit VLDL production²⁹. This observation suggests that neural mechanisms are involved in the control of VLDL metabolism. Thus, in analogy with hypotheses pertaining to the effect of CNTO736 on glucose metabolism discussed above, CNTO736 may modulate VLDL release via neural routes that involve the hypothalamus, brain stem, and vagal nerve. It is unclear why chronic treatment with Ex-4 did not result in similar effects on basal EGP and VLDL production. In this context, it is interesting to note that Ex-4 may have less affinity for the vagal GLP-1R than GLP-1 itself³⁹. It is tempting to speculate, that this explains the difference in effect of chronic treatment with CNTO736 and Ex-4 on EGP and VLDL production.

The pathophysiological consequence of the capacity of CNTO736 to inhibit VLDL secretion is uncertain, since circulating TG levels were not affected. It is also important to note, that the beneficial impact of CNTO736 on VLDL production is not simply due to a reduction of body weight, since a robust effect was also evident after chronic treatment with the lowest dose of the drug, which did not impact on body weight.

The current study suggests that CNTO736 has properties to provide better metabolic control in patients with T2DM. Chronic administration of the drug clearly

reinforces insulin action on glucose disposal and production in insulin resistant mice. Moreover, chronic CNTO736 treatment inhibits fasting EGP and VLDL synthesis in the same experimental context. Administration of Ex-4 has similar effects on insulin mediated glucose metabolism, suggesting that common, GLP-1-like effects of these compounds underlie their glucoregulatory action. In contrast, Ex-4 treatment did not impact fasting EGP and VLDL production. This could imply that CNTO736 has a unique property that is responsible for inhibiting glucose production and VLDL release. We speculate that CNTO736 acts via GLP-1 receptors on vagal afferents and hypothalamic neurons to modulate glucose and VLDL metabolism.

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Chapter 4

GLP-1 Mimetibody, a GLP-1 receptor agonist, decreases
VLDL-TG production rate and plasma cholesterol
concentration in high-fat-fed APOE*3-Leiden mice

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In preparation

Abstract

GLP-1 Mimetibody (GLP-1M) is a glucagon-like peptide-1 (GLP-1) receptor agonist that incorporates a GLP-1 peptide analogue linked to the Mimetibody™ platform, which has been shown to inhibit VLDL triglyceride production. In the present study, we further evaluate the potential of this compound to modulate lipid metabolism. High-fat-fed APOE*3-Leiden transgenic mice were administered daily i.p. injections of GLP-1M or placebo for 4 weeks. Plasma cholesterol concentrations, lipoprotein profiles, VLDL-TG and VLDL-[³⁵S]apoB production rates and expression of genes involved in hepatic lipid metabolism were determined. GLP-1M lowered total plasma cholesterol levels (GLP-1M: 3.7 ± 0.6 ; Control: 4.6 ± 0.7 mM, $P < 0.05$) and lipoprotein fractionation showed increased cholesterol levels in the HDL fraction. Moreover, GLP-1M decreased the production rate of VLDL-TG (GLP-1M: 163 ± 33 ; Control 264 ± 49 $\mu\text{mol/kg/h}$, $P < 0.05$) and VLDL-[³⁵S]apoB (GLP-1M: $67 \times 10^3 \pm 18 \times 10^3$; Control: $107 \times 10^3 \pm 32 \times 10^3$ dpm/ml/h, $P < 0.05$). Finally, GLP-1M decreased the hepatic expression of *apob* (-36%, $P < 0.05$ vs. control). In conclusion, these results indicate that GLP-1M improves VLDL metabolism in high-fat-fed APOE*3-Leiden mice and suggest the potential to ameliorate dyslipidemia in patients with type 2 diabetes.

Introduction

Glucagon-like peptide-1 (GLP-1), an incretin hormone released by the gut in response to meals, stimulates insulin release, inhibits glucagon secretion, slows gastric emptying, and induces satiety¹⁻⁵. Although these features hold promise for the treatment of type 2 diabetes mellitus, native GLP-1 cannot be used as a drug because of its short plasma half-life (several minutes). GLP-1 analogues that are resistant to hydrolysis by dipeptidyl peptidase IV (DPP-IV) have been developed to circumvent this problem⁶. Indeed, clinical studies show that they effectively improve glucose metabolism in T2DM patients^{7, 8} and several compounds are currently on the verge of marketing as tools to treat the disease. Byetta™ (exendin-4 (Ex-4)) was approved in this context in 2005^{9, 10}.

Lipid metabolism is also disturbed in patients with T2DM. Increased very low density lipoprotein triglyceride (VLDL-TG) concentrations and decreased high-density lipoprotein (HDL) cholesterol in plasma significantly contribute to the long term risk of macrovascular disease in these patients^{11, 12}. We have recently shown that chronic treatment with the GLP-1 receptor agonist, CNTO736, apart from its glycemic effects, inhibits VLDL-TG production by the liver in C57Bl/6 mice maintained on a high fat diet¹³. Interestingly, Ex-4 did not impact on VLDL-TG production in this insulin resistant mouse model, despite the fact that both Ex-4 and CNTO736 equally and profoundly ameliorated glycemic control. Therefore, CNTO736 may have compound-specific effects on VLDL metabolism that potentially reinforce its capacity to prevent macrovascular disease in high fat-fed mice (and humans). However, several issues need to be clarified before firm conclusions can be drawn with respect to the potential of the drug to modify VLDL-TG metabolism and, thereby, reduce macrovascular risk.

The objective of this study was to further explore the inhibitory effect of CNTO736 on VLDL-TG production. We set out to compare the chronic effects of CNTO736 with the latest version of the molecule, from now on referred to as GLP-1 Mimetibody follow-up molecule (GLP-1M), and Ex-4 on lipid metabolism in APOE*3-Leiden transgenic mice. These mice express the human APOE*3-Leiden gene, resulting in a lipoprotein profile reminiscent of that of patients with dysbetalipoproteinemia, marked by elevated plasma cholesterol and triglyceride levels that are mainly confined to the VLDL/LDL-sized lipoprotein fraction¹⁴. In contrast to other mice, APOE*3-Leiden transgenics respond to various hypolipidemic drugs in a similar way as humans¹⁵ and are therefore considered a “human-like” animal model for studies evaluating the impact of drugs on hyperlipidemia and atherosclerosis^{14, 16, 17}.

Methods

Animals and diet. Male APOE*3-Leiden transgenic mice (10-11 weeks old) were housed in a temperature and humidity-controlled environment and were fed a high-fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) with free access to water for 12 weeks to induce insulin resistance¹⁸. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Drugs. GLP-1M was constructed by fusing a GLP-1 peptide analogue to a flexible Gly/Ser linker and a fragment of a V region heavy chain (VH) domain linked directly to the CH2 and CH3 domains of an Fc as described previously for CNTO736¹⁹. Ex-4 (molecular weight = 4186.57 g/mol) was purchased from Sigma (St. Louis, MO).

Treatment. After 8 weeks of high-fat-feeding, mice were divided into 6 groups, matched for body weight and fasting plasma triglyceride (TG) concentration. The groups received daily subcutaneous (s.c.) doses of GLP-1M (0.3, 0.1, or 0.03 mg/kg, dissolved in PBS), CNTO736 (0.3 mg/kg, dissolved in PBS), Ex-4 (7.1 µg/kg, dissolved in PBS), or PBS in a volume of 100 µl at 08.00 a.m. during the last 4 weeks on diet. At 08.00 a.m. on the last day of the 12 week high-fat diet period, animals were given a last s.c. dose and the experiments were initiated 7 hours later.

Plasma lipid and lipoprotein analysis. Blood was collected by tail bleeding into chilled paraoxon-coated capillary tubes (Sigma) to prevent ongoing *in vitro* lipolysis²⁰. The tubes were placed on ice and centrifuged, and the obtained plasma was snap-frozen in liquid nitrogen and stored at -20°C. Plasma was assayed for glucose, FFA, TG, and cholesterol using commercially available enzymatic kits (Instruchemie, Delfzijl, The Netherlands), and insulin concentrations were measured by ELISA (Mercodia AB, Uppsala, Sweden). Lipid distribution over plasma lipoproteins by fast-performance liquid chromatography was determined as described before²¹. Fractions of 50 µl were collected and assayed for TG and cholesterol as described above.

Hepatic VLDL particle production. Mice were fasted for 7 hours during the light period and anesthetized with an intraperitoneal injection of acepromazine 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). Mice received an intravenous injection of 100 µl PBS containing 150 µCi Tran³⁵S label (GE Healthcare, Little Chalfont, U.K.) which binds to newly produced

apolipoprotein B (apoB) required for the generation of liver-derived VLDL. After 30 min, the animals received a 15% (by volume) intravenous injection of Triton WR-1339 (500 mg/kg body weight; Tyloxapol, Sigma) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG²². Blood samples were drawn before (time point 0) and 10, 20, 40, and 60 min after injection. Plasma was assayed for TG as described above. After the last sampling, mice were sacrificed by cervical dislocation and exsanguinated via the retro-orbital plexus for isolation of VLDL. Livers were immediately removed from the mice and snap-frozen in liquid nitrogen for determination of hepatic lipid content. VLDL was quantitatively isolated from plasma after density gradient ultracentrifugation at $d < 1.006 \text{ g/ml}$ by aspiration²³. TG and total cholesterol were determined as described above. Phospholipids were determined using a standard commercial kit (Spinreact, Sant Esteve de Bas, Spain). VLDL-apoB was selectively precipitated with 2-propanol²⁴ and counted for incorporated ³⁵S.

Hepatic lipid content. Liver tissue samples were homogenized in PBS (approx. 10% wet w/v), and the protein content was measured according to the method of Lowry *et al*²⁵. For analysis of liver lipids, 0.4 ml aliquots of tissue were homogenized with 1.5 ml methanol-chloroform (2:1). The homogenate was then centrifuged (2,000 rpm for 5 min) and 0.5 ml chloroform and 0.5 ml distilled water were added to the liquid phase, and the samples were vortexed. Phase separation was accomplished by centrifugation (2,000 rpm for 5 min), where after the bottom phase was removed to a new tube and the samples were dried in a nitrogen flow at 50°C. The pellet was dissolved in 100 μl 2% Triton X-100 in chloroform. TG and cholesterol concentrations were determined as described above.

Real-time PCR. Real-time polymerase chain reaction (RT-PCR) was used to measure mRNA expression levels of liver genes known to play important roles in TG and cholesterol homeostasis (fatty acid synthase (*fas*), acetyl-Coenzyme A carboxylase alpha (*acaca*), *apob*, microsomal triglyceride transfer protein (*mttp*), ATP-binding cassette, sub-family A (ABC1), member 1 (*abca1*), sterol regulatory element binding protein 1 (*srebf1*), *srebf2*, farnesoid X-activated receptor (*fxr*), low density lipoprotein receptor (*ldlr*), diacylglycerol O-acyltransferase 1 (*dgat1*), cytochrome P450, family 7, subfamily a, polypeptide 1 (*cyp7a1*), liver X receptor α (*lxra*), *lxr\beta*, peroxisome proliferative activated receptor, alpha (*ppara*), *ppary*, and *acyl-Coenzyme A oxidase* (*acox1*). Therefore, 2 additional groups of APOE*3-Leiden mice were used, which were not subjected to Triton injection, to isolate livers. These animals were also maintained on a high fat diet for 12 weeks. During the last 4 weeks on diet, mice received daily s.c. injections of GLP-1M (0.3 mg/kg) or PBS. At the end of the treatment period of 4 weeks, mice were fasted for 7 hours during the light period, sacrificed by cervical dislocation, and liver samples were frozen in liquid nitrogen.

Total RNA was extracted from liver samples using Trizol. The amount of RNA was determined by Nanodrop. The quality was checked by the ratio of absorption at 260 nm and absorption at 280 nm. cDNA was synthesized from 2 µg of total RNA using RevertAid™ First Strand cDNA synthesis kit (Fermentas, Hanover, MD, U.S.A.). For RT-PCR, primer assays were ordered by Qiagen, Venlo, the Netherlands. PCR amplification was performed in a total reaction volume of 25 µl. The reaction mixture consisted of QuantiFast SYBR Green PCR Master Mix (Qiagen), Primer assay, nuclease free water, and cDNA. An identical cycle profile was used for all genes: 95°C for 5 min, followed by 95°C for 10 sec and 60°C for 30 sec 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/HPRT/Cyclophilin) and relative to the control samples is given by $2^{-\Delta\Delta Ct}$. For each gene, all samples were run together in duplex allowing relative comparisons of the samples of a given gene.

Statistical analysis. Differences between groups were determined with the Kruskal–Wallis non-parametric test for k independent samples. When significant differences were found, the Mann–Whitney non-parametric test was used as a follow-up test to determine differences between two independent groups. A p -value of less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD.

Results

Plasma metabolites, body weight, and hepatic content. Body weight, fasting plasma glucose, insulin, TG, and cholesterol concentrations are shown in table 1. Chronic treatment did not alter body weight in any group. Fasting plasma glucose concentration was significantly decreased for all drug-treated groups compared to baseline values after chronic administration. Insulin levels increased during the last 4 weeks on diet for the control group and GLP-1M (0.03 and 0.1 mg/kg), whereas no difference was found for the other groups. TG levels decreased in all treated groups compared to baseline values. However, significance was found for the lowest dose of GLP-1M only. The groups did not differ with respect to fasting plasma FFA levels. Plasma cholesterol levels significantly decreased after 4 weeks of treatment with 0.3 mg/kg GLP-1M and CNT0736 compared to baseline values.

Lipoprotein fractionation by fast performance liquid chromatography showed no differences in distribution for TG concentration (figure 1a). However, cholesterol concentration was considerably lower in the VLDL fraction of 0.3 mg/kg GLP-1M and CNT0736 treated animals compared to controls. In addition, cholesterol concentration

was increased in the HDL fraction for the GLP-1M and CNTO736 treated mice compared to controls (Figure 1b).

Hepatic TG and total cholesterol content did not differ between groups (table 2).

Table 1. Plasma parameters in APOE*3-Leiden mice at the start and after 2 and 4 weeks of chronic injections with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNTO736, or exendin-4.

	Control			GLP-1M (0.03 mg/kg)			GLP-1M (0.1 mg/kg)			GLP-1M (0.3 mg/kg)			CNTO736 (0.3 mg/kg)			Exendin-4		
	start	2 wks	4 wks	start	2 wks	4 wks	start	2 wks	4 wks	start	2 wks	4 wks	start	2 wks	4 wks	start	2 wks	4 wks
Body weight (g)	30.4 ± 3.5	30.3 ± 3.6	30.5 ± 3.5	30.8 ± 2.8	30.8 ± 2.8	31.2 ± 3.3	29.9 ± 4.4	30.0 ± 4.0	29.8 ± 3.8	29.6 ± 2.1	29.2 ± 1.8	29.1 ± 1.7	31.6 ± 4.1	29.8 ± 4.0	29.7 ± 3.4	29.5 ± 2.9	29.3 ± 2.2	28.5 ± 1.9
Glucose (mM)	10.5 ± 2.0	11.0 ± 1.3	11.0 ± 0.9	9.8 ± 1.8	9.8 ± 1.1	8.2 ± 1.8*	10.1 ± 1.5	7.2 ± 2.0**	6.5 ± 1.9**	9.8 ± 1.5	6.6 ± 1.1**	6.0 ± 1.0**	10.2 ± 1.5	6.7 ± 1.6**	6.0 ± 1.1**	9.7 ± 1.3	8.3 ± 2.1	6.9 ± 1.7**
Insulin (ng/ml)	1.1 ± 0.2	1.5 ± 0.4*	1.8 ± 0.5*	1.0 ± 0.5	1.3 ± 0.6	1.8 ± 0.5*	0.9 ± 0.2	1.1 ± 0.4	1.3 ± 0.3*	0.9 ± 0.4	1.1 ± 0.5	1.2 ± 0.4	1.0 ± 0.6	0.8 ± 0.2	1.0 ± 0.4	0.9 ± 0.2	1.0 ± 0.3	1.0 ± 0.4
TG (mM)	1.1 ± 0.6	0.9 ± 0.7	0.9 ± 0.4	1.2 ± 0.4	0.9 ± 0.4	0.7 ± 0.3*	1.3 ± 0.5	1.2 ± 0.5	0.8 ± 0.3	1.3 ± 0.8	1.2 ± 0.4	0.9 ± 0.3	1.2 ± 0.5	1.1 ± 0.7	0.8 ± 0.2	1.3 ± 0.4	1.1 ± 0.4	0.8 ± 0.2
FFA (mM)	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	0.6 ± 0.1	0.7 ± 0.2	0.8 ± 0.4	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.2	0.7 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	0.9 ± 0.2
Cholesterol (mM)	5.0 ± 1.1	5.2 ± 1.0	4.6 ± 0.7	4.7 ± 1.2	4.9 ± 1.3	4.3 ± 1.2	4.5 ± 1.7	4.2 ± 1.1	3.9 ± 1.0	4.8 ± 0.8	4.5 ± 1.0	3.7 ± 0.6*	4.6 ± 0.8	4.0 ± 1.0	3.4 ± 1.0*	4.6 ± 0.7	4.8 ± 0.9	4.1 ± 0.9

Values represent mean ± SD for at least 8 mice per group. *P<0.05 vs. start treatment. **P<0.01 vs. start treatment.

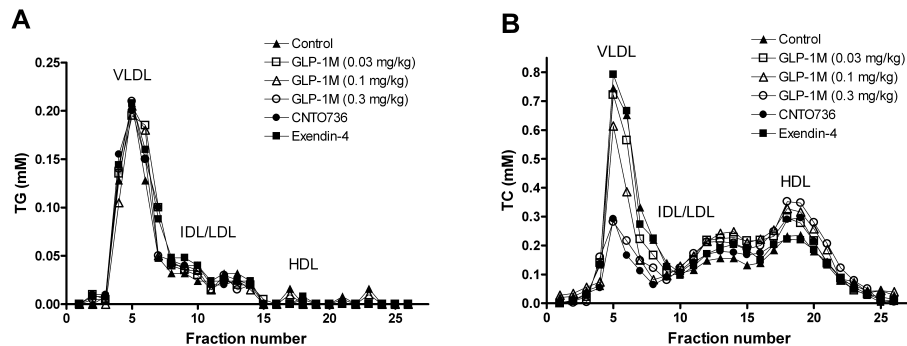


Figure 1. Effect of chronic treatment with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNTO736, or exendin-4 on the distribution of triglyceride (TG; A) and total cholesterol (TC; B) among lipoproteins in APOE*3-Leiden mice.

Table 2. Effect of chronic treatment with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNTO736, or exendin-4 on hepatic lipid content.

Intervention	Triglycerides ($\mu\text{g}/\text{mg}$ protein)	Total cholesterol ($\mu\text{g}/\text{mg}$ protein)
Control	563 $\pm$ 129	152 $\pm$ 50
GLP-1M (0.03 mg/kg)	490 $\pm$ 91	131 $\pm$ 22
GLP-1M (0.1 mg/kg)	478 $\pm$ 166	131 $\pm$ 29
GLP-1M (0.3 mg/kg)	486 $\pm$ 178	152 $\pm$ 56
CNTO736 (0.3 mg/kg)	534 $\pm$ 104	148 $\pm$ 26
Exendin-4	492 $\pm$ 120	155 $\pm$ 43

Values represent mean $\pm$ SD for at least 8 mice per group.

VLDL production. Chronic treatment with GLP-1M and CNTO736 reduced plasma TG concentrations after Triton injection, a measure of VLDL-TG production rate, compared to control animals. Ex-4 treatment had no effect whatsoever on VLDL production (figure 2). Analysis of the composition of the VLDL particles revealed no differences between groups (table 3). The *de novo* total apoB production rate in newly synthesized VLDL particles was significantly lower in mice treated with 0.3 mg/kg GLP-1M and CNTO736 compared to controls. No effect on apoB production was found for Ex-4 treated animals compared to controls (figure 3).

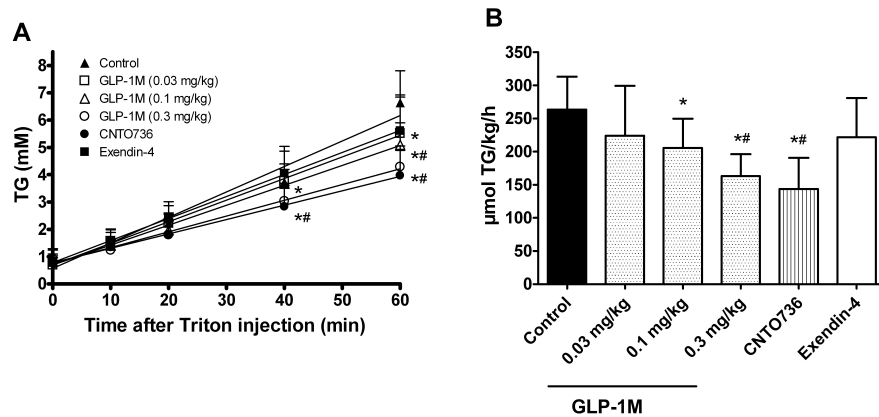
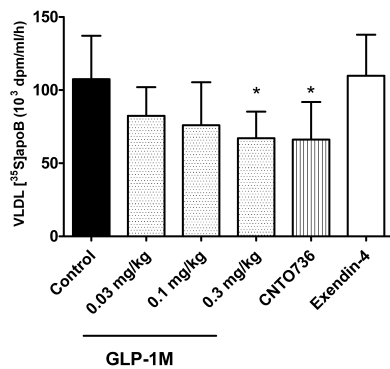
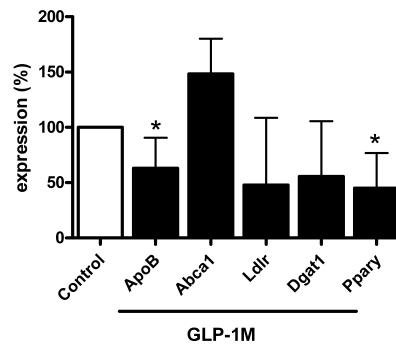


Figure 2. TG concentrations (A) and VLDL-TG production rate (B) in APOE*3-Leiden mice that received chronic injections with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNTO736, or exendin-4 after Triton injection. Values represent mean $\pm$ SD for at least 8 mice per group. * $P < 0.05$ vs. control. # $P < 0.05$ vs. exendin-4.

Table 3. Lipid composition of nascent VLDL of APOE*3-Leiden mice that received chronic injections with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNT0736, or exendin-4.

Intervention	Cholesterol	Triglycerides	Phospholipids
	% of total lipid		
Control	20 ± 3	67 ± 4	13 ± 2
GLP-1M (0.03 mg/kg)	21 ± 4	67 ± 5	12 ± 4
GLP-1M (0.1 mg/kg)	19 ± 5	68 ± 3	13 ± 2
GLP-1M (0.3 mg/kg)	17 ± 5	71 ± 7	12 ± 6
CNT0736 (0.3 mg/kg)	19 ± 5	73 ± 6	10 ± 5
Exendin-4	19 ± 5	68 ± 7	13 ± 2

Values represent mean ± SD for at least 8 mice per group.

**Figure 3.** VLDL-³⁵S]apoB production in APOE*3-Leiden mice that received chronic injections with vehicle, GLP-1M (0.03, 0.1, or 0.3 mg/kg), CNT0736, or exendin-4 after Triton injection. Values represent mean ± SD for at least 8 mice per group. *P<0.05 vs. control and exendin-4**Figure 4.** mRNA fold expression levels of different proteins in liver of APOE*3-Leiden mice that received chronic injections with vehicle or GLP-1M (0.3 mg/kg). Values represent mean ± SD for 6 mice per group. *P<0.05 vs. control.

Real-Time PCR. Chronic treatment with GLP-1M (0.3 mg/kg) resulted in a significant downregulation of *apob* and *pparγ* (figure 4). A trend towards downregulation was seen for *ldlr* ($p=0.09$) and *dgat1* ($p=0.09$) and a trend towards upregulation for *abca1* ($p=0.10$). However, the difference with the control group for these latter genes did not reach significance. No differences were found for the other genes ($p>0.30$).

Discussion

Here we show that GLP-1M, a GLP-1 receptor agonist, dose-dependently inhibits VLDL-TG release in diet-induced insulin resistant APOE*3-Leiden mice. The rate of appearance of apoB, an important component of VLDL particles, was diminished as well, indicating that the drug curtails the number of particles produced. The reduction of apoB output was corroborated by a decline of *apob* mRNA expression in the liver. The composition of VLDL particles was not affected by GLP-1M. Remarkably, Ex-4 did not impact on VLDL-TG production, although it did reduce plasma glucose levels to an extent that was similar to that brought about by both other drugs. Finally, chronic treatment with GLP-1M (but not Ex-4) decreased circulating (fasting) cholesterol levels and shifted the distribution of cholesterol among distinct plasma lipoprotein fractions toward HDL.

GLP-1M appears to inhibit VLDL-TG production in APOE*3-Leiden mice maintained on a high fat diet. Changes in VLDL-TG production are due to alterations in the amount of TG per VLDL particle, by the number of particles secreted (as reflected by apoB output), or both²⁶. The lipid composition of nascent VLDL did not differ between groups. However, the VLDL-apoB production rate was significantly decreased by GLP-1M. These data suggest that GLP-1M particularly inhibits the production of particles, leaving their composition unaffected. Interestingly, overproduction of VLDL particles of normal size and composition is the hallmark of diabetic dyslipidemia in humans¹¹.

The effect of GLP-1M on VLDL-TG production may be attributable to a direct effect of the drug on *apob* gene expression. The availability of apoB and substrate play key roles in the control of VLDL production²⁷ and insulin inhibits VLDL particle assembly at multiple levels^{28, 29}. GLP-1M did not reduce hepatic triglyceride stores to explain its impact on VLDL particle production. Our high-fat-fed mice were insulin resistant, as reflected by high plasma glucose and insulin levels in fasting condition, and insulin resistance is associated with increased VLDL-apoB production in mice and humans^{30, 31}. The mechanistic details of insulin action on apoB metabolism are not completely known, but may involve the modulation of apoB mRNA translation³². Thus, it is conceivable, that GLP-1M inhibits the production of VLDL particles in high-fat-fed mice by rescuing hepatic insulin sensitivity, thereby reducing the efficacy of apoB mRNA translation. However, Ex-4 did not affect VLDL-TG production, while it reduced circulating glucose concentrations to a similar extent as GLP-1M did. Moreover, in a previous study we showed that CNT0736 inhibits VLDL-TG release whereas Ex-4 (in a dose of 7.1 µg/kg similar to the dose used in the present study) does not, even though both drugs have similar effects on insulin sensitivity of glucose metabolism¹³. It needs to be emphasized, that our current data suggest that the dose of Ex-4 we used may have been too low to fully mimic the impact of

the highest dose of GLP-1M on insulin sensitivity, given the (not significantly) lower plasma glucose levels during GLP-1M treatment. Nevertheless, we consider it unlikely that GLP-1M inhibits VLDL-TG production *primarily* through its beneficial effect on insulin action. GLP-1M clearly diminished *apob* expression in the liver. We speculate that this pharmacological feature of the drug is primarily responsible for its effect on VLDL particle production. Indeed, GLP-1 was shown to inhibit lymphatic apoB output, supporting a role of this peptide in the control of apoB production³³. The lack of effect of Ex-4 in this context warrants further study.

The inhibition of VLDL-TG production by GLP-1M did not result in decreased plasma TG levels, which is also true for CNT0736¹³. Thus, other mechanisms that determine plasma VLDL-TG levels (e.g. rates of endothelial binding and subsequent lipolysis by LPL, and/or the rates of removal of small VLDL from the circulation or its conversion to IDL) must have been affected by GLP-1M as well. The drug did not affect the distribution of TG among the various lipoprotein subfractions, suggesting that it does not impact on VLDL to IDL conversion. Various factors, including the triglyceride and apoE content of VLDL particles, modulate epithelial binding and subsequent lipolysis³⁴. Although the TG content of VLDL particles was not affected by GLP-1M treatment, the drug may have modified other features of VLDL particles that determine clearance. Also, uptake of small VLDL particles by the liver may have been affected. Obviously, evaluation of these hypotheses requires further study.

Chronic treatment with 0.3 mg/kg GLP-1M and CNT0736 decreased total cholesterol levels in plasma, and Ex-4 (in the current dose) did not appear to exert this effect. Cholesterol is removed from the circulation by uptake of lipoproteins in various tissues via receptor mediated pathways³⁵. Uptake in the liver occurs primarily via the LDL receptor. Subsequently, cholesterol enters the entero-hepatic bile acid circulation to be partly disposed of via the faeces. *Ldlr* expression tended to be *diminished* by GLP-1M, suggesting that clearance via this receptor does not explain the drug's effect on circulating cholesterol concentrations. Alternatively, excess cholesterol was directed towards the liver via HDL lipoproteins to be disposed of via bile. Hepatic cholesterol content was not affected by GLP-1M, suggesting that increased supply of (HDL) cholesterol was offset by enhanced removal via the bile³⁶. Preliminary studies indicate that both sterol and bile acid secretion were increased indeed by chronic treatment with GLP-1M (Parlevliet *et al.*, data not shown).

GLP-1M (and CNT0736) shifted the distribution of cholesterol among distinct lipoprotein fractions toward HDL, suggesting that the drug somehow promotes efficient clearance of cholesterol from chylomicrons, VLDL, and IDL by extrahepatic tissues to form mature HDL. Again, Ex-4 in the current dose does not appear to exert this effect. The

export of hepatic cholesterol towards nascent HDL by ABCA1 is rate-limiting in the process of HDL formation³⁷. *Abca1* expression in the liver was increased in GLP-1M treated animals as compared with placebo treated controls, but the difference did not reach statistical significance. HDL metabolism is also controlled by the concerted impact of a number of plasma proteins such as phospholipid transfer protein, lecithin-cholesterol acyltransferase, endothelial-bound enzymes LPL and hepatic lipase, and GLP-1M may modulate the production and/or activity of these peptides to shift cholesterol in plasma from VLDL towards HDL lipoproteins³⁸⁻⁴⁰.

Our data suggest that GLP-1M may have powerful anti-atherogenic properties in insulin resistant subjects. Atherosclerosis is driven by increased (VLDL) cholesterol and triglyceride levels, often accompanied by low circulating HDL concentrations⁴¹. HDL particles are anti-atherogenic, partly because of their role in reverse cholesterol transport, but also because of their anti-oxidative, anti-inflammatory, anti-thrombotic, and anti-apoptotic properties⁴²⁻⁴⁴. Therefore, the reduction of plasma cholesterol concentration by GLP-1M as well as its capacity to increase HDL cholesterol levels holds promise as pharmacological features preventing atherosclerosis in high risk metabolic conditions.

The current study suggests that GLP-1M has properties not reported for a GLP-1 analogue thus far. Chronic administration of the drug in high-fat-fed APOE*3-Leiden mice clearly inhibited VLDL-TG production. Moreover, chronic GLP-1M administration decreased total cholesterol levels and increased HDL in the same experimental context. Chronic administration with Ex-4 did not impact on VLDL or cholesterol metabolism, suggesting that the GLP-1M effects on lipid metabolism are not mediated via its impact on insulin sensitivity.

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Chapter 5

GLP-1 treatment reduces endogenous insulin resistance
via activation of central GLP-1 receptors in mice fed a
high-fat diet

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Abstract

Glucagon-like peptide-1 (GLP-1) improves insulin sensitivity in humans and rodents. It is currently unknown to what extent the (metabolic) effects of GLP-1 treatment are mediated by central GLP-1 receptors. We studied the impact of central GLP-1 receptor (GLP-1R) antagonism on the metabolic effects of peripheral GLP-1 administration in mice. High-fat-fed insulin resistant C57Bl/6 mice were treated with continuous subcutaneous infusion of GLP-1 or saline (PBS) for 2 weeks, while the GLP-1R antagonist exendin-9 (EX-9) or cerebral spine fluid (CSF) were simultaneously infused in the left lateral cerebral ventricle (icv). Glucose and glycerol turnover were determined during a hyperinsulinemic euglycemic clamp. VLDL-triglyceride (VLDL-TG) production was determined in hyperinsulinemic conditions. Our data show that the rate of glucose infusion necessary to maintain euglycemia was significantly increased by GLP-1. Simultaneous icv infusion of EX-9 diminished this effect by 62%. The capacities of insulin to stimulate glucose disposal and inhibit glucose production were reinforced by GLP-1. Simultaneous icv infusion of EX-9 significantly diminished the latter effect. Central GLP-1R antagonism alone did not affect glucose metabolism. Also, GLP-1 treatment reinforced the inhibitory action of insulin on VLDL-TG production. In conclusion, peripheral administration of GLP-1 reinforces the ability of insulin to suppress endogenous glucose- and VLDL-TG production (but not lipolysis), and boosts its capacity to stimulate glucose disposal in high-fat-fed C57Bl/6 mice. Activation of central GLP-1 receptors contributes substantially to the inhibition of endogenous glucose production by GLP-1 treatment in this animal model.

Introduction

Glucagon-like peptide-1 (GLP-1) is produced by entero-endocrine L-cells in response to food intake^{1, 2}. Circulating GLP-1 plays a major role in the control of postprandial metabolism, as it augments nutrient induced insulin release^{1, 3}, inhibits glucagon secretion⁴, and reduces endogenous glucose production (EGP) through (peripheral?) mechanistic routes that appear to be independent of islet hormones⁵. Circulating GLP-1 also inhibits food intake, probably via vagal afferents activating neurons in the nucleus of the solitary tract that subsequently project to hypothalamic neurons to blunt appetite^{6, 7}. Thus, circulating GLP-1 has multiple effects on postprandial behaviour and metabolism to coordinate the systemic response to food intake.

GLP-1 is also produced in a discrete population of neurons in the hind brain^{8, 9}, and GLP-1 fibers terminate in the arcuate and paraventricular nucleus of the hypothalamus⁹, brain areas that play key roles in the control of metabolism¹⁰. GLP-1 receptors are abundantly expressed in these nuclei^{11, 12}, and recent evidence indicates that activation of arcuate GLP-1 receptors sensitizes the liver to insulin, at least as far as its impact on glucose production is concerned¹³. Despite compelling evidence that GLP-1 can cross the blood brain barrier¹⁴, peripheral and central GLP-1 signaling systems are generally considered as separate regulatory circuits. To date, GLP-1 effects on insulin action have only been reported as a result of central GLP-1 receptor activation^{13, 15}. We have recently shown that peripheral administration of a novel GLP-1 agonist ameliorates insulin resistance of glucose and VLDL-triglyceride (VLDL-TG) metabolism in high-fat-fed C57Bl/6J mice¹⁶. It is currently unknown to what extent the (metabolic) effects of GLP-1 treatment are mediated by central GLP-1 receptors. Here, we aimed to evaluate the impact of blocking central GLP-1 receptors on the metabolic effects of peripherally administered GLP-1. Our data show beneficial effects of GLP-1 treatment on glucose and lipid metabolism in insulin resistant high-fat-fed mice. GLP-1 enhanced insulin's action to inhibit glucose production via activation of central GLP-1 receptors.

Material and Methods

Animals and diet. Male C57Bl/6J mice (12 weeks old) (Charles River, Maastricht, the Netherlands) were housed in a temperature and humidity-controlled environment on a 12-h light-dark cycle (lights on from 7:00 – 19:00) and were fed a high-fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) with free access to water for 16 weeks to induce insulin resistance^{17, 18}. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Drugs. GLP-1 (7-36) (human, bovine, guinea pig, mouse, rat; molecular weight: 3297.68 g/mol) and EX-9 (molecular weight: 3369.80 g/mol) were purchased from Bachem (Weil am Rhein, Germany).

Surgical procedures. For intracerebroventricular (icv) cannula implantation, mice were anaesthetized with 0.5 mg/kg medetomidine (Orion Corp. Espoo, Finland), 5.0 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.05 mg/kg fentanyl (Bipharma, Weesp, The Netherlands). A 30-gauge guide cannula (Brain infusion kit 3, Alzet, DURECT Corp., Cupertino, CA) was stereotactically implanted in the left lateral ventricle using the following coordinates from Bregma: 0.46 mm posterior, 1.0 mm lateral, and 2.2 mm ventral. The guide cannula was connected to an osmotic minipump (model 1004, Alzet) via a catheter. This catheter was filled with artificial cerebral spine fluid (CSF, Harvard Apparatus, Natick, MA, US) to delay the start of delivery of the drug by 5 days. A small air bubble was introduced to separate the drug from the CSF. The minipump was placed sc in the right back region, for the continuous delivery of 0.5 pmol/kg/min EX-9 (dissolved in CSF) or CSF at a rate of 0.11 μ l/h as a control. This dose of EX-9 is known to specifically block the central (but not peripheral) GLP-1 receptors¹⁵. The anaesthesia was antagonized using 2.5 mg/kg antipamezol (Pfizer, Capelle a/d IJssel, The Netherlands), 0.5 mg/kg flumazenil (Roche), and 1.2 mg/kg naloxon (Orpha, Purkersdorf, Austria). Post-operative analgesia was provided by sc administration of 0.05 mg/kg buprenorphine (Temgesic®, Schering-Plough, Amstelveen, The Netherlands). In addition, antibiotic (cefazoline, 50 mg/kg) was sc injected. After 5 days of recovery (on the day the icv EX-9/CSF treatment started), mice were matched for body weight and non-fasting glucose levels and a second minipump (model 1002, Alzet) was implanted subcutaneously in the left back region under light isoflurane anaesthesia for the continuous sc delivery of 3 pmol/kg/min GLP-1 (dissolved in PBS) to increase plasma GLP-1 levels¹⁹ or PBS as a control. We chose a two times higher dose of GLP-1 than referred to, since that dose did not result in a significant decrease in non-fasting glucose levels throughout the treatment. After the surgical procedures, we ended up with four groups, from now on referred to by the routes of administration (icv/sc) and names of the compounds: CSF/PBS, CSF/GLP-1, EX-9/PBS, and EX-9/GLP-1. After 2 weeks of intervention experiments were performed as described below. At the end of the experiment, dye (0.5% Evans Blue) was injected through the cannula to confirm its position in the ventricle. Only mice with correct placement of cannulas were included in the final analysis.

Hyperinsulinemic euglycemic clamp. Mice were fasted prior to the clamp for 16 hours overnight with food withdrawn at 1700 h the day before the study. Hyperinsulinemic euglycemic clamp experiment was performed as described before^{20, 21}. During the experiment, mice were sedated with 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands). First, basal rates of glucose and glycerol turnover were determined by isotope dilution methodology using a primed (p) continuous (c) iv infusion of [1-14C]Glucose (p: 0.2 μ Ci; c: 0.3 μ Ci/h, GE Healthcare, Little Chalfont, U.K.) and [1-(3)-3H]Glycerol (p: 0.6 μ Ci; c: 0.9 μ Ci/h, GE Healthcare) for 60 minutes. The choice of these tracers may not be that common. However, in the past we did not find any important differences with other papers and theoretically do not expect any problems by using these tracers. We are aware that the disadvantage of most 14C-glucose tracers is that they are reversible isotopes, i.e. a fraction of the labelled breakdown products is reincorporated in the mother compound, resulting in an underestimation of the rate of appearance. However, it is important to also recognize that other tracers have their own problems. With 3H-glucose for example, recycling occurs with unlabeled glucose via glucose cycling from fructose-6-phosphate etc. Also, part of the glucose is lost in the hexose monophosphate pathway²². After the basal period, insulin (Actrapid, Novo Nordisk, Denmark) was administered in a primed (4.5 mU) continuous (6.8 mU/h) iv infusion for 90 minutes to attain steady state circulating insulin levels of $\sim$ 5 ng/ml. A variable iv infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10 min intervals via tail bleeding (< 3 μ l) (Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 μ l) were taken via tail bleeding during the basal period (after 50 and 60 min) and during the clamp period (after 70, 80, and 90 min) to determine plasma concentrations of glucose, non-esterified fatty acids (NEFA), insulin, glycerol, and plasma [1-14C]Glucose and [1-(3)-3H]Glycerol specific activities. At the end of the clamp, VLDL-production was quantified.

VLDL-TG production. VLDL-TG production was determined during continuous insulin infusion (6.8 mU/h) directly after the clamp experiment. At t=0 min blood was taken via tail bleeding and mice were iv injected with 500 mg of tyloxapol (Triton WR-1339, Sigma-Aldrich) per kg body weight as a 10% (w/w) solution in sterile saline in a total volume of 150 μ l. This completely blocked VLDL clearance from serum²³. Additional blood samples (20 μ l) were taken at t=10, 20, 40, and 60 min after tyloxapol injection and used for determination of plasma TG concentration. After the last sampling, mice were sacrificed by cervical dislocation.

Analytical procedures. Commercially available kits were used to determine plasma levels of glucose, NEFA, TG (Instruchemie, Delfzijl, The Netherlands), and free glycerol (Sigma, MO, US). Plasma insulin concentration was measured by ELISA (Crystal Chem. Inc., Downers Grove, IL, USA). Total plasma [1-14C]Glucose and [1-(3)-3H]Glycerol were determined in 8 μ l plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water.

Calculations. The turnover rates of glucose and glycerol (μ mol/min/kg) were 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 [1-14C]Glucose or [1-(3)-3H]Glycerol (dpm/ μ mol). 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 (GIR). Hepatic VLDL-TG production rates (μ mol/h/kg) were calculated from the linear increase in plasma TG concentrations in time. All metabolic parameters were expressed per kg of body weight.

Statistical analysis. Statistical analysis was performed using SPSS. Differences between groups were determined with the Kruskal–Wallis non-parametric test for k independent samples. When significant differences were found, the Mann–Whitney non-parametric test was used as a follow-up test to determine differences between two independent groups. Effects on the suppression of glycerol turnover between groups were assessed by repeated measure ANOVA. A *P*-value of less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD.

Results

Body weight and plasma parameters. Fasting body weights, plasma glucose, insulin, glycerol, and NEFA concentrations in basal and hyperinsulinemic conditions are shown in Table 1. Body weight did not differ and plasma glucose and insulin levels were similar in all groups, both in basal and hyperinsulinemic conditions. Insulin infusion reduced plasma glycerol and NEFA concentrations to a similar extent in all groups.

Table 1. Body weight and plasma parameters in basal and hyperinsulinemic conditions in mice that received chronic intracerebroventricular/subcutaneous infusion of CSF/PBS, CSF/GLP-1, EX-9/PBS, or EX-9/GLP-1. Values represent mean $\pm$ SD for at least 9 mice per group.

		CSF/PBS	CSF/GLP-1	EX-9/PBS	EX-9/GLP-1
Body weight (g)		33.2 $\pm$ 2.7	32.2 $\pm$ 2.3	33.0 $\pm$ 4.0	33.3 $\pm$ 2.6
Glucose (mM)	Basal	5.11 $\pm$ 0.71	4.39 $\pm$ 0.81	5.16 $\pm$ 0.88	5.20 $\pm$ 0.83
	Hyperinsulinemic	5.04 $\pm$ 0.89	4.31 $\pm$ 0.65	5.37 $\pm$ 1.10	5.16 $\pm$ 0.90
Insulin (ng/ml)	Basal	0.87 $\pm$ 0.44	0.73 $\pm$ 0.24	0.98 $\pm$ 0.76	0.85 $\pm$ 0.57
	Hyperinsulinemic	4.84 $\pm$ 0.92	5.33 $\pm$ 1.32	4.88 $\pm$ 1.02	5.22 $\pm$ 1.61
Glycerol (mM)	Basal	0.12 $\pm$ 0.03	0.14 $\pm$ 0.04	0.13 $\pm$ 0.04	0.14 $\pm$ 0.05
	Hyperinsulinemic	0.09 $\pm$ 0.03	0.09 $\pm$ 0.02	0.10 $\pm$ 0.03	0.10 $\pm$ 0.03
FFA (mM)	Basal	0.67 $\pm$ 0.13	0.68 $\pm$ 0.18	0.68 $\pm$ 0.14	0.68 $\pm$ 0.12
	Hyperinsulinemic	0.39 $\pm$ 0.10	0.36 $\pm$ 0.07	0.41 $\pm$ 0.08	0.39 $\pm$ 0.10

Glucose turnover. In basal conditions, glucose turnover was not different between groups (CSF/PBS: 36 $\pm$ 8; CSF/GLP-1: 34 $\pm$ 6; EX-9/PBS: 35 $\pm$ 7; EX-9/GLP-1: 36 $\pm$ 6 μ mol/min/kg). During hyperinsulinemia, plasma glucose levels were successfully clamped at $\sim$ 5 mM (figure 1A). The GIR required to maintain euglycemia was more than twice as high in GLP-1 treated animals compared to controls (CSF/PBS: 17 $\pm$ 10; CSF/GLP-1: 43 $\pm$ 9 μ mol/min/kg, $P < 0.01$), indicating that GLP-1 reinforces whole body insulin action in this experimental context (figure 1B). Blocking the central GLP-1 receptors by icv infusion of EX-9 clearly blunted the effect of GLP-1 on GIR by 62% (EX-9/GLP-1: 27 $\pm$ 11 μ mol/min/kg, $P < 0.01$ vs. CSF/GLP-1). Central GLP-1 receptor antagonism by itself did not affect GIR (EX-9/PBS: 20 $\pm$ 13 μ mol/min/kg).

Hyperinsulinemia increased glucose disposal in all groups (figure 2A). However, the disposal rate was significantly higher in GLP-1 treated animals compared to controls (CSF/PBS: 46 $\pm$ 7; CSF/GLP-1: 53 $\pm$ 6 μ mol/min/kg, $P < 0.05$). Simultaneous icv infusion of EX-9 had no effect (EX-9/GLP-1: 53 $\pm$ 10 μ mol/min/kg). Also, icv infusion of EX-9 alone did not affect insulin mediated glucose disposal (EX-9/PBS: 43 $\pm$ 7 μ mol/min/kg).

EGP during insulin infusion is shown in figure 2B. GLP-1 treatment enhanced insulin's capacity to inhibit EGP (CSF/PBS: 28 $\pm$ 12; CSF/GLP-1: 13 $\pm$ 9 μ mol/min/kg, $P < 0.01$) and simultaneous icv infusion of EX-9 significantly blunted this effect by 61% (EX-9/GLP-1: 22 $\pm$ 9 μ mol/min/kg vs. CSF/GLP-1, $P < 0.05$). Icv infusion of EX-9 alone did not affect EGP (EX-9/PBS: 26 $\pm$ 11 μ mol/min/kg).

Glycerol turnover. Lipolysis, as reflected by basal rates of glycerol turnover, was not different between groups in basal condition (CSF/PBS: 3.0 ± 0.7 ; CSF/GLP-1: 3.5 ± 0.9 ; EX-9/PBS: 3.1 ± 0.8 ; EX-9/GLP-1: 3.4 ± 1.3 $\mu\text{mol}/\text{min}/\text{kg}$). Glycerol turnover was significantly reduced during hyperinsulinemia only in GLP-1 treated animals (CSF/PBS: 2.9 ± 1.1 ; CSF/GLP-1: 2.7 ± 0.7 ; EX-9/PBS: 2.8 ± 1.1 ; EX-9/GLP-1: 2.9 ± 0.9 $\mu\text{mol}/\text{min}/\text{kg}$; figure 3). However, the effect expressed as percentage reduction of baseline values was not significantly different between interventions (CSF/PBS: 18 ± 15 ; CSF/GLP-1: 20 ± 17 ; EX-9/PBS: 19 ± 21 ; EX-9/GLP-1: 19 ± 20 % reduction from baseline)

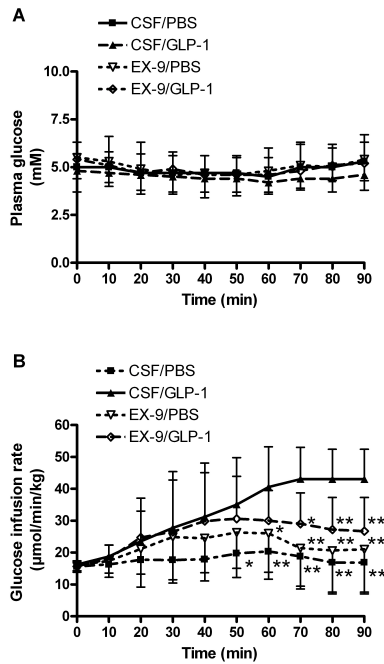


Figure 1. Plasma glucose (A) and glucose infusion rate (B) during a hyperinsulinemic euglycemic clamp in mice that received chronic intracerebroventricular/ subcutaneous infusion of CSF/PBS, CSF/GLP-1, EX-9/PBS, or EX-9/GLP-1. Values represent mean $\pm$ SD for at least 9 mice per group. *p < 0.05 vs. control. **p < 0.01 vs. control.

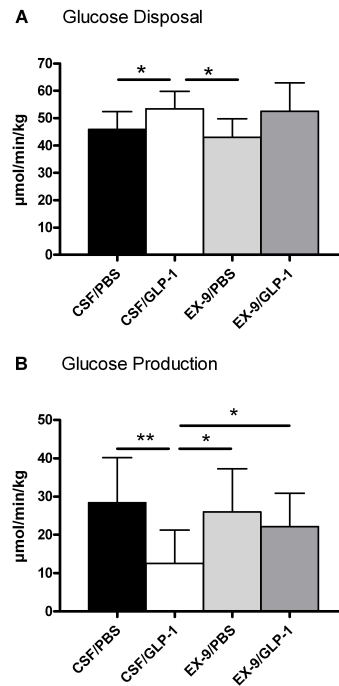


Figure 2. Glucose uptake (A) and glucose production (B) during a hyperinsulinemic euglycemic clamp in mice that received chronic intracerebroventricular/ subcutaneous infusion of CSF/PBS, CSF/GLP-1, EX-9/PBS, or EX-9/GLP-1. Values represent mean $\pm$ SD for at least 9 mice per group. *p < 0.05 vs. control. **p < 0.01 vs. control.

VLDL-TG production. Plasma TG levels (figure 4) and hence, VLDL-TG production rate in hyperinsulinemic condition were significantly reduced by GLP-1 treatment (CSF/PBS: 252 ± 47 ; CSF/GLP-1: 205 ± 31 $\mu\text{mol}/\text{h}/\text{kg}$, $P < 0.05$). Simultaneous icv infusion of EX-9 did not

significantly impact on this effect (EX-9/GLP-1: $216 \pm 39 \mu\text{mol/h/kg}$) and icv infusion of EX-9 alone did not affect VLDL-TG production (EX-9/PBS: $249 \pm 40 \mu\text{mol/h/kg}$).

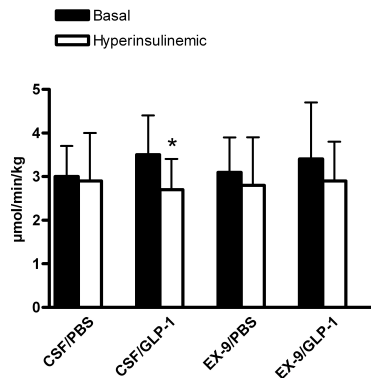


Figure 3. Glycerol turnover before (basal) and after (hyperinsulinemic) the initiation of a hyperinsulinemic euglycemic clamp in mice that received chronic intracerebroventricular/subcutaneous infusion of CSF/PBS, CSF/GLP-1, EX-9/PBS, or EX-9/GLP-1. Values represent mean $\pm$ SD for at least 9 mice per group. *p < 0.05 vs. control.

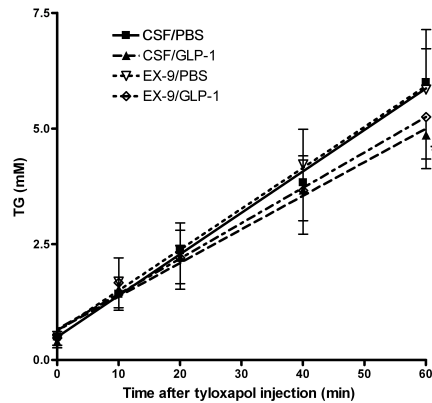


Figure 4. Triglyceride (TG) concentrations during hyperinsulinemia after tyloxapol infusion in mice that received chronic intracerebroventricular/ subcutaneous infusion of CSF/PBS, CSF/GLP-1, EX-9/PBS, or EX-9/GLP-1. Values represent mean $\pm$ SD for at least 9 mice per group. *p < 0.05 vs. control.

Discussion

This study shows that chronic subcutaneous administration of GLP-1 enhances whole body insulin sensitivity of glucose metabolism in high-fat-fed insulin resistant C57Bl/6J mice. This is brought about by the composite stimulatory effects of GLP-1 on the capacity of insulin to suppress EGP and stimulate glucose disposal. Moreover, GLP-1 enhanced insulin's ability to inhibit VLDL-TG production in this experimental context. Simultaneous icv infusion of EX-9, blocking central GLP-1 receptors, abolished the inhibition of EGP, whereas it did not affect VLDL-TG production. Icv infusion of EX-9 alone did not affect insulin action in any way. These data show that GLP-1 treatment ameliorates insuline resistance in high-fat-fed mice and strongly suggest that it reinforces insulin action to suppress endogenous glucose production via central GLP-1 receptors.

To the best of our knowledge, this is the first study to focus on the role of central GLP-1 receptors in the metabolic effects of (pharmacologic levels of) circulating GLP-1. All other studies evaluating the behavioural and metabolic effects of central GLP-1 receptor signaling have quantified the impact of central administration of GLP-1 (analogues) or

GLP-1 receptor antagonists (e.g. refs^{13, 15, 24}). Our data suggest that peripheral GLP-1 treatment modulate insulin action via central receptors, as far as insulin's ability to inhibit glucose production is concerned. This inference is consistent with data indicating that circulating GLP-1 has access to the brain^{11, 14, 25}. The fact that icv administration of EX-9 alone did not impact on glucose or lipid metabolism in this experimental context suggest that central GLP-1 receptors do not play a major role in the control of (postprandial) fuel flux by endogenous GLP-1 (and thereby suggest that a supraphysiological plasma concentration of GLP-1 is required to activate central receptors. Although we did not measure plasma GLP-1 levels, which is a limitation of this study, we assume GLP-1 levels to be considerably increased as observed in a previous experiment of similar design¹⁹). Gut-derived circulating GLP-1 is rapidly degraded and blood levels rise only for a limited period of time in response to a meal²⁶. The kinetics of postprandial GLP-1 production may not allow for substantial activation of central receptors in high-fat-fed insulin resistant mice, whereas continuous GLP-1 infusion might. This issue clearly requires further study. Our data do suggest that GLP-1 analogues, with extended plasma half-lives for therapeutic use, act to improve metabolism in part via the brain.

The beneficial effects of GLP-1 on glucose metabolism corroborate the results of a previous study investigating the therapeutic potential of a novel GLP-1 analogue in insulin resistant mice¹⁶. They are also in keeping with earlier reports indicating that GLP-1 and its analogues ameliorate whole-body insulin resistance in obese animal models^{27, 28} and in T2DM patients²⁹. They further extend our knowledge of the precise actions of GLP-1 on distinct components of glucose flux in insulin-resistant animals, inasmuch as they show that it enhances insulin action on both glucose disposal and production in the long term. In apparent contrast, however, a recent paper indicates that central administration of GLP-1 hampers insulin mediated glucose disposal in male Long Evans rats, albeit not to a major extent¹³. Moreover, another paper consistently reported that icv exendin-4 (a GLP-1 receptor agonist) acutely impairs insulin's action on glucose uptake in normal C57Bl/6J mice (but only in hyperglycemic conditions)¹⁵. The same investigators also reported that chronic (4 wk) infusion of EX-9 (in a dose that was equal to ours) into the lateral ventricle of high-fat-fed male C57Bl/6J mice rescues insulin sensitivity and prevents the onset of glucose intolerance in these animals, again suggesting that central GLP-1 receptor activation impairs insulin action³⁰. Our data unequivocally indicate that peripheral administration of GLP-1 enhances insulin sensitivity in the same insulin resistant animal model. In particular, they indicate that activation of central GLP-1 receptors contributes substantially to the effect of GLP-1 treatment on glucose production. This inference agrees with at least one aforementioned study, showing that activation of central GLP-1 receptors reinforces insulin's capacity to suppress EGP¹³. It is difficult to reconcile the

conflicting results of these studies. One major difference between the design of all other experiments and ours is the fact that we studied the effects of peripherally injected GLP-1, whereas others evaluated the impact of centrally administered compounds (where circulating GLP-1 levels were presumably low). It is conceivable that centrally injected GLP-1 activates neuronal circuits that are distinct from those activated by circulating GLP-1, and therefore exerts differential metabolic effects. However, it remains difficult to understand that icv EX-9 alone appears to have no effect on insulin sensitivity in our studies, whereas it reinforced insulin action in virtually the same experiment reported by Knauf et al³⁰. Our study design differed from Knauf's in that EX-9 was given icv for 4 weeks in their experiment, 2 weeks longer than in ours. Thus, the effects observed by Knauf et al may have been (indirect) effects of longer term EX-9 treatment. Also, the timing and composition of the dietary intervention was somewhat different in the studies by Knauf. Experiments were done after 4 weeks of a virtually carbohydrate free diet, while we studied animals after 16 weeks on a diet containing approximately 20% of carbohydrate. The longer period of high fat feeding may have reduced basal GLP-1 levels and diminished the GLP-1 response to food intake³¹. Obviously, a reduction of circulating GLP-1 will blunt its impact on insulin action and therefore the effect of (central) GLP-1 receptor antagonism on insulin sensitivity. The data clearly warrant further exploration of this issue. Finally, the period of fasting prior to the hyperinsulinemic euglycemic clamp was different; a 6h fasting period by Knauf vs. a 16h fast in our study, which might have contributed to a different peripheral phenotype³².

GLP-1 reinforced the capacity of insulin to inhibit VLDL-TG production. This finding adds to our knowledge of the effects of GLP-1 on lipid metabolism. Our observations are fully consistent with those reported in one of our previous papers, delineating the metabolic effects of a novel GLP-1 analogue in the same animal model¹⁶. Also, native GLP-1 reduces postprandial plasma TG levels in healthy, normal-weight humans and T2DM patients^{33, 34}. The assembly of VLDL particles in the endoplasmic reticulum of hepatocytes is dependent on the intracellular presence of TG, other lipids, and apolipoprotein B (apoB) as its major components. Therefore, the availability of apoB and substrates play key roles in the control of VLDL production³⁵. Insulin inhibits VLDL assembly in multiple ways. It limits the flow of fatty acid substrates to the liver by inhibiting (adipocyte) lipolysis and probably also modulates apoB mRNA translation³⁶. However, GLP-1 did not affect lipolysis or insulin's capacity to suppress this process and, accordingly, it did not change plasma NEFA concentrations. Therefore, inhibition of lipolysis is not the route through which GLP-1 modifies VLDL-TG secretion in high-fat-fed insulin resistant mice. Thus, GLP-1 may strengthen the ability of insulin to restrain VLDL-TG production directly by diminishing the availability of apoB, as it was shown to inhibit (intestinal) apoB production³⁷. Simultaneous

icv administration of EX-9 did not significantly affect the ability of insulin to suppress VLDL-TG production. This observation suggests that GLP-1 treatment reinforces the impact of insulin on VLDL-TG secretion via peripheral receptors in our experimental set-up. In this respect, it is interesting to note the direct role of GLP-1 on human hepatocytes to reduce steatosis³⁸. However, it is doubtful whether rodent hepatocytes express GLP-1 receptors^{39, 40}. Further investigation of the mechanisms driving GLP-1's effect on lipid metabolism is warranted.

Despite its unequivocal effects on glucose and VLDL-TG metabolism, GLP-1 did not significantly impact on the ability of insulin to inhibit lipolysis. This is consistent with the results of a previous study, showing that CNTO736, a novel GLP-1 analogue, ameliorates insulin resistance of glucose and VLDL-TG metabolism, but not lipolysis in high-fat-fed C57Bl6/J mice¹⁶. In concert, these studies strongly suggest that GLP-1 exerts tissue specific effects on insulin action in high-fat-fed C57Bl/6 mice.

The neuronal circuits that are activated by circulating GLP-1 and mediate its metabolic effects remain to be identified. Larger, blood-born molecules can access the arcuate nucleus through local, functional gaps in the blood-brain-barrier⁴¹. Arcuate neuropeptide Y (NPY) and pro-opiomelanocortin neurons are intimately involved in the control of fuel flux. Indeed, icv injection of NPY impairs insulin's ability to suppress glucose production²¹. Animal models of obesity and type 2 diabetes (including diet-induced obesity) are marked by elevated NPY expression in hypothalamic nuclei^{42, 43} (although this has been debated^{44, 45}), and these exceedingly active NPY neurons may drive overproduction of glucose in these models. GLP-1 has been reported to prevent the orexigenic effects of NPY possibly by reducing hypothalamic NPY mRNA expression^{7, 46, 47}). In analogy, peripheral administered GLP-1 may have inhibited arcuate NPY neurons to explain the findings on glucose turnover presented here.

In conclusion, we here show that GLP-1 treatment ameliorates insulin resistance of glucose and VLDL-TG metabolism in high-fat-fed mice. In particular, peripheral administration of GLP-1 reinforces the capacity of insulin to stimulate glucose disposal and inhibit glucose and VLDL-TG production in this model, where central GLP-1 receptors are required for the effect on glucose production.

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Chapter 6

Obinepitide, a novel Y2/Y4 agonist, and TM30339, a novel Y4 agonist, reduce insulin resistance in high-fat-fed C57Bl/6J mice

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In preparation

Abstract

Obinipitide, a dual-analogue of peptide YY₃₋₃₆ and pancreatic polypeptide (PP), and TM30339, a PP analogue, were developed as anti-obesity drugs. Pre-clinical studies have shown long-term effects on decreased body weight in human and animals. Here, we further evaluate the potential of these drugs in terms of insulin sensitivity of glucose and lipid metabolism. Diet-induced insulin resistant C57Bl/6J mice were daily subcutaneously injected with Obinipitide (0.3 and 3.0 mg/kg), TM30339 (0.3 and 3.0 mg/kg), or vehicle for 4 weeks. Glucose and glycerol turnover were determined during a hyperinsulinemic euglycemic clamp. Very low density lipoprotein-triglyceride (VLDL-TG) production was determined in basal and hyperinsulinemic conditions after intravenous injection of tyloxapol. 3.0 mg/kg Obinipitide and both doses of TM30339 significantly enhanced insulin mediated glucose disposal (Obinipitide: 100 ± 26 , $p < 0.01$; 0.3 mg/kg TM30339: 90 ± 25 , $p < 0.05$; 3.0 mg/kg TM30339: 116 ± 19 , $p < 0.01$; control: 62 ± 25 % stimulation). In addition, both drugs affected insulin's capacity to inhibit endogenous glucose production (Obinipitide: 51 ± 24 , $p < 0.05$; 0.3 mg/kg TM30339: 60 ± 23 , $p < 0.01$; 3.0 mg/kg TM30339: 68 ± 24 , $P < 0.01$; control: 30 ± 26 % inhibition). Insulin's capacity to inhibit glycerol turnover was not affected by any of the treatments. In basal conditions, none of the drugs affected VLDL-TG production. However, during insulin infusion VLDL-TG production was decreased in 3.0 mg/kg treated mice only (3.0 mg/kg TM30339: 134 ± 17 ; control: 167 ± 29 $\mu\text{mol/h/kg}$; $p < 0.01$). The data suggest that Obinipitide and TM30339 appear to be useful tools to improve glucose metabolism in diet-induced insulin resistant C57Bl/6J mice.

Introduction

Peptide YY (PYY) and pancreatic polypeptide (PP) are closely related 36-amino acid peptides. PYY is synthesized in the intestinal L-cells, which are predominantly located in the distal part of the gastrointestinal tract. PP is synthesized in the so-called F-cells, located in the pancreatic islet of Langerhans. Both PYY and PP are released into the circulation in response to nutrient intake in proportion to caloric intake^{1, 2}. PYY circulates primarily as the N-terminally truncated form of the full length peptide, PYY₃₋₃₆³. After being released PYY₃₋₃₆ and PP bind and activate a class of G-protein coupled receptors belonging to the neuropeptide Y receptor family. To date 5 subtypes have been identified: Y1R, Y2R, Y4R, Y5R, and Y6R⁴. PYY₃₋₃₆ is known to exert its effects predominantly via the Y2R, which are highly expressed in the arcuate nucleus⁵, whereas PP effects are thought to be mediated via the Y4R subtype, expressed at many sites in the brain⁶.

The central Y receptors are involved in the control of a diverse set of regulatory processes, including appetite and body weight. Food intake is significantly reduced after peripheral administration of PYY₃₋₃₆ and PP in both rodents and humans (7-10). Chronic PYY₃₋₃₆ administration reduces body weight^{11, 12}. Moreover, PYY overexpression protects against diet-induced obesity in mice, whereas ablation of PYY leads to the development of obesity^{13, 14}. In addition to their roles in feeding and body weight regulation, PYY₃₋₃₆ and PP affect insulin sensitivity in animal models of obesity and type 2 diabetes^{12, 15-17}. Specifically, for PYY₃₋₃₆ this is the result of an enhanced insulin mediated glucose disposal^{16, 17}.

The aforementioned studies suggest that the Y receptors are interesting targets for the treatment of obesity and type 2 diabetes mellitus (T2DM). Selective Y2/Y4 agonists may have metabolic effects similar to those of PYY₃₋₃₆ and PP. In this respect, Obinipitide (dual Y2/Y4 receptor agonist) and TM30339 (Y4 receptor agonist), were developed by 7TM Pharma (Copenhagen, Denmark) as anti-obesity drugs^{18, 19}. Pre-clinical studies have shown that Obinipitide reduces the body weight of DIO mice more than native PYY₃₋₃₆. Furthermore, daily sc injections of Obinipitide in obese humans inhibited food intake at a statistically significant level up to 9 h after dosing²⁰. TM30339 also reduces the body weight of DIO mice²¹.

Here, we further explore the pharmacological characteristics of Obinipitide and TM30339 in terms of their metabolic effects. In particular, we evaluate the chronic impact of these compounds on insulin sensitivity of glucose and very low density lipoprotein-triglyceride (VLDL-TG) metabolism in diet-induced insulin resistant C57Bl/6 mice. Overproduction of VLDL-TG, which is a prominent feature of diabetic dyslipidemia (as the proximate cause of hypertriglyceridemia), contributes significantly to cardiovascular risk in T2DM patients²². Neuropeptide Y (NPY), also acting via the Y receptors, has been shown to stimulate VLDL production^{23, 24}. Since PYY₃₋₃₆ inhibits NPY expression via presynaptic Y2

receptors^{7, 25} (and possibly PYY exerts the same effects on NPY via the Y4R), we therefore hypothesized that Obinipitide and TM30339 would inhibit VLDL-TG production.

Methods

Animals. 12 week old male C57BL/6J mice (Charles River, Maastricht, The Netherlands) were housed in a temperature-controlled room on a 12-hour light-dark cycle (lights on from 7:00 – 19:00) and fed a high fat diet (44 energy% fat derived from bovine fat, Hope Farms, Woerden, The Netherlands) for 16 weeks with free access to water to induce insulin resistance²⁶. All animal experiments were approved by the Animal Ethics Committee from the Leiden University Medical Center, Leiden, The Netherlands.

Drugs. Obinipitide and TM30339 were supplied by 7TM Pharma. These peptide analogues of pancreatic polypeptide were synthesized by solid phase peptide synthesis (SPPS) and purified by reversed phase high performance liquid chromatography. The peptides were dissolved in sterile 0.9% saline for injection and the solution pH adjusted to approximately pH 7.4 before being filtered through a 0.22 µm filter.

Treatment. Mice were injected subcutaneously (sc) with phosphate buffered saline (PBS) twice daily (BID) for 11 days for habituation to daily injections. Thereafter, at 12 weeks of high-fat-feeding, the mice were divided into 2 x 5 groups. The groups were matched in fasting condition for body weight and plasma glucose levels for clamp experiments and body weight and plasma TG levels for VLDL production experiments (period of fasting was 16 hours for clamp study and 4 hours for VLDL study). Each group received sc injections of Obinipitide BID (0.3 or 3.0 mg/kg), TM30339 once daily (OD) (0.3 mg/kg or 3.0 mg/kg), or saline BID for 4 weeks. The first dose was given at the beginning of the light period and the second dose directly before the lights went out. TM30339 was administered at the end of the light period. Mice receiving TM30339 also received vehicle on the first dosing occasion to mirror vehicle control. Before, after 2 weeks, and by the end of treatment, body weight, plasma glucose, insulin, free fatty acids (FFA), and TG concentrations were determined in fasting condition. On the day of study, mice received a last sc dosing of Obinipitide (0.3 or 3.0 mg/kg), TM30339 (0.3 or 3.0 mg/kg), or saline 20 min prior to the experiments.

Hyperinsulinemic euglycemic clamp. Mice had free access to high fat food and water until 16 hours prior to the clamp (i.e. 17.00h the day before the clamp). Hyperinsulinemic euglycemic clamps were performed under 6.25 mg/kg acepromazine (Alfasan, Woerden, The Netherlands), 6.25 mg/kg midazolam (Roche, Mijdrecht, The Netherlands), and 0.3125 mg/kg fentanyl (Janssen-Cilag, Tilburg, The Netherlands) anesthesia to quantify glucose

and glycerol turnover. First, basal rates of glucose and glycerol turnover were determined by giving a primed (p) continuous (c) intravenous (iv) infusion of [1-¹⁴C]-glucose (p: 0.2 µCi; c: 0.3 µCi/h, Amersham, Little Chalfont, U.K.) and [1-(3)-³H]-glycerol (p: 0.6 µCi; c: 0.9 µCi/h, Amersham) for 60 minutes. Subsequently, insulin (Actrapid, Novo Nordisk, Denmark) was administered in a primed (4.5 mU) continuous (6.8 mU/h) iv infusion for 90 minutes to attain steady state circulating insulin levels of ~4 ng/ml. A variable iv infusion of a 12.5% D-glucose solution was used to maintain euglycemia, as determined at 10 min intervals via tail bleeding (< 3 µl, Accu-chek, Sensor Comfort, Roche Diagnostics GmbH, Mannheim, Germany). Blood samples (60 µl) were taken via tail bleeding during the basal period (after 50 and 60 min) and during the clamp period (20 and 10 minutes prior to- and at the end of the clamp) to determine the plasma concentration of glucose, glycerol, FFA, and insulin and plasma [1-¹⁴C]-glucose and [1-(3)-³H]-glycerol specific activities. At the end of the clamp, VLDL-TG production was quantified.

VLDL-production. Basal VLDL production experiments were performed after a 4 hour fast (09.00-13.00h) and under 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag) anesthesia. Mice received an iv injection of 100 µl PBS containing 150 µCi Tran³⁵S label (GE Healthcare, Little Chalfont, U.K.) to label newly produced apoB. After 10 minutes, the animals received a sc dosing of Obinipitide (0.3 or 3.0 mg/kg), TM30339 (0.3 or 3.0 mg/kg), or saline. Twenty minutes thereafter, mice received a 15% (by volume) iv injection of tyloxapol (500 mg/kg body weight; Triton WR-1339, Sigma-Aldrich Corp., St. Louis, MO, US) to prevent systemic lipolysis of newly secreted hepatic VLDL-TG. Blood samples were taken before (time point 0) and 15, 30, 60, and 90 min after tyloxapol injection. Thirty minutes after the last sampling, mice were sacrificed by cervical dislocation and exsanguinated via the retro-orbital plexus.

Hyperinsulinemic VLDL-TG production was determined during continuous insulin infusion (6.8 mU/h) directly after the clamp study. Mice received a 15% (by volume) iv injection of tyloxapol (500 mg/kg body weight; Triton WR-1339, Sigma). Blood samples were taken before (time point 0) and 10, 20, 40, and 60 min after tyloxapol injection. After the last sampling, mice were sacrificed

DEXA scan. Body composition was measured by dual-energy X-ray absorptiometry (DEXA) using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland, Hampshire, UK). During measuring, mice were anesthetized with a mixture of 6.25 mg/kg acepromazine (Alfasan), 6.25 mg/kg midazolam (Roche), and 0.3125 mg/kg fentanyl (Janssen-Cilag).

Analytical procedures. Commercially available kits were used to determine plasma levels of glucose, FFA, TG (Instruchemie, Delfzijl, The Netherlands), and free glycerol (Sigma). Plasma insulin concentration was measured by ELISA (Crystal Chem. Inc., Downers Grove, IL, USA). Total plasma [1-¹⁴C]-glucose and [1-(3)-³H]-glycerol were determined in 8 µl plasma and in supernatants after trichloroacetic acid (20%) precipitation and water evaporation to eliminate tritiated water. VLDL was quantitatively isolated from plasma after density gradient ultracentrifugation at $d < 1.006$ g/ml by aspiration. VLDL-apoB was selectively precipitated with 2-propanol and counted for incorporated ³⁵S.

Calculations. Turnover rates of glucose (µmol/min/kg) were calculated in basal and hyperinsulinemic conditions as the rate of tracer infusion (dpm/min) divided by the plasma specific activity of [1-¹⁴C]-glucose and [1-(3)-³H]-glycerol (dpm/µmol). The ratio was corrected for body weight. Endogenous glucose production (EGP) was calculated as the difference between glucose disappearance and the infusion rate of glucose. Hepatic VLDL-TG production rates (µmol/h/kg) were calculated from the linear increase in plasma TG concentrations in time.

Statistical analysis. Statistical analysis was performed using SPSS (version 16.0; SPSS Inc., Chicago, IL, USA). Differences between groups were determined with the Kruskal–Wallis non-parametric test for k independent samples. When significant differences were found, the Mann–Whitney non-parametric test was used as a follow-up test to determine differences between two independent groups. A p-value of less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD.

Results

Body weight and DEXA scan. 3.0 mg/kg Obinipitide and both doses of TM30339 significantly reduced body weight (figure 1). The decrease was most prominent during the first week of treatment. However, the difference with control animals remained till the end of the treatment period. Body weight reduction was fully accounted for by a reduction in fat mass, as shown by DEXA scan analysis (figure 2).

Glucose metabolism

Body weight and plasma parameters. Fasting body weights, plasma glucose, and insulin concentrations before and during treatment and in hyperinsulinemic conditions are shown in table 1. Chronic administration of 3.0 mg/kg Obinipitide and both doses of TM30339

reduced fasting plasma glucose levels. Basal plasma insulin levels were not affected by any of the treatments.

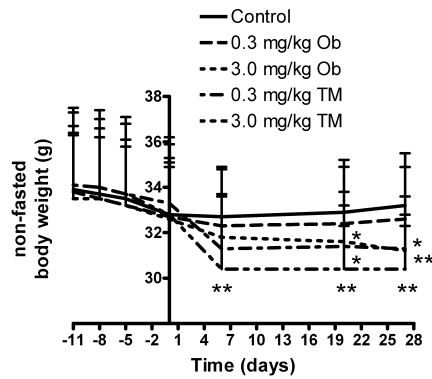


Figure 1. Non-fasted bodyweight of mice after treatment with Obinipitide (Ob) (0.3 or 3.0 mg/kg), TM30339 (TM) (0.3 or 3.0 mg/kg), or vehicle for 4 weeks. Values represent mean $\pm$ SD for at least 21 mice per group. * p < 0.05 vs. control. ** p < 0.01 vs. control.

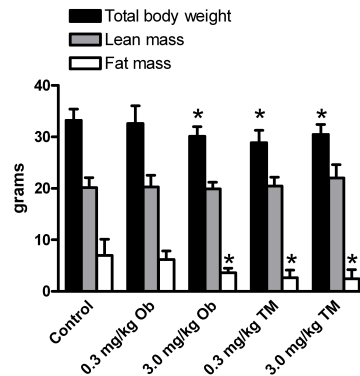


Figure 2. Body composition of mice after treatment with Obinipitide (Ob) (0.3 or 3.0 mg/kg), TM30339 (TM) (0.3 or 3.0 mg/kg), or vehicle for 4 weeks. Values represent mean $\pm$ SD for at least 6 mice per group. * p < 0.05 vs. control.

Hyperinsulinemic euglycemic clamp. Basal glucose turnover, glucose disposal and EGP in hyperinsulinemic conditions after 4 weeks of treatment are shown in table 2. In basal conditions, there was no difference in glucose turnover between groups. Chronic administration of 3.0 mg/kg Obinipitide and both doses of TM30339 increased the rate of glucose infusion necessary to maintain euglycemia during insulin administration, indicating that both drugs enhanced whole body insulin sensitivity (figure 3).

Glucose disposal during insulin infusion was significantly increased in 3.0 mg/kg Obinipitide and 3.0 mg/kg TM30339 treated mice (table 2). If expressed as a percentage from baseline values, also 0.3 mg/kg TM30339 treatment increased insulin's action to stimulate glucose disposal (figure 4). Glucose production during insulin infusion was significantly decreased in TM30339 treated animals (table 2). If expressed as a percentage from baseline values, the highest dose of Obinipitide also reinforced the capacity of insulin to suppress EGP (figure 5). The lowest dose of Obinipitide did not significantly affect glucose turnover.

Table 1. Plasma parameters in basal (fasting) condition and during hyperinsulinemia, before and during chronic treatment with Obinepitide (BID) (0.3 or 3.0 mg/kg), TM30339 (OD) (0.3 or 3.0 mg/kg), or control. Values represent mean $\pm$ SD for at least 10 mice per group. **p<0.01 vs. control.

		Control (saline BID)	Obinepitide (BID)		TM30339 (OD)	
			0.3 mg/kg	3.0 mg/kg	0.3 mg/kg	3.0 mg/kg
Body weight (g)	Start treatment	31.2 $\pm$ 2.5	30.9 $\pm$ 2.1	31.3 $\pm$ 2.5	31.1 $\pm$ 2.9	31.3 $\pm$ 3.0
	2 wks treatment	31.3 $\pm$ 2.2	30.1 $\pm$ 1.9	29.5 $\pm$ 1.0	28.8 $\pm$ 1.8**	28.2 $\pm$ 1.4**
	4 wks treatment	31.5 $\pm$ 2.3	30.3 $\pm$ 1.5	28.6 $\pm$ 0.6**	28.6 $\pm$ 1.5**	27.9 $\pm$ 1.2**
	Hyperinsulinemic	-	-	-	-	-
Glucose (mmol/l)	Start treatment	6.9 $\pm$ 1.2	6.7 $\pm$ 0.6	6.7 $\pm$ 1.0	7.0 $\pm$ 1.0	6.5 $\pm$ 0.9
	2 wks treatment	6.8 $\pm$ 1.1	6.3 $\pm$ 0.8	5.0 $\pm$ 1.8**	5.6 $\pm$ 0.9**	5.1 $\pm$ 1.2**
	4 wks treatment	6.0 $\pm$ 1.3	6.0 $\pm$ 1.5	5.1 $\pm$ 0.7**	5.0 $\pm$ 0.6**	4.8 $\pm$ 0.6**
	Hyperinsulinemic	5.9 $\pm$ 1.3	6.2 $\pm$ 1.1	5.4 $\pm$ 0.8	5.1 $\pm$ 0.7	4.7 $\pm$ 0.6
Insulin (ng/ml)	Start treatment	0.5 $\pm$ 0.3	0.5 $\pm$ 0.2	0.4 $\pm$ 0.2	0.5 $\pm$ 0.2	0.5 $\pm$ 0.3
	2 wks treatment	0.5 $\pm$ 0.2	0.5 $\pm$ 0.2	0.3 $\pm$ 0.2	0.5 $\pm$ 0.3	0.3 $\pm$ 0.2
	4 wks treatment	0.7 $\pm$ 0.3	0.6 $\pm$ 0.3	0.4 $\pm$ 0.2	0.6 $\pm$ 0.4	0.4 $\pm$ 0.2
	Hyperinsulinemic	3.9 $\pm$ 1.1	4.1 $\pm$ 1.1	3.5 $\pm$ 0.7	3.6 $\pm$ 1.0	3.4 $\pm$ 0.9
FFA (mmol/l)	Start treatment	0.9 $\pm$ 0.2	0.8 $\pm$ 0.2	1.0 $\pm$ 0.3	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2
	2 wks treatment	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	1.0 $\pm$ 0.2	0.9 $\pm$ 0.2	0.8 $\pm$ 0.3
	4 wks treatment	0.8 $\pm$ 0.2	0.7 $\pm$ 0.2	0.7 $\pm$ 0.2	0.8 $\pm$ 0.3	0.7 $\pm$ 0.2
	Hyperinsulinemic	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1

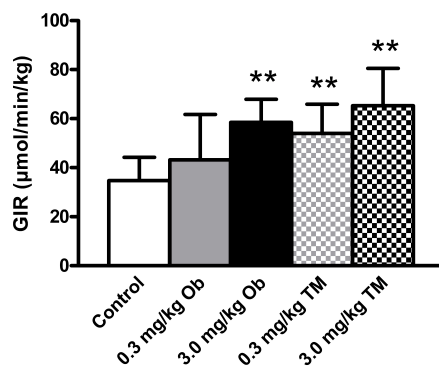


Figure 3. Glucose infusion rate (GIR) during a hyperinsulinemic euglycemic clamp in mice after treatment with Obinepitide (Ob) (0.3 or 3.0 mg/kg), TM30339 (TM) (0.3 or 3.0 mg/kg), or control for 4 weeks. Values represent mean $\pm$ SD for at least 10 mice per group. **p < 0.01 vs. control.

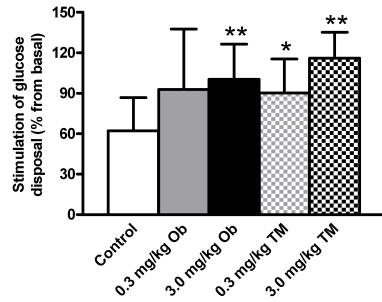


Figure 4. Stimulation of glucose disposal during a hyperinsulinemic euglycemic clamp in mice after treatment with Obinepitide (Ob) (0.3 or 3.0 mg/kg), TM30339 (TM) (0.3 or 3.0 mg/kg), or control for 4 weeks. Values represent mean $\pm$ SD for at least 10 mice per group. * p < 0.05 vs. control. ** p < 0.01 vs. control.

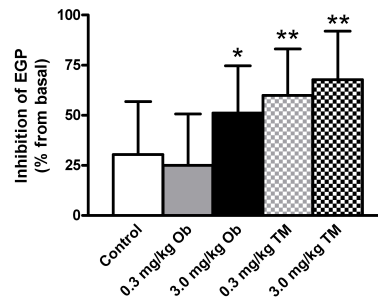


Figure 5. Inhibition of endogenous glucose production (EGP) during a hyperinsulinemic euglycemic clamp in mice after treatment with Obinepitide (Ob) (0.3 or 3.0 mg/kg), TM30339 (TM) (0.3 or 3.0 mg/kg), or control for 4 weeks. Values represent mean $\pm$ SD for at least 10 mice per group. * p < 0.05 vs. control. ** p < 0.01 vs. control.

Table 2. Glucose turnover in basal and hyperinsulinemic conditions in mice that received treatment for 4 weeks with Obinepitide (BID) (0.3 or 3.0 mg/kg), TM30339 (OD) (0.3 or 3.0 mg/kg), or control. Values represent mean $\pm$ SD for at least 10 mice per group. * p < 0.05 vs. control ** p < 0.01 vs. control.

	Control (saline BID)	Obinepitide (BID)		TM30339 (OD)	
		0.3 mg/kg	3.0 mg/kg	0.3 mg/kg	3.0 mg/kg
Basal glucose turnover (μ mol/min/kg)	38.6 $\pm$ 7.8	38.7 $\pm$ 8.8	39.2 $\pm$ 7.4	36.7 $\pm$ 5.7	34.8 $\pm$ 5.0
Insulin mediated glucose disposal (μ mol/min/kg)	62.4 $\pm$ 15.0	74.0 $\pm$ 22.9	77.4 $\pm$ 11.2*	68.9 $\pm$ 7.4	74.9 $\pm$ 10.3*
Hyperinsulinemic endogenous glucose production (μ mol/min/kg)	27.9 $\pm$ 12.5	31.6 $\pm$ 15.7	19.8 $\pm$ 11.6	14.1 $\pm$ 7.6**	10.9 $\pm$ 8.3**

VLDL metabolism

Neither drug affected VLDL-apoB or VLDL-TG production rate in basal (fasting) conditions (table 3). Remarkably, both drugs appeared to elevate plasma TG concentrations in basal condition, where the increase was statistically significant (compared to control) for the highest dose of each drug. During insulin infusion, plasma TG concentrations rose more slowly after tyloxapol in mice that received chronic injections of 3.0 mg/kg TM30339 (figure 6), which translates into a decreased VLDL-TG production rate in these animals (table 3).

Table 3. Bodyweight, plasma TG levels and VLDL production rate in basal (fasting) condition and during hyperinsulinemia before and during chronic treatment with Obinipitide (BID) (0.3 or 3.0 mg/kg), TM30339 (OD) (0.3 or 3.0 mg/kg), or control. Values represent mean $\pm$ SD for at least 7 mice per group. *p < 0.05 vs. control. **p < 0.01 vs. control. #p < 0.01 vs. start treatment.

		Control (saline BID)	Obinipitide (BID)		TM30339 (OD)	
			0.3 mg/kg	3.0 mg/kg	0.3 mg/kg	3.0 mg/kg
Body weight (g)	Start treatment	31.0 $\pm$ 2.2	31.7 $\pm$ 3.9	31.0 $\pm$ 2.4	31.4 $\pm$ 3.8	31.4 $\pm$ 4.4
	2 wks treatment	31.4 $\pm$ 2.2	31.5 $\pm$ 3.2	30.1 $\pm$ 1.8	29.8 $\pm$ 2.9	28.8 $\pm$ 2.9
	4 wks treatment	32.4 $\pm$ 2.1	32.2 $\pm$ 3.5	29.8 $\pm$ 1.6	30.1 $\pm$ 3.1	29.7 $\pm$ 3.0
TG (mmol/l)	Start treatment	0.54 $\pm$ 0.11	0.53 $\pm$ 0.10	0.49 $\pm$ 0.12	0.50 $\pm$ 0.13	0.46 $\pm$ 0.11
	2 wks treatment	0.62 $\pm$ 0.07	0.61 $\pm$ 0.07	0.58 $\pm$ 0.08	0.55 $\pm$ 0.16	0.40 $\pm$ 0.08
	4 wks treatment	0.66 $\pm$ 0.17	0.79 $\pm$ 0.20#	0.99 $\pm$ 0.18**#	0.85 $\pm$ 0.18#	0.88 $\pm$ 0.17*#
VLDL-apoB production ($\times 10^3$ dpm/ml/h)	Basal	56 $\pm$ 14	59 $\pm$ 21	58 $\pm$ 13	66 $\pm$ 14	65 $\pm$ 17
VLDL-TG production (μ mol/h/kg)	Basal	282 $\pm$ 49	282 $\pm$ 69	263 $\pm$ 37	291 $\pm$ 47	273 $\pm$ 48
	Hyperinsulinemic	167 $\pm$ 29	169 $\pm$ 48	159 $\pm$ 10	172 $\pm$ 27	134 $\pm$ 17**

Lipolysis

Obinipitide or TM30339 did not affect lipolysis (glycerol R_a) (figure 7) or circulating FFA concentrations in basal (fasting) conditions (table 1). Although insulin infusion reduced glycerol turnover significantly in the 3.0 mg/kg Obinipitide and TM30339 groups, insulin's capacity to suppress glycerol turnover (expressed as percentage of baseline values) was not affected by any of the interventions (data not shown). Also, the decline of plasma FFA concentrations induced by insulin was not affected by either drug (table 1).

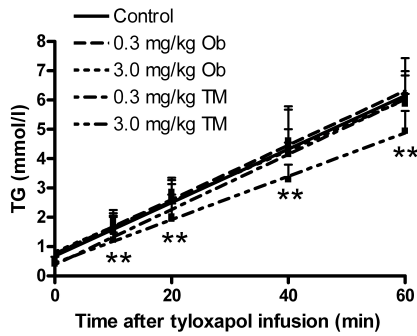


Figure 6. Triglyceride (TG) concentration after tyloxapol injection in hyperinsulinemic conditions in mice after treatment with Obinipitide (0.3 or 3.0 mg/kg), TM30339 (0.3 or 3.0 mg/kg), or vehicle for 4 weeks. Values represent mean $\pm$ SD for at least 10 mice per group. **p < 0.01 vs. control.

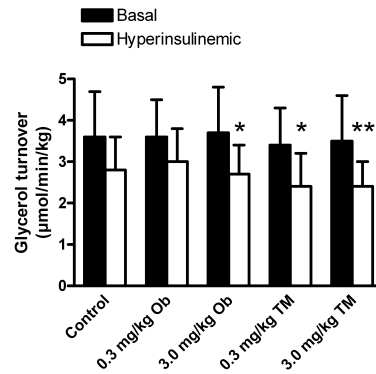


Figure 7. Glycerol turnover during a hyperinsulinemic euglycemic clamp in mice after treatment with Obinipitide (0.3 or 3.0 mg/kg), TM30339 (0.3 or 3.0 mg/kg), or vehicle for 4 weeks. Values represent mean $\pm$ SD for at least 10 mice per group. *p < 0.05 vs. basal. **p < 0.01 vs. basal.

Discussion

This study shows that Obinipitide and TM30339 may be useful tools to improve glucose metabolism in diet-induced insulin resistant C57Bl/6J mice. In particular, chronic administration of Obinipitide (3.0 mg/kg) and TM30339 (0.3 and 3.0 mg/kg) reduced fasting plasma glucose in these animals. Moreover, chronic treatment with these drugs clearly enhanced whole body insulin sensitivity. Both compounds can reinforce insulin's capacity to stimulate glucose disposal and inhibit glucose production. In addition, insulin's capacity to inhibit VLDL-TG production was reinforced by chronic treatment with 3.0 mg/kg TM30339.

The beneficial effects on body weight corroborate the results of previous pre-clinical studies investigating the anti-obesity potential of Obinipitide and TM30339^{20, 21}. All weight was lost in the first week of treatment, which is consistent with earlier findings documenting a transient effect on food intake and body weight of chronic PYY₃₋₃₆ treatment^{11, 27}. Clearly, in time, the body compensates via yet unknown mechanisms to restore energy homeostasis. However, despite the loss of this acute effect, body weight remained lower during treatment with either drug. The decline of body weight was fully accounted for by a reduction of fat mass. Neither Obinipitide nor TM30339 affected lipolysis or insulin's capacity to suppress this process and, accordingly, neither drug changed plasma FFA concentrations. Therefore, the reduction of fat mass can not be

attributed to a lipolytic effect of the drugs. Alternatively, the decline of fat mass may have been the result of a shift in the balance of fuel use in favor of fat oxidation^{11, 17}.

Besides affecting body weight, both drugs clearly ameliorated insulin resistance. In particular, Obinipitide enhanced insulin mediated glucose disposal, an observation that is in line with previous findings of chronic PYY₃₋₃₆ treatment^{16, 17}. Interestingly, our findings also show that agonism of Y4R reinforces insulin's ability to promote glucose disposal, indicating a novel role for this receptor. Moreover, both drugs reinforced insulin action to inhibit EGP. Previous studies showed that activation of Y2 receptors by PYY₃₋₃₆ reinforces insulin mediated glucose disposal, but not its capacity to suppress EGP. Therefore, the impact of Obinipitide on EGP might be mediated by the Y4R. In this respect, it is interesting to note that PP infusion in patients with chronic pancreatitis, a condition accompanied by decreased PP levels, ameliorates insulin resistance of EGP²⁸.

The mechanism underlying the effects of both compounds on insulin sensitivity of glucose metabolism remains to be established. A single injection of both drugs did not affect glucose metabolism in the same experimental context (data not shown). It is conceivable that the drugs work via their impact on food intake and/or body weight²⁹. However, chronic PYY₃₋₃₆ administration enhances insulin-mediated glucose disposal via a mechanism that is independent of food intake and body weight¹⁷. Whatever the mechanism, and highly significant, the present data suggest that chronic treatment with Obinipitide or TM30339 has beneficial metabolic effects in insulin resistant DIO mice, which holds promise for their potential as tools to treat obesity and type 2 diabetes in humans.

These drugs do not appear to modulate VLDL metabolism in basal conditions. However, insulin's capacity to inhibit VLDL-TG production was reinforced by chronic treatment with 3.0 mg/kg TM30339, suggesting that the drug may be able to improve postprandial control of TG production. It is rather surprising that only the highest dose of TM30339 was effective, since the lower dose of TM30339 and 3.0 mg/kg Obinipitide have a similar impact on body weight and glucose metabolism. The mechanism underlying the capacity of TM30339 to reinforce insulin action to reduce VLDL-TG production needs to be clarified in more detail.

Unexpectedly, both Obinipitide and TM30339 increased fasting plasma TG levels after 4 weeks of treatment. In view of the fact that VLDL-TG production in basal condition was not affected by either one of the drugs, this observation suggests that both compounds hamper clearance of VLDL particles from the circulation. Since hypertriglyceridemia is a cardiovascular risk factor, this feature of the drugs is worrisome if confirmed in future (clinical) experiments. It is important to note that comparison between plasma TG levels at 2 vs. 4 weeks of intervention is difficult, since the timing of

drug administration was somewhat different. At 2 weeks, the last injection was given 4 hours before blood sampling, compared to 20 minutes before blood sampling during the VLDL production experiment. The latter may indicate that Obinipitide and TM30339 acutely increased plasma TG levels. Increased TG levels have been observed previously in chow-fed transgenic mice over-expressing PYY¹⁴. When these mice were put on a high-fat diet, TG levels decreased to the level observed in control animals. PYY over-expressing mice on an ob/ob background have decreased plasma TG levels compared to controls. These observations suggest that the metabolic background modulates the impact of Y2R activation (Y2R is the primary receptor of PYY₃₋₃₆) on TG levels. Therefore, it is conceivable that Obinipitide and/or TM30339 impact on plasma TG concentrations in obese animal models differently than in the animal model used here (and perhaps obese humans as well).

In conclusion, the current study indicates that Obinipitide and TM30339 reduce body weight and ameliorate insulin resistance of glucose metabolism in C57Bl/6J mice maintained on a high fat diet. The drugs did not impact on lipolysis, and only the highest dose of TM30339 reinforced the capacity of insulin to inhibit VLDL-TG production. These data hold promise for the potential of these drugs as tools to treat obesity and type 2 diabetes mellitus in humans.

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Chapter 7

Summary and conclusions

Type 2 diabetes mellitus has now become a global epidemic with increasing number of patients every year according to figures of the World Health Organization¹. It is a disorder characterized by high blood glucose concentrations in the context of insulin resistance and relative insulin deficiency. While it is often initially managed by increasing exercise and dietary modification, medications are typically needed as the disease progresses. The past decade hormones from the gastrointestinal tract have gained much interest as potential new therapeutic drug targets in the battle against type 2 diabetes. Gut peptides play an important role in regulating food intake and energy homeostasis. In addition, via overlapping pathways, they are able to impact on insulin sensitivity. Therefore, and with the increasing incidence of obesity, which can lead to several chronic diseases, including type 2 diabetes, these peptides became even more interesting for therapeutic interventions. Since the physical properties (mainly their short-half lives) of native gut hormones do not ultimately allow for therapy, pharmaceutical companies are now developing peptide analogues with improved qualities. The present thesis focuses on modulation of insulin sensitivity of glucose and lipid metabolism by different gut hormones or their analogues.

In the current thesis all experiments were performed in wild-type male C57Bl/6 mice, except for chapter 4, in which APOE*3-Leiden mice were used. The C57Bl/6 mice are very susceptible to diet-induced insulin resistance, and therefore a suitable model to study modulation of insulin sensitivity by peptide/drug interventions. Insulin sensitivity was measured by the hyperinsulinemic euglycemic clamp technique, considered the golden standard method for quantification of insulin action. VLDL production was determined after blocking lipolysis.

Recent findings indicate that gut hormones, like PYY₃₋₃₆ and GLP-1, target the brain to regulate food intake, and in addition, impact on insulin sensitivity. Another gut hormone, OXM, has also been shown to affect food intake and appetite. Its effects on glucose metabolism are rather unexplored. In **chapter 2** we demonstrate that OXM beneficially affects glucose metabolism in diet-induced insulin-resistant C57Bl/6 mice. These effects were found at circulating insulin levels that were more than double during insulin infusion in OXM-treated mice, complicating the interpretation of the data. Indeed, per unit of ambient insulin concentration, glucose uptake and EGP were not different in OXM and placebo-treated animals. The exact mechanism via which OXM affects glucose metabolism remains to be elucidated. OXM is co-released with GLP-1 from intestinal L-cells into the blood in response to food ingestion in amounts proportional to caloric content. After being released, it has to bind its receptor to initiate a reaction; however no unique receptor for OXM has been identified yet. Since the actions of OXM resemble those

described for GLP-1 (it also inhibits meal-stimulated gastric acid secretion and food intake), OXM is believed to act via the GLP-1 receptor, although it also, with a much lower affinity, can bind the glucagon receptor (Gcgr). The anorectic actions of OXM require the GLP-1 receptor only, as centrally administered OXM inhibits food intake in mice that lack the Gcgr, but the anorectic effects of OXM are completely absent in GLP-1R^{-/-} mice². Furthermore, OXM exerts its effects on the islet β -cell in a GLP-1R dependent manner; it stimulates glucose-dependent insulin secretion and activates cell survival pathways leading to reduced β -cell apoptosis³. In addition, the amelioration of glucose tolerance we found was confirmed to be mediated via the GLP-1 receptor³. Although the precise mechanisms utilized by OXM for transduction of various biological actions remain unclear, a few studies demonstrate a dissociation between actions of OXM and those of GLP-1^{4,5}. Therefore, the importance of OXM as a biologically active peptide would be greatly strengthened by the identification of a separate OXM receptor, or by studies employing specific OXM antagonists or immunoneutralizing antisera that block actions of OXM but not glucagon or GLP-1. However, the fact that OXM can improve glucose tolerance in high fat fed, insulin resistant mice indicates that this peptide (or any novel peptidomimetic) may be useful in the treatment of obesity and type 2 diabetes, like other proglucagon derived peptides. Unexpectedly, OXM enhanced glucose production in the basal condition, probably by virtue of its capacity to activate the glucagon (or a yet unidentified OXM) receptor in the liver. This augmented increase in basal glucose turnover by OXM has not been described before for GLP-1. Indeed, in chapter 3, the GLP-1 analogue CNT0736 even decreases glucose turnover in basal conditions. Obviously, stimulation of glucose production is an undesirable quality of drugs used to treat insulin resistance that requires careful evaluation. At the end of the day, if it turns out to be a consistent metabolic effect of treatment with OXM (mimetics), excessive glucose production clearly limits the clinical applicability of this peptide.

The actions of native GLP-1 (it enhances glucose-stimulated insulin secretion, improves blood glucose profiles of type 2 diabetes patients, reduces body weight and food intake, and slows gastric emptying) were the main reasons for the physiological and pharmacological interest in this peptide. Clinical trials have shown that exendin-4, a potent GLP-1 receptor agonist, is useful in the regulation of glucose homeostasis in people with type 2 diabetes mellitus. A disadvantage of Byetta, the marketing name of Exenatide (the synthetic version of exendin-4), is the twice daily injection within 60 minutes of food intake. Therefore, the development of GLP-1 analogues continues. CNT0736 is one of those novel analogues. It retains many activities of native GLP-1 yet has a significantly enhanced pharmacokinetic profile. Moreover, it has a longer half-life than exendin-4 in mice (15-20h vs. ~2h) and therefore might allow for fewer injections in patients. In

chapter 3 the effects of this novel GLP-1 analogue were investigated with regards to its impact on insulin sensitivity of glucose and lipid metabolism. Our data corroborate earlier reports indicating that GLP-1 and its analogues in the long run ameliorate whole body insulin resistance in obese animal models and in T2DM patients⁶⁻⁹. They further extend our knowledge of the precise actions of GLP-1 analogues on distinct components of glucose flux in insulin resistant animals, inasmuch as they show that these compounds acutely reinforce insulin's ability to promote glucose disposal and boost insulin action on both glucose uptake and production in the long term. Since insulin resistance is a complex disease and is associated with obesity and cardiac disease, drug intervention that can handle multiple aspects of the disease are preferable. Therefore, the observations that chronic CNTO736 treatment decreases body weight and inhibits VLDL-TG synthesis are very promising. Since the C57Bl/6 mice are not a suitable model for studying lipid metabolism, we further explored the inhibitory effect of GLP-1M (the latest version of the CNTO molecule) on VLDL-TG production in **chapter 4** in APOE*3-Leiden mice. APOE*3-Leiden mice express the human APOE*3-Leiden gene, resulting in a lipoprotein profile reminiscent of that of patients with dysbetalipoproteinemia, marked by elevated plasma cholesterol and triglyceride levels that are mainly confined to the VLDL/LDL-sized lipoprotein fraction. In contrast to other mice, APOE*3-Leiden transgenics respond to various hypolipidemic drugs in a similar way as humans and are therefore considered a "human-like" animal model for studies evaluating the impact of drugs on hyperlipidemia^{10, 11}. Chronic administration of GLP-1M in high-fat-fed APOE*3-Leiden mice clearly inhibited VLDL-TG production. Moreover, chronic administration of the drug decreased total cholesterol levels and increased HDL in the same experimental context. Our data suggest that GLP-1M may have powerful anti-atherogenic properties in insulin resistant subjects. Atherosclerosis is driven by increased (VLDL) cholesterol and TG levels, often accompanied by low circulating HDL concentrations. HDL particles are anti-atherogenic, partly because of their role in reverse cholesterol transport, but also because of their anti-oxidative, anti-inflammatory, anti-thrombotic, and anti-apoptotic properties¹²⁻¹⁴. Therefore, the reduction of plasma cholesterol concentration by GLP-1M as well as its capacity to increase HDL cholesterol levels holds promise as pharmacological tool to prevent atherosclerosis in high risk metabolic conditions. However, the pathophysiological consequence of the capacity of GLP-1M to inhibit VLDL secretion is uncertain, because circulating TG levels were not affected, perhaps as a result of a diminished clearance of TG from plasma.

Notably, although exendin-4 did impact on insulin sensitivity to a similar extent as CNTO736 in **chapter 3**, it did not impact on VLDL-synthesis in our experimental set-up. This suggests that GLP-1M may have compound-specific effects on lipid metabolism that

potentially reinforce its capacity to prevent macrovascular disease in high fat-fed mice (and humans). On the other hand, it might be related to the serum half-life of GLP-1M, which is considerably longer than exendin-4. This issue needs to be clarified before a firm conclusion can be drawn on the effects of GLP-1M on VLDL metabolism.

All peripheral signals from the gastrointestinal tract convey metabolic information to the brain. The main structures involved in the control of glucose homeostasis are the hypothalamus and brainstem. The hypothalamus is the first integration centre of hormonal information. Specifically, the arcuate nucleus (ARC), located in the mediobasal hypothalamus, contains neurons sensitive to both hormones (ghrelin, leptin, GLP-1) and nutrients (glucose, fatty acids). Downstream targets of the ARC include the paraventricular and dorsomedial hypothalamus nuclei. Other projections of the ARC target the medialpreoptic area, lateral hypothalamus and ventromedian area. Finally, the structure of the limbic system and cortex also interact with the hypothalamus. It is currently unknown to what extent the (metabolic) effects of circulating GLP-1 are mediated by central GLP-1 receptors. In **chapter 5**, we show that GLP-1 treatment ameliorates insulin resistance of glucose metabolism in high-fat-fed mice in part via activation of central GLP-1 receptors. The neuronal circuits that are activated by peripheral administered GLP-1 and mediate its metabolic effects remain to be identified.

One possibility is that GLP-1 in the hypothalamus modulates the neuropeptide Y (NPY) pathway. It has been shown previously that intracerebroventricular administration of GLP-1 completely prevents the orexigenic effects of NPY, suggesting that GLP-1 acts by blocking NPY transmission to inhibit food consumption¹⁵. Intracerebroventricular administration of NPY acutely impairs insulin's ability to suppress endogenous glucose production¹⁶. In addition, animal models of obesity and type 2 diabetes (including diet-induced obesity) are marked by elevated NPY expression in hypothalamic nuclei^{17, 18}. For these reasons, it is conceivable that these exceedingly active NPY neurons are involved in the pathophysiology of enhanced endogenous glucose production in the face of hyperinsulinemia in these models (and obese humans). GLP-1 has been reported to reduce hypothalamic NPY neuronal activity¹⁹. Therefore, chronic administration of GLP-1 or any of its analogues may have antagonized NPY-induced insulin resistance to explain the findings presented here.

The hypothalamus is, however, not solely responsible for the integration of peripheral information originating from the gut. The brainstem, in addition to interacting with hypothalamic circuits, also plays a principal role in the regulation of energy homeostasis. Located in the brainstem, the nucleus of the solitary tract (NTS) receives vagal afferents from the gastrointestinal tract. Therefore, GLP-1 (or its analogues) may

activate neurons in the NTS in the brain stem via afferent vagal inputs to modulate glucose metabolism. GLP-1 evokes vagal afferent nerve activity to initiate a hepatopancreatic reflex that is critically involved in the control of insulin release²⁰. It is conceivable that vagal afferent output in this context is not limited to the pancreas also but affects liver and other visceral organs to modulate glucose production. Moreover, vagal ablation attenuates the anorexigenic effects of peripheral GLP-1 administration²¹. In analogy, activation of vagal afferents by GLP-1 may affect glucose metabolism. Besides affecting afferent fibres, peripheral GLP-1 may also impact directly on the brainstem. Like the ARC, the NTS is in close anatomical proximity to a circumventricular organ with an incomplete blood-brain-barrier - the area postrema (AP) - and is therefore in an ideal position to respond to peripheral circulating signals, such as GLP-1. Indeed, administration of GLP-1 results in a significant increase in signal intensity in the AP of fasted mice, reflecting an increase in neuronal activity within the brainstem²².

However, despite the compelling evidence of the importance of the gut-brain axis in the control of glucose homeostasis, it remains possible that GLP-1 acts directly on the liver and other peripheral tissues via a structurally and functionally distinct GLP-1R. Indeed, GLP-1 was reported to have insulin-like actions in liver and skeletal muscle, which are not mediated by the classical GLP-1R^{23, 24}.

Finally, in **chapter 6**, the potential of two novel developed analogues, Obinipitide and TM30339, was explored. The results show that chronic treatment with these drugs beneficially impacts on insulin resistance in high-fat-fed mice, with respect to insulin mediated glucose disposal and production. Both compounds, in analogy with its property as anti-obesity drugs, also reduced body weight (by decreasing total fat mass) in our experimental set-up. Since insulin sensitivity and weight loss are strongly correlated²⁵, the effect on the later might be the underlying cause for the reduction in insulin resistance. Pair-feeding studies may provide the answer. Regardless of the mechanism, and highly significant, our results indicate that the metabolic effects of Obinipitide and TM30339 do not vanish during chronic treatment, making them valuable drugs to treat both obesity and insulin resistance.

In conclusion, we have shown a variety of gut hormones or its peptidomimetics to beneficially impact on glucose metabolism. However, our animal models still raise questions, which need to be clarified in more detail, before the drugs can be used in the clinical setting. Long-term treatment may also have side-effects, related to gastric emptying and nausea, which have been reported before for GLP-1 and related molecules. Also, in response to post-marketing reports of acute pancreatitis in patients using

Exenatide, the U.S. Food and Drug Administration (FDA) added a warning to the labeling of Byetta in 2007. In August 2008, four additional deaths from pancreatitis in users of Exenatide were reported to the FDA; while no definite relationship had been established, the FDA was reportedly considering additional changes to the labeling of this drug.

Over the past decade a growing amount of evidence indicates that the gut-brain axis is a key player in the control of glucose homeostasis. Peptides such as GLP-1 have emerged as key factors able to convey metabolic information to the brain and also to modulate endogenous glucose production and disposal. Elucidating more precisely the molecular events in the brain that underlie the effects of these hormones could lead to the identification of new (or improved) therapeutic agents.

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Samenvatting en conclusies

Type 2 diabetes mellitus (suikerziekte) is aan het uitgroeien tot een wereldwijde epidemie met een toenemend aantal patiënten elk jaar volgens cijfers van de Wereldgezondheidsorganisatie¹. Het is een aandoening gekarakteriseerd door een hoge bloedsuikerspiegel (hyperglycemie) in combinatie met ongevoeligheid voor insuline (insulineresistentie) en een betrekkelijk insulinetekort. Daar waar de ziekte in het beginstadium onder controle kan worden gehouden door meer beweging en een wijziging van het voedselpatroon, is medicatie nodig wanneer de ziekte verergert. Het afgelopen decennium hebben hormonen van het maag-darmstelsel veel interesse geworven om mogelijk als nieuwe therapeutische medicijnen te dienen in the strijd tegen type 2 diabetes. Darmhormonen spelen een belangrijke rol in het reguleren van de voedselinname en de energiehuishouding. Daarnaast, kunnen ze, via overlappende paden, invloed uitoefenen op de insulinegevoeligheid. Om deze redenen, en met het oog op het toegenomen aantal gevallen met obesitas (zwaarlijvigheid), wat tot verscheidene chronische ziektes kan leiden, waaronder type 2 diabetes, werden deze eiwitten nog interessanter voor therapeutische interventies. Omdat de fysische eigenschappen (in het bijzonder hun korte halfwaardetijden) van lichaamseigen darmhormonen vaak niet geschikt zijn om voor therapie te kunnen dienen, worden er nu door farmaceutische bedrijven analogen van deze eiwitten ontwikkeld met verbeterde kwaliteiten. Dit proefschrift richt zich op het beïnvloeden van de insulinegevoeligheid van de glucose- en vetstofwisseling door verschillende darmhormonen en hun analogen.

In dit proefschrift zijn alle experimenten uitgevoerd in mannelijke C57Bl/6 muizen, behalve voor hoofdstuk 4, waarin de APOE*3-Leiden muis werd gebruikt. De C57Bl/6 muis is zeer gevoelig voor dieetgeïnduceerde insulineresistentie en daarom een geschikt model om de effecten op de insulinegevoeligheid na medicijntoediening te bestuderen. De mate van insulinegevoeligheid werd gemeten met de hyperinsulinemische euglycemische clamp techniek, welke beschouwd wordt als de gouden standaard voor het bepalen hiervan. De productie van VLDL (een deeltje dat vet uit de lever naar weefsels transporteert) werd bepaald na het blokkeren van de klaring van vet.

Recente bevindingen laten zien dat darmhormonen, zoals peptideYY₃₋₃₆ en glucagon-like peptide-1 (GLP-1), de voedselinname reguleren via de hersenen, en daarnaast ook, invloed uitoefenen op de insulinegevoeligheid. Van een ander darmhormoon, oxyntomoduline (OXM), is ook bekend dat het de voedselinname en het hongergevoel kan beïnvloeden. Echter, de effecten van OXM op de glucosestofwisseling zijn weinig onderzocht. In **hoofdstuk 2** laten wij zien dat OXM de glucosestofwisseling positief kan beïnvloeden in dieetgeïnduceerde insuline resistente C57Bl/6 muizen. Deze effecten werden gevonden bij circulerende insulineconcentraties die meer dan verdubbeld waren

in OXM behandelde muizen vergeleken met de controle groep, wat de interpretatie van de data gecompliceerd maakt. Inderdaad, wanneer de glucose opname en productie gecorrigeerd werden voor de rondculerende insulineconcentratie, werd geen verschil gevonden tussen OXM en placebo behandelde dieren. Het exacte mechanisme hoe OXM de glucosetofwisseling beïnvloedt moet nog worden opgehelderd. OXM wordt samen met GLP-1 vrijgelaten in het bloed uit de zogenoemde L-cellen van de darm in reactie op voedselinname in hoeveelheden proportioneel aan de calorische hoeveelheid. Na te zijn vrijgekomen, moet OXM zijn receptor binden om een reactie te initiëren; echter tot op heden is er geen unieke OXM receptor gevonden. Omdat de acties van OXM veel gelijkenis tonen met hetgeen beschreven is voor GLP-1 (het remt maaltijdgestimuleerde maagzuursecretie en voedselinname), wordt aangenomen dat OXM via de GLP-1 receptor (GLP-1R) werkt, hoewel het ook, alleen met veel minder affiniteit, aan de glucagon receptor (Gcgr) kan binden. De anorectische acties van OXM vereisen alleen de GLP-1 receptor, aangezien OXM, gegeven in het brein, de voedselinname remt in muizen die geen Gcgr hebben. De anorectische effecten van OXM waren volledig afwezig in muizen zonder GLP-1R². Daarnaast oefent OXM zijn effecten op de β -cel van de alveesklier uit op een GLP-1R afhankelijke manier; het stimuleert glucoseafhankelijk de insulinesecretie en activeert cel overlevingspaden die leiden tot een afname van sterfte van de β -cel³. Ook van de verbetering van de glucosetolerantie die wij vonden, werd bevestigd dat deze via de GLP-1R gestuurd wordt³. Hoewel het precieze mechanisme achter verscheidene biologische acties van OXM nog onduidelijk blijft, zijn er een aantal studies die laten zien dat er ook verschillen zijn tussen de acties van OXM en die van GLP-1^{4, 5}. Daarom zal het belang van OXM als een biologisch actief eiwit flink versterkt worden door het identificeren van een eigen OXM receptor of door studies die gebruik maken van specifieke OXM antagonisten of immumoneutraliserende antisera die de werking van OXM, maar niet van glucagon of GLP-1, blokkeren. Echter, het feit dat OXM de glucosetolerantie in hoog vet gevoede insuline resistente muizen kan verbeteren, geeft aan dat dit eiwit (of elk nieuw ontwikkeld analoog) bruikbaar kan zijn in de behandeling van obesitas en type 2 diabetes, net als andere van proglucagon afkomstige eiwitten (o.a. GLP-1). Echter, geheel onverwacht, verhoogde OXM de glucose productie in nuchtere condities, waarschijnlijk middels zijn capaciteit om de Gcgr (of een nog ongeïdentificeerde OXM receptor) in de lever te activeren. Deze verhoging in glucose productie door OXM is niet beschreven voor GLP-1. In tegenstelling, in **hoofdstuk 3**, verlaagt de GLP-1 analoog CNTO736 zelfs de glucoseconcentratie in basale condities. Het is onmiskenbaar dat de stimulatie van de glucose productie een ongewenste eigenschap is van een medicijn bedoeld om insulineresistentie mee te behandelen en vraagt daarom zorgvuldig vervolgonderzoek. Als aan het eind van de dag blijkt dat dit een consistent effect van OXM

(analogen) behandeling is, dan limiteert overmatige glucose productie de klinische potentie van deze stof.

De eigenschappen van GLP-1 (het verhoogt de glucose-gestimuleerde insuline secretie, verbetert bloedglucose profielen van type 2 diabetes patiënten, vermindert lichaamsgewicht en voedselinname en vertraagt de maaglediging) waren de belangrijkste redenen voor de fysiologische en farmacologische belangstelling van dit eiwit. Klinische studies hebben aangetoond dat exendin-4, een van nature voorkomende GLP-1 analoog, zeer bruikbaar is bij de regulering van de glucosehuishouding in mensen met type 2 diabetes mellitus. Een nadeel van Byetta, de merknaam van exenatide (de synthetische versie van exendin-4), is het tweemaal daags injecteren binnen 60 minuten van de voedselinname. Daarom gaat de ontwikkeling van GLP-1 analogen verder. CNTO736 is één van die nieuwe analogen. Het bezit de vele activiteiten van het oorspronkelijke GLP-1, echter heeft het een aanzienlijk verbeterd farmacokinetisch profiel. Bovendien heeft het een langere halfwaardetijd dan exendin-4 in muizen (15-20 uur vs ~2 uur) wat kan bijdragen aan minder injecties bij patiënten. In **hoofdstuk 3** werden de effecten van deze nieuwe GLP-1-analoog onderzocht met betrekking tot het effect op de insulinegevoeligheid van de glucose- en vetstofwisseling. Onze data bevestigen eerdere rapporten waaruit blijkt dat GLP-1 en zijn analogen op de lange termijn insulineresistentie van het hele lichaam in obese diervormen en in patiënten met type 2 diabetes verbeteren⁶⁻⁹. Verder breiden zij onze kennis uit over de precieze werking van GLP-1 analogen op de afzonderlijke componenten van de glucosehuishouding in insuline resistente dieren, voor zover zij aantonen dat deze stoffen acuut het vermogen van insuline om glucose te verwijderen versterken en op de lange termijn de werking van insuline bevorderen op zowel glucose opname als productie. Aangezien insulineresistentie een complexe ziekte is en geassocieerd is met obesitas en hartziekten, is medicijnbehandeling die meerdere aspecten van de ziekte aangrijpt wenselijk. Daarom zijn de bevindingen dat chronische behandeling met CNTO736 leidt tot een daling van het lichaamsgewicht en een remming van de VLDL-triglyceride productie, veelbelovend. Aangezien de C57BL/6 muis geen geschikt model is voor het bestuderen van de vetstofwisseling, hebben wij het remmende effect van GLP-1M (de nieuwste versie van het CNTO molecuul) op de VLDL-triglyceride productie in **hoofdstuk 4** verder onderzocht in APOE*3-Leiden muizen. APOE*3-Leiden muizen brengen het menselijk APOE*3-Leiden gen tot expressie, wat resulteert in een lipoproteïnen profiel gelijkend aan die van patiënten met dysbetalipoproteinemia, gekenmerkt door verhoogde plasma cholesterol en triglyceriden die voornamelijk beperkt zijn tot de VLDL/LDL-lipoproteïne-fractie. In tegenstelling tot andere muizen, reageren APOE*3-Leiden transgenen op verschillende medicijnen die lipiden niveaus verlagen op een vergelijkbare manier als in de mens en

worden daarom beschouwd als een "humaanachtig" diermodel voor onderzoek naar de effecten van medicijnen op verhoogde vetgehaltes in het bloed^{10, 11}. Chronische toediening van GLP-1M in hoog vet gevoede APOE*3-Leiden muizen remt duidelijk de VLDL-triglyceride productie. Bovendien leidde chronische toediening van het geneesmiddel tot verlaagde cholesterol concentraties en een verhoogd HDL in dezelfde experimentele context. Onze gegevens suggereren dat GLP-1M krachtige anti-atherogene eigenschappen kan hebben in insuline resistente personen. Atherosclerose wordt gedreven door toegenomen (VLDL), cholesterol en triglyceride niveaus, vaak gepaard met lage HDL circulerende concentraties. HDL-deeltjes zijn anti-atherogeen, deels vanwege hun rol in omgekeerd cholesterol transport, maar ook vanwege hun anti-oxidatieve, anti-ontsteking, anti-trombotische en anti-apoptotische eigenschappen¹²⁻¹⁴. Daarom is de verlaging van de plasma cholesterolconcentratie door GLP-1M evenals de capaciteit om HDL-cholesterol niveaus te verhogen, beloftevol voor GLP-1M om als farmacologisch middel aderverkalking te voorkomen in metabole omstandigheden met een verhoogd risico daarop. Echter, de pathofysiologische gevolgen van de capaciteit van GLP-1M om de VLDL secretie te remmen is onzeker, omdat circulerende triglyceride spiegels onaangedaan waren, misschien als gevolg van een verminderde klaring van triglyceride uit het plasma.

Belangrijk te vermelden is dat exendin-4 in **hoofdstuk 3** op een vergelijkbare mate invloed heeft op de insulinegevoeligheid als CNT0736, maar geen invloed heeft op de VLDL-productie in onze experimentele opzet. Dit suggereert dat GLP-1M stofspecifieke effecten kan hebben op de vetstofwisseling die de capaciteit versterken om macrovasculaire ziektes in hoog vet gevoede muizen (en mensen) te voorkomen. Aan de andere kant kan het worden gerelateerd aan de halfwaardetijd van GLP-1M in het bloed, welke aanzienlijk langer is dan exendin-4. Dit vraagstuk moet worden opgehelderd voordat een kordate conclusie kan worden getrokken over de effecten van GLP-1M op de vetstofwisseling.

Alle perifere signalen van het maag-darmkanaal brengen metabole informatie over naar de hersenen. De belangrijkste structuren die betrokken zijn bij de controle van de glucosehuishouding zijn de hypothalamus en de hersenstam. De hypothalamus is het eerste integratiecentrum van hormonale informatie. Met name de nucleus arcuatus (ARC), gelegen in de mediobasale hypothalamus, bevat neuronen die gevoelig zijn voor zowel hormonen (ghreline, leptine, GLP-1) als voedingsstoffen (glucose, vetzuren). Verdere doelstructuren van de ARC zijn de kernen van de paraventriculaire en dorsomediale hypothalamus. Andere projecties van de ARC hebben het mediale preoptische gebied, de laterale hypothalamus en het ventromediane gebied als doel. Ten slotte hebben de structuur van het limbische systeem en de cortex ook interactie met de hypothalamus.

Het is momenteel niet bekend in hoeverre de (metabole) effecten van circulerend GLP-1 worden gereguleerd door centrale GLP-1-receptoren. In **hoofdstuk 5** laten we zien dat GLP-1 behandeling de insulineresistentie van de glucosetofwisseling in hoog vet gevoede muizen vermindert voor een deel via de activering van GLP-1 receptoren gelegen in de hersenen. De neuronale circuits die worden geactiveerd door de toediening van GLP-1 en zijn metabole effecten reguleren, moeten nog worden geïdentificeerd.

Een mogelijkheid is dat GLP-1 in de hypothalamus de neuropeptide Y (NPY) route moduleert. Het is eerder gebleken dat directe toediening van GLP-1 in de hersenen de stimulerende effecten van NPY op de voedselinname volledig voorkomt, wat suggereert dat GLP-1 werkt door het blokkeren van NPY signalen om voedselconsumptie te remmen¹⁵. Acute toediening van NPY in de hersenen vermindert het vermogen van insuline om de endogene glucose productie te onderdrukken¹⁶. Bovendien worden diermodellen van obesitas en type 2 diabetes (met inbegrip van dieetgeïnduceerde zwaarlijvigheid) gekenmerkt door verhoogde NPY expressie in de hypothalamaire kernen^{17, 18}. Om deze redenen is het denkbaar dat deze verhoogd actieve NPY neuronen betrokken zijn bij de pathofysiologie van verhoogde endogene productie van glucose in de aanwezigheid van hyperinsulinemie in deze modellen (en obese mensen). Eerder is laten zien dat GLP-1 in de hypothalamus de neuronale activiteit van NPY kan verminderen¹⁹. Daarom kan chronische toediening van GLP-1 of één van zijn analogen de door NPY geïnduceerde insulineresistentie hebben tegengewerkt om de bevindingen die we hier laten zien te verklaren.

De hypothalamus is echter niet alleen verantwoordelijk voor de integratie van perifere informatie afkomstig uit de darm. De hersenstam, in aanvulling op de interactie met de hypothalamaire paden, speelt ook een belangrijke rol in de regulering van de energiehuishouding. Gelegen in de hersenstam, ontvangt de nucleus tractus solitarius (NTS) via de vagus zenuw signalen uit het maag-darmkanaal. Daarom is het mogelijk dat GLP-1 (of zijn analogen) neuronen in de NTS in de hersenstam activeert via deze signalen om de glucosetofwisseling te moduleren. Ook veroorzaakt GLP-1 vagale afferente zenuwactiviteit om een hepatopancreatische reactie te veroorzaken, welke een belangrijke rol speelt bij de controle van het vrijkomen van insuline²⁰. Het is denkbaar dat vagale afferente activiteit in dit verband niet beperkt is tot de alvleesklier, maar ook van invloed is op de lever en andere organen om de viscerale productie van glucose te reguleren. Bovendien vermindert vagale ablatie de anorectische effecten van perifere GLP-1 toediening²¹. In analogie, kan activering van de vagale afferentie door GLP-1 de glucosetofwisseling beïnvloeden. Naast invloed op afferente vezels, kan perifeer GLP-1 ook rechtstreeks invloed hebben op de hersenstam. Net als de ARC, is de NTS gelegen in nauwe anatomische nabijheid van een circumventriculair orgaan met een onvolledige

bloed-hersen-barrière - het area postrema (AP) - en is daardoor in een ideale positie om te reageren op circulerende perifere signalen, zoals GLP-1. Inderdaad, toediening van GLP-1 leidt tot een aanzienlijke toename van de signaalintensiteit in de AP van nuchtere muizen, wat duidt op een toename van de neuronale activiteit in de hersenstam²².

Ondanks de toenemende data van het belang van de darm-hersen-as in het controleren van de glucosehuishouding, blijft het mogelijk dat GLP-1 rechtstreeks op de lever en andere perifere weefsels werkt via een structureel en functioneel verschillende GLP-1R. Eerder is gerapporteerd dat GLP-1 insulineachtige acties heeft in lever en skeletspieren, die niet toe te schrijven zijn aan de bekende GLP-1R^{23, 24}.

Ten slotte werd in **hoofdstuk 6**, het potentieel van twee nieuwe ontwikkelde analogen, Obinipitide en TM30339, onderzocht. De resultaten laten zien dat chronische behandeling met deze geneesmiddelen gunstige effecten heeft op de insulineresistentie in hoog vet gevoede muizen, met betrekking tot de insuline gereguleerde glucose opname en productie. Beide stoffen, in overeenstemming met hun eigenschappen als anti-obesitas geneesmiddelen, verminderden ook het lichaamsgewicht (door het verlagen van de totale vetmassa) in onze experimentele opzet. Aangezien insulinegevoeligheid en gewichtsverlies sterk gecorreleerd zijn²⁵, zou het effect van het laatste de onderliggende oorzaak kunnen zijn voor de vermindering van de insulineresistentie. Gepaard gevoede studies kunnen hierop het antwoord geven. Echter, onafhankelijk van het mechanisme, en zeer belangrijk, laten onze resultaten zien dat de metabole effecten van Obinipitide en TM30339 niet afzwakken tijdens chronische behandeling, wat ze waardevolle therapieën maken om zowel obesitas als insulineresistentie te behandelen.

Concluderend, hebben we van een verscheidenheid aan darmhormonen of analogen daarvan aangetoond dat ze gunstige effecten hebben op de glucosetofwisseling. Onze diermodellen roepen echter nog steeds vragen op die in meer detail moeten worden verduidelijkt, alvorens de stoffen kunnen worden gebruikt in de klinische setting. Langdurige behandelingen kunnen ook bijwerkingen uiten, welke gerelateerd zijn aan maaglediging en misselijkheid, die gemeld zijn voor GLP-1 en verwante moleculen. In reactie op de postmarketing meldingen van acute alvleesklierontsteking bij patiënten die exenatide gebruiken, heeft de Amerikaanse Food en Drug Administration (FDA) een waarschuwing aan de etikettering van Byetta toegevoegd in 2007. In augustus 2008 werden vier extra sterfgevallen als gevolg van alvleesklierontsteking bij gebruikers van exenatide gerapporteerd aan de FDA. Hoewel er geen duidelijke relatie werd vastgesteld, overweegt de FDA naar verluidt aanvullende wijzigingen op de etiketten van dit medicijn.

In de afgelopen tien jaar wijst een toenemende hoeveelheid bewijsmateriaal erop dat de darm-hersen-as een belangrijke speler is in het controleren van de glucosehuishouding. Peptiden zoals GLP-1 zijn aan het licht gekomen als belangrijke factoren die metabole informatie kunnen overbrengen naar de hersenen en bovendien de endogene glucose productie en opname reguleren. Het meer in het detail ophelderen van de moleculaire gebeurtenissen in de hersenen die ten grondslag liggen aan de effecten van deze hormonen kan leiden tot de identificatie van nieuwe (of betere) therapeutische medicijnen.

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Curriculum Vitae



Edwin Tijmen Parlevliet werd geboren op 4 oktober 1981 te Katwijk (Zuid-Holland). In 2000 behaalde hij zijn VWO diploma aan het Andreas College locatie Pieter Groen te Katwijk. In datzelfde jaar begon hij aan de studie Biomedische Wetenschappen aan de Universiteit Leiden. Hiervoor werd in 2001 de propedeuse behaald. In 2003 werd het Bachelor of Science behaald. In het kader van de Master of Science opleiding volgde hij in 2003 een stage op de afdeling Endocrinologie van het Leids Universitair Medisch Centrum (LUMC), waarin hij onderzoek deed naar nieuwe target genen van PTHrP tijdens de botgroei onder begeleiding van Drs. J. Hoogendam en prof. M. Karperien. In 2004 volgde een afstudeerstage bij de afdeling Biomedisch onderzoek van TNO Kwaliteit van Leven in Leiden, onder begeleiding van Drs. A.C. Heijboer. Deze wetenschappelijke stage richtte zich op effecten van het darmhormoon oxyntomoduline op het glucose metabolisme in de muis, waar over ook de afstudeerscriptie werd geschreven.

Na zijn afstuderen in 2005 werd hij in oktober van dat jaar aangesteld als promovendus op de afdeling Endocrinologie van het LUMC. Onder begeleiding van Prof. Dr. H. Pijl werd het in het proefschrift beschreven onderzoek verricht. In 2008 ontving hij de EFSD/ Amylin Paul Langerhans Grant.

