EXPLORING THE ROLE OF GLUCAGON IN GLUCOSE HOMEOSTASIS

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CHAPTER 1

General introduction and outline of the thesis

GLUCOSE HOMEOSTASIS

The pancreatic islets of Langerhans secrete the reciprocal hormones insulin and glucagon which are responsible for controlling of the glucoregulatory feedback-loop and tightly control blood glucose levels (Figure 1). Insulin is produced by the ß-cells of the pancreas and was first discovered in 1921 by Dr. Frederick Banting and Charles Best. Shortly after the discovery of insulin, glucagon hormone, which is secreted from pancreatic α-cells, was discovered by Kimball and Murlin [1].

INSULIN REGULATES CARBOHYDRATE, PROTEIN AND FAT METABOLISM IN THE BODY

Insulin binds to tyrosine kinase receptors at the cell surface. Insulin inhibits hepatic glucose production and stimulates glycogenesis, the process of glycogen synthesis for storage in liver and muscles. Glucose uptake is increased by insulin, which stimulates the transport of vesicles containing glucose transporters towards the cell membrane. Furthermore, insulin inhibits glucagon secretion from pancreatic α-cells, thereby decreasing hepatic glucose production (gluconeogenesis and glycogenolysis). Adipose tissue is exquisitely sensitive to the inhibitory effect of insulin on lipolysis. Insulin decreases the release of nonesterified fatty acids and glycerol from adipose tissue and gluconeogenic precursors from skeletal muscles, thus causing a decrease in precursor supply for hepatic gluconeogenesis.

GLUCAGON PROTECTS AGAINST HYPOGLYCEMIA

The main role of glucagon is to protect the body and in particular the brain from low glucose levels during periods of fasting. In healthy subjects, glucagon levels are only elevated in the fasting state, because low glucose levels are the most important physiologic stimulator of glucagon secretion. Glucagon mediates its effects by binding to and activating the glucagon receptor (GCGR), a member of the class B family of heptahelical GTP-binding protein coupled receptors [2]. Stimulation of these receptors results in activation of adenylate cylase and increased levels of intracellular cyclic adenosine monophosphate (cAMP). Glucagon receptors are mainly expressed in the liver and kidney with lesser amounts in heart, adipose tissue, adrenal glands, pancreas, cerebral cortex and gastrointestinal tract [2;3]. The role of GCGR in glucose homeostasis has been studied in mice lacking the receptor, which show slightly reduced plasma levels of glucose and insulin. Agonism at the glucagon receptor results in glycogenolysis, gluconeogenesis, proteolysis and lipolysis.

INCRETINS

There are two main incretin hormones in humans, GLP-1 (glucagon-like peptide-1) and GIP (glucose-dependent insulinotropic peptide, also known as gastric inhibitory peptide). Both hormones are secreted by endocrine cells that are located in the epithelium of the small intestine. Food intake, and also stimulation of the sympathetic nervous system (for example physical exercise), stimulate the secretion of GLP-1 and is rapidly inactivated by enzyme DPP-IV (Dipeptidyl peptidase-IV). The mechanism of incretin action is outlined in Figure 2. GLP-1 stimulates the production and secretion of insulin, the release of somatostatin, and glucose utilization by increasing insulin sensitivity. It inhibits glucagon release, gastric emptying, appetite, and food intake via the central nervous system [4].

BALANCE BETWEEN GLUCOSE PRODUCTION AND UTILIZATION

In the postabsorptive state plasma glucose levels are the result the balance between the rates of glucose production and glucose utilization [5]. Each of these processes is tightly regulated by the levels of hormones and substrates in blood. Glucose is produced by both the liver (90%) and the kidneys (10%). The kidneys take up ~10% of the glucose produced, so that in a net sense they do not supply glucose to the other tissues of the body. Therefore, the liver is responsible for providing glucose to both insulin-insensitive (neural tissues, formed elements of the blood, skin, smooth muscle, etc.) and insulin-sensitive (skeletal muscle and fat) tissues. The control of hepatic glucose production (HGP) by the liver serves as a primary regulatory event of glucose homeostasis. In normal physiology the liver maintains blood glucose homeostasis by rapid clearance of glucose from the portal vein in the absorptive state after a meal (glycogenesis), and by controlled production of glucose (gluconeogenesis & glycogenolysis) in the fasted state at a sufficient rate to maintain euglycemia (blood glucose level 4.5-6.5 mmol/L) [6].

DIABETES MELLITUS TYPE 2 (T2DM)

Type 2 diabetes mellitus (T2DM) is a characterized by a combination of resistance to insulin action in target tissues and inadequate compensatory insulin secretion. In the absence of a defect in ß-cell function, individuals can compensate for insulin resistance with appropriate hyperinsulinemia. However, in a later stage of the disease, a decline in pancreatic ß-cell function (relative insulin deficiency) ultimately leads to postprandial and fasting hyperglycemia

that characterizes T2DM. Insulin resistance prolongs the duration of postprandial hyperglycemia, which can be marked when both hepatic and extra-hepatic insulin resistance are present [7]. Studies using hyperinsulinemic euglycemic clamps show impaired suppression of hepatic glucose production by hyperglycemia [8] and by elevated insulin levels [9], indicating that intrahepatic changes in glucose metabolism and in responsiveness to hyperglycemia and insulin contribute to the increase in the hepatic glucose threshold and insulin resistance.

In addition, subjects with T2DM have elevated fasting glucagon concentrations that do not decrease appropriately, and can even paradoxically increase, after food ingestion [10-13]. During fasting conditions in T2DM patients, hyperglucagonemia sustains gluconeogenesis and glycogenolysis in the liver, contributing to increased fasting blood glucose levels [14]. Similarly, increased glucagon responses after food ingestion, result in inadequate suppression of hepatic glucose production, contributing to increased postprandial glucose levels [11]. This paradoxical glucagon response may be explained by an impaired suppressive effect of glucose on the α-cell in T2DM and by gastrointestinal factors as reduced incretin effect observed in patients with T2DM [15]. Knop *et al.* showed attenuated and delayed glucagon suppression in T2DM after oral ingestion of glucose, where intravenous administration of the same amount of glucose results in normal suppression of glucagon, supporting this hypothesis [15].

GLYCAEMIC CONTROL / TREATMENT OF T2DM

The Diabetes Control and Complications Trial [16] and the U.K. Prospective Diabetes Study [17;18] have documented that strict glycemic control effectively reduces the risk of developing microvascular (diabetic nephropathy, neuropathy, and retinopathy) and, to a lesser extent, macrovascular (coronary artery disease, peripheral arterial disease, and stroke) complications of diabetes. Current treatments for T2DM are focused on increasing insulin secretion or improving insulin sensitivity. Lifestyle modifications such as diet, are considered the first line of treatment to halt or delay further progression of the disease [19]. In addition to energy intake restriction, specific food components like amino acids and proteins can be applied to more directly modulate glycemic control. The possibility that substances other than glucose could stimulate insulin secretion was first reported by Cochrane *et al*. in 1956 [20]. Subsequently, many studies have demonstrated that the combined intake of carbohydrate and protein induced a higher insulin response than the intake of carbohydrate alone [21;22], both in healthy subjects [23-25] and in T2DM patients [26-31].

CURRENT INSULIN-BASED THERAPIES

Standard pharmacological, insulin-based, treatment regimens for T2DM are oral blood glucose lowering medication as biguanides, sulfonylureas, and/or thiazolidinediones. In the 1950's, the biguanide drug metformin was introduced for the treatment of diabetes as insulin sensitizer by increasing the efficiency of glucose transporters and lowering glycated hemoglobin (HbA1c) by 1–2% [32]. Gastrointestinal side-effects occur to varying degrees in up to 30% of patients. Sulphonylureas act mainly by stimulating insulin release from the ß-cells of the pancreas and may also improve insulin resistance in peripheral target tissues. These drugs reduce concentrations of HbA1c by 1–2% and fasting plasma glucose (FPG) concentrations by 3.3–3.9 mmol/L [33]. Hypoglycemia is the most worrisome side effect of the sulfonylureas. Thiazolidinediones (TZD), such as pioglitazone, have been associated with a 0.5 to 1.5 % reduction in HbA1c levels and 1.4 to 2.8 mmol/L reductions in FPG levels. TZD's are contraindicated in patients with (a history of) heart failure and should be used with caution in women at high risk of fractures [34]. Currently, TZDs are hardly prescribed any more in the Netherlands. In a more advanced stage of the disease, exogenous subcutaneous insulin therapy or a combination of subcutaneous insulin with oral drugs will be prescribed. Although insulin therapy results in a 1-2% reduction of HbA1c, it is accompanied by weight gain, a significant risk of hypoglycemia and an increased risk of cancer in patients taking long-acting insulin [32].

CURRENT INCRETIN-BASED THERAPIES

Almost 10 years ago, incretin-based therapies (GLP-1 analogs or DPP-IV inhibitors) were introduced on the market for overweight T2DM patients [35]. T2DM patients display an impaired incretin effect and higher GLP-1 levels can be achieved using GLP-1 analogs, GLP-1 receptor agonists produced by recombinant DNA technology or DDP-IV inhibitors which prolong the half-life of endogenous GLP-1 by preventing its enzymatic degradation. In 2005 exenatide was the first FDA (Food and Drug Administration, USA) approved drug that uses the 'incretin effect'. Currently available GLP-1 analogs exenatide and liraglutide have to be administered subcutaneously; exenatide twice daily and liraglutide once a day. Recently, exenatide has been developed in an extended-release formulation which can be used once weekly. Clinical trials with exenatide [36-39] showed significant reductions in HbA1c of approximately 1.0–1.2% when compared to placebo, and a modest reduction in fasting plasma glucose (FPG) of approximately 1.0–1.4 mM. The average weight loss amounted to 1.6 kg in the exenatide-treated groups. Liraglutide significantly lowered HbA1c by 0.8–1.5%, FPG with up to 2.6 mM, and induced a weight loss in the range of 2 to 3 kg compared to the placebo-treated group [39-41]. In LEAD 6 study both available

GLP-analogs were compared [42]. The mean reduction in HbA1c levels was significantly higher with liraglutide 1.8 mg once daily than with exenatide 10 mg twice daily (−1.12% versus −0.79%; p<0.001). The most common adverse events associated with GLP-1 mimetics are gastrointestinal. Safety concerns have been raised during the development of liraglutide; a small number of cases of pancreatitis have been reported [43], while the initial concerns regarding thyroid C-cell tumors have not been confirmed in humans [44]. In contrast to GLP-1 receptor agonists, DPP-IV inhibitors are orally available and have a longer duration of action, requiring only once daily dosing. Sitagliptin, vildagliptin, saxagliptine, and linagliptine as monotreatment and also combined with metformin are currently available in the Netherlands. These drugs control hyperglycemia, reduce HbA1c concentrations by ~1%, and improve pancreatic ß-cell function. DPP-IV inhibitors are generally safe and well-tolerated with a low risk of hypoglycemia, but do not reduce appetite or cause weight loss such as GLP-1 agonists [45]. The long-term safety and effects of GLP-1 analogs and DPP-IV inhibitors have not been established yet.

SGLT2 INHIBITORS IN THE TREATMENT OF TYPE 2 DIABETES

Agents that inhibit sodium glucose co-transporter 2 (SGLT2) in the kidney represent a novel class of drugs, which has become available for treatment of T2DM since 2012. The SGLT2 transporter protein is found only in renal epithelium cells of the proximal tubule, and mediates the majority (~90%) of glucose reabsorption along the nephron. Pharmacological inhibition of SGLT2 increases urinary glucose excretion and decreases plasma glucose levels in an insulin-independent manner [46]. Hypoglycemic episodes are less likely, because of the insulin independence of their action plus the fact that these compounds only lower the glucose re-absorption threshold without completely blocking renal glucose reabsorption. In November 2012 the first SGLT2 inhibitor dapagliflozine was introduced on the market in Europe, followed by canagliflozine in September 2013. In the USA canagliflozin became the first SGLT2 inhibitor (approved March 2013), followed by dapagliflozine which was approved in January 2014 by the FDA. Clinical trials on these two agents have shown significant and sustained HbA1c reduction of 0.5-1% when used as monotherapy or in combination with other antidiabetic agents [47]. Comparing other antidiabetic drugs, the major disadvantage of SGLT2 inhibitors is the increased risk of genital mycotic infections and urinary tract infections, particularly in women. Additionally, since the efficacy of SGLT2 inhibitors requires adequate filtered load of glucose in the kidney, their efficacy diminishes in renal impairment. Multiple other SGLT2 inhibitors are currently in clinical development such as small molecules empagliflozin, ipragliflozin, LX4211 (a novel dual inhibitor of SGLT1 and SGLT2 glucose transporters), and antisense oligonucleotide targeting the human SGLT2 transporter [48].

NOVEL MECHANISM(S) OF ACTION FOR THE TREATMENT OF DIABETES TYPE 2

Despite all currently available pharmacological therapies, treatment of patients with diabetes is not completely successful in restoring control of glucose metabolism. Thus, there remains a need for agents with novel mechanism(s) of action. Nowadays, there is more attention to the role of liver in the pathogenesis of diabetes, reduction of hepatic glucose production has been targeted as a strategy for diabetes treatment and can be achieved through counteracting the action of glucagon. Attenuation of the action of glucagon could be a viable therapeutic strategy for T2DM in combination with insulin-based antidiabetic drugs that are currently on the market (combined targeting of two sites within the liver).

GLUCAGON RECEPTOR ANTAGONISTS

Pharmacological antagonism of glucagon action may be a potential therapeutic approach for T2DM. Different mechanisms to lower glucagon levels are: inhibition of glucagon secretion, GCGR receptor blockers and (antisense) inhibition of GCGR expression. Peptide antagonists and monoclonal antibodies against the GCGR attenuated hyperglycemia in animal models [49-51], suggesting a potential to treat hyperglycemia in T2DM through the inhibition of glucagon function. Peptides were the earliest GCGR antagonists that were designed by structural modification of the native hormone [52] . However, oral bioavailability and long half-life appeared to be hurdles that have not been overcome. The development of small molecules against the GCGR has not been very successful due to limited drug selectivity, cross-species differences and lack of sustained effects after non-competitive blockade [53]. Only one phase 1 study has been published describing the acute effects of Bay 27-9955, a small molecule glucagon receptor inhibitor [54] that blunted hyperglucagonemia-induced hyperglycemia. However, long-term antidiabetic benefits, as well as side effects, of this compound in patients with T2DM remain unknown.

ANTISENSE THERAPY

Antisense oligonucleotides (ASOs) as therapeutical agents are relatively new, and only one antisense compound has been approved by the FDA and European authorities until now [55]. Many antisense compounds are currently in preclinical or clinical development phases. ASOs are short, single-stranded molecules which are complementary to a target messenger ribonucleic acid (mRNA). Upon Watson-Crick hybridization with their target mRNA, ASOs inhibit

translation through the activation of endogenous RNase H enzymes and other mechanisms, for example via alternative splicing (Figure 3) [56].

CLINICAL USE OF ANTISENSE OLIGONUCLEOTIDES (ASOS)

For clinical use of ASOs, chemical modified ASOs were used. The first generation oligonucleotides contain a phosphorothioate modification to protect the molecule from rapid degradation by nucleases. Second-generation 2'-O-methyl (2'-OME) and 2'-methoxyethyl (2'-MOE) oligonucleotides were developed to further increase nuclease resistance, thereby improving pharmacokinetics and to increase target affinity. Third-generation oligonucleotides represent a heterogeneous group of ASOs that are most often DNA and RNA analogs with modified phosphate linkages or riboses. These modifications also lead to improved nuclease resistance, affinity and pharmacokinetics. The different classes of ASOs have different toxicological, pharmacokinetic and pharmacological properties [57;58]. Studies with species-specific GCGR antisense drugs in rodent models of T2DM have demonstrated selective inhibition of hepatic and adipose tissue glucagon receptor expression and normalization of blood glucose levels without development of hypoglycemia or weight gain [59;60]. In addition to hepatic effects, in preclinical studies glucagon receptor antisense therapy increased the levels of active GLP-1 levels and improved pancreatic beta cell function [60].

In conclusion, the pathophysiology of T2DM is characterized not only by insulin resistance and ß-cell dysfunction, but also with elevated fasted and postprandial plasma glucagon levels. It has been suggested that the diabetic α-cell exhibits a reduced glucose sensitivity and/or insulin resistance. The overall aim of this thesis was to gain more insight in the role of glucagon in glucose homeostasis in health and disease, and to explore glucagon antagonism as therapeutic potential for the treatment of T2DM.

OUTLINE OF THIS THESIS

This thesis is comprised of a variety of human studies designed to investigate the role of glucagon in glucose homeostasis in health and disease.

CHAPTER 2 studied the effects of a single protein hydrolysate meal replacement (insuVida™) on postprandial serum glucose, insulin and glucagon levels in patients with type 2 diabetes.

The aim of the study in CHAPTER 3 was to characterize the applicability of the glucagon challenge test as a tool in diabetes research, by assessing the inter- and intra-individual variabilities of the glucagon challenge test and investigating the activity of the autonomic nervous system (ANS) during the challenge, as this might have an indirect impact on glucose homeostasis.

Furthermore, we determined whether human adipose tissue expresses glucagon receptor MRNA.

In CHAPTER 4 the effects of a glucagon challenge in T2DM patients were explored. A stable isotope glucose tracer technique was applied to determine hepatic glucose production. The influence of oral antidiabetic drugs on the response to hyperglucagonemia was investigated by using a cross-over study design. We compared the glucagon challenge data of healthy volunteers with T2DM.

Increased fasting and post-meal glucagon concentrations cause excessive hepatic glucose production (HGP) in patients with type 2 diabetes, suggesting that attenuation of hepatic glucagon action could be a promising therapeutic strategy for T2DM. CHAPTER 5 shows the results of the phase I double-blind, placebo-controlled, dose-escalation study, which evaluated the safety, tolerability, PK and pharmacodynamics of single and multiple dose administrations of placebo or antisense glucagon receptor antagonist (ISIS 325568) at 4 dose levels in healthy subjects. In the multiple dose cohorts at each dose level, 8 subjects received 8 doses over 6-weeks (3 IV doses in week 1 followed by 5 weekly SC doses) and underwent a glucagon challenge procedure (glucagon infusion that doubled both plasma glucagon and glucose levels) at baseline and at the end of 6-week treatment.

CHAPTER 6 describes the development of a semi-mechanistic model simultaneously describing glucagon, plasma glucose, insulin and glucagon receptor internalization. This model was build using data from our glucagon challenge study in healthy volunteers (chapter 3).

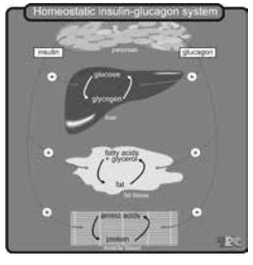
Finally, CHAPTER 7 combines the results and conclusions from the previous chapters and places these in a broader perspective. The role of glucagon in glucose homeostasis in health and disease is discussed and suggestions for future research are given.

FIGURE 1 Homeostatic insulin-glucagon system
Diagram of the homeostatic (insulin-glucagon) system. Insulin secretion is stimulated by high blood glucose levels and glucagon is stimulated by low blood glucose levels.

FIGURE 2 Enteroinsulinar axis
After food intake (1) the complex sugars are broken down in the small intestine into glucose molecules. L-cells in the distal intestinal wall secrete the incretin GLP-1 upon stimulation by food/glucose (2). This glucagon-like peptide-1 is released proportional to the amount of food post-prandial in the distal intestine. GLP-1 is key player in many processes after a meal. It stimulates the release of insulin by the ß-cells in the pancreas (3). Similarly, GLP-1 inhibits the release of glucagon by the α-cells (4). The rise in insulin will stimulate the glucose uptake from the blood into the cells (8). Further, GLP-1 inhibits gastric emptying (5) and induces the feeling of satiety (6) in order to reduce further carbohydrate intake. GLP-1 is metabolised by the enzyme dipeptidyl peptidase 4 (DDP-IV) which is present in the cell wall (7).

FIGURE 3 Antisense oligonucleotides
RNase H-dependent antisense mechanism. Single-stranded oligonucleotides are transported across the plasma membrane, by either poorly characterized natural processes or by the use of facilitators such as cationic lipids (step 1). Once in the cytoplasm, single-stranded oligonucleotides rapidly accumulate in the cell nucleus (steps 2 and 3), where they bind to their targeted RNA (step 4). Once bound to the RNA, RNase H recognizes the oligonucleotide/RNA duplex as a substrate, cleaving the RNA strand and releasing the antisense oligonucleotide (step 5). Although the cleavage of the RNA by RNase H is shown to occur in the nucleus, RNase H is also present in the cytosol, allowing for cleavage to occur in that cellular compartment as well.

See inside of the backcover for the figures in full colour.



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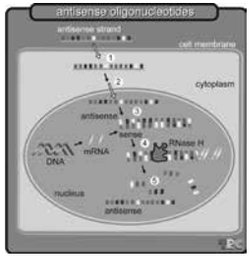

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CHAPTER 2

Hydrolysed casein decreases postprandial glucose concentrations in T2DM patients, irrespective of leucine content

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ABSTRACT

BACKGROUND Lifestyle modifications, including diet, are important in the prevention and management of type 2 diabetes mellitus (T2DM). However, limited information is available on the effects of single doses of meal replacements, particularly with regard to their effect on postprandial glucose. Therefore a study was performed comparing the effects of a single meal replacement in T2DM patients on postprandial serum glucose, insulin and glucagon.

METHODS This randomized, double-blind, partial cross-over study was performed in 36 T2DM patients who continued their oral anti-diabetic medication. Each patient received three out of four treatments separated by 7 days. The treatments were a proprietary casein hydrolysate (insuVida™) alone or with additional leucine, unhydrolysed casein or placebo. Blood sampling was done for 4 hrs. Treatments were compared using repeated measures ANOVA. Results are given as an estimate of the difference (%) for the 4 hrs epoch.

RESULTS Glucose concentrations were lowered by 4.7% by insuVida™ and insuVida™ plus added leucine compared to placebo (95%CI: -1.6% to -7.7%) while the effect of unhydrolysed casein was -1.7% (-4.8% to 1.5%). Addition of leucine to insuVida™ induced the greatest increase in insulin (51.8%; 41.1% to 63.4%). All three treatments increased glucagon concentrations by 14% (8% to 20%) compared to placebo.

CONCLUSIONS A single dose of insuVida™ with or without addition of leucine significantly lowered plasma glucose compared to placebo and intact casein in T2DM patients. This is most likely due to an insulinotropic effect of insuVida™. The data suggest that this type of intervention may be a viable treatment strategy in T2DM.

INTRODUCTION

Lifestyle modifications such as diet, have beneficial effects on glucose metabolism in type 2 diabetes mellitus (T2DM) and are considered the first line of action to halt or delay further progression of the disease [1]. The development of ingredients for a functional food could be an interesting addition to the current dietary recommendations. Patients with T2DM can be characterised in part by impaired insulin response upon intake of carbohydrates such as glucose. Therefore, insulin secretagogues such as sulfonylurea derivatives are an important class of antidiabetic medication [2]. Amino acids are known to have insulinotropic properties as well [3-5]. The possibility that substances other than glucose could stimulate insulin secretion was first reported by Cochrane *et al*. in 1956, who showed that casein ingestion could induce acute hypoglycemia in children with familial idiopathic hypoglycemia [6]. Subsequently, many studies have demonstrated that the combined intake of carbohydrate and protein induced a higher insulin response than the intake of carbohydrate alone [7;8], both in healthy subjects [9-11] and in T2DM patients [12-17].

Specific amino acids, such as leucine, have been shown to further augment the insulin response of protein co-ingestion in healthy subjects [11;18] and type 2 diabetes mellitus patients [17;19]. Leucine stimulates insulin secretion in pancreatic ß-cells by increasing mitochondrial metabolism by activation of glutamate dehydrogenase (GDH) and increasing ATP (adenosinetrifosfaat) production by transamination of leucine [20;21]. Although the hypoglycaemic effect of proteins was first noted in the 1960s [7;8], a practical application of this effect has only been explored more thoroughly during the last ten years. One of these applications is the use of a protein hydrolysate as a meal replacement. Research with the proprietary casein hydrolysate insuVida™ (insV, formerly known as InsuVital™) has shown that ingestion of this product with carbohydrate augments the insulin response and enhances glucose disposal in patients with long-standing T2DM [12;15;19], which makes it an attractive food-based intervention. However, some of these studies were performed using a fairly high amount of protein (25-85 gram protein). Hence, these interventions were associated with a high protein load which would be undesirable in a subset of patients with diabetes mellitus who may have an impaired renal function. The addition of a protein hydrolysate could be used in a functional or clinical food for diabetic subjects if lower amounts of protein would also produce significant benefits in terms of glucose management. However, efficacy at a lower dose has not previously been demonstrated and therefore further research is needed to compare intact protein versus protein hydrolysate and on the additional effect of leucine on glucose homeostasis. Therefore, in a randomized, double-blind, placebo-controlled, partial cross-over study, we compared the insulinotropic, glycaemic and glucagonaemic effects of a low dose of a proprietary casein hydrolysate, with and without added leucine, against its native intact protein in individuals with stable-treated type 2 diabetes. The patients continued their medication in order to demonstrate whether the effects of the hydrolysate could add beneficial effects on top of medication.

METHODS

The study was conducted in accordance with the Declaration of Helsinki and Guideline for Good Clinical Practice. The protocol of this study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC). After informed consent was obtained, all patients were screened approximately three weeks before start of the study to assess eligibility. Screening consisted of a medical examination, ECG, vital signs, standard urine analysis and, hematology, virology, chemistry and HbA1c laboratory tests.

SUBJECTS

The study was performed in 36 patients of either gender, between 50 and 70 years of age and with an established diagnosis of T2DM as evidenced by the use of oral antidiabetic medication for at least one year and a fasting plasma glucose concentration of ≥ 7.0 mmol/L at screening after abstaining from oral antidiabetic medication for at least two days. Exclusion criteria were use of insulin, body mass index (BMI) > 35 kg/m², (recent) pregnancy, significant history of ischemic heart disease or congestive heart failure, uncontrolled hypertension, severe diabetic retinopathy, impaired liver function or renal function. After inclusion, patients were randomized according to the procedure described by Wakeling and MacFie [22]. A partial cross-over design was chosen in which each participant received three out of the four possible treatments with wash-out periods of 7 days. This was chosen to decrease the burden for the patient without under-powering the study.

TREATMENTS

The interventions were offered as a breakfast meal replacement shake that was freshly prepared prior to use by dissolving a sachet with 300 mL cold water. The treatments were administered between 9.00 and 9.30 AM. The energy content of the shakes was standardized and comparable with a normal breakfast. No information on the composition of the contents in the sachets was visible for either the participants or the research staff. Three out of the following four treatments were given (Table 1):

- Placebo
- Unhydrolysed casein
- insuVida™ (insV)
- insuVida™+leucine (insV+leu)

For each of the treatments the amount of carbohydrate, fat and vitamins was identical, except for the addition of 0.25 mg chromium picolinate and minerals for the insV treatments. All beverages were uniformly lemon flavoured. insuVida™ (DSM Food Specialties, Delft, The Netherlands) is a casein hydrolysate which is obtained by enzymatic hydrolysis of sodium caseinate. The amount and composition of the amino acids of both the intact and the hydrolyzed protein is the same (15 gram).

PROTOCOL

Patients continued to take their antidiabetic medication after screening. They maintained their normal dietary and physical activity patterns throughout the entire experimental period, but refrained from heavy physical labour and exercise training for at least 2 days before each study day. The enrolled patients participated on three study days in total with a wash-out periods of one week between two occasions.

In the evening prior to each study day, the patients took a standardized meal with a total energy content of 2676 kJ. The macro-nutrient energy distribution was 55 energy % carbohydrate, 28 energy % fat, and 17 energy % protein. This was followed by an overnight fast of at least 12 hours. During study days, patients remained fasted and refrained from xanthine-containing products as long as measurements continued. Lunch was offered after completion of the last measurement.

BLOOD SAMPLING AND ANALYSIS

Blood samples were drawn by sampling from an intravenous cannula inserted into a forearm vein. Blood was collected at -5, 0, 10, 20, 30, 40, 50, 60, 90, 120, 150, 180, 210 and 240 minutes after treatment ingestion for glucose, insulin and glucagon. Blood samples for insulin and glucose were collected in non-additive tubes. The samples were allowed to clot for 30-45 minutes and subsequently centrifuged at 4°C for 10 minutes at 2000 g. Samples were stored below -20°C until analysis. Blood samples for glucagon were collected in pre-chilled EDTA tubes. The samples were put on ice and within one minute 50 µl, 500 KIE Trasylol (Bayer, Germany) was added, centrifuged at 4°C for 10 minutes at 2000 g and within 30 minutes after collecting stored at a minimum of -20°C until analysis.

Glucose and insulin concentrations were measured in an automated assay using a fully automated Immulite analyzer (Immulite 2500 Analyzer Assay, EURO/DPC, UK). Glucagon was measured using a radioimmunoassay (RIA, Linco Research Inc., St Charles, MO, USA). The assays were performed at the Central Laboratories for Clinical Chemistry of LUMC. All assays were validated to manufacturer standards prior to study sample analysis. All samples from the same subject were processed within the same batch.

POWER CALCULATION

As data from former studies did not yield enough information for a cross-over power calculation, a conservative power calculation with relatively high standard deviation and a power of 90% was conducted. This resulted in a necessary effect size of 21.7 mmol/L*4hrs , assuming a standard deviation of differences of 39.0, using a paired t-test with a 0.05 two-sided significance level for 36 subjects.

STATISTICAL ANALYSIS

All data were entered into the analyses. Pharmacodynamic endpoints (postprandial serum glucose, insulin and glucagon levels) were analyzed separately by mixed model analysis of variance with treatment, occasion visit, time and treatment by time as fixed effects, with subject, subject by time and subject

by treatment as random effect (subjects received three of four possible treatments), and with the (average) baseline value as covariate. Variables were analyzed after log transformation and least square means (LSM) estimates were calculated within the model. All data handling and statistics were performed using SAS for Windows version 9.1.2 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

SUBJECT CHARACTERISTICS

Sixty subjects were screened for the study. Twenty-four subjects were excluded from participation because they did not meet the inclusion criteria. Thirty-six patients entered the study; 27 males and 9 females. Demographics of the patients are shown in Table 2. Eleven of 15 patients on monotherapy for T2DM used a biguanide drug.

Twelve patients used a combination of a biguanide and sulfonylurea derivative, and six a biguanide and a thiazolidinedione drug. Three patients were on triple therapy. The majority of the patients received multi-modality treatment because of the increased cardiovascular risk associated with T2DM; 19 patients used statins, 23 antihypertensive medication and 10 patients received anticoagulant or antiplatelet therapy. Occasional use of analgesics or antacids was also reported, but none of concomitantly used drugs were considered to interfere with the objectives of the study (Table 3).

TOLERABILITY

No significant adverse events (AE) were recorded during this study. The most frequently occurring AE was gastrointestinal-related and consisted of abdominal cramps with or without diarrhea (n=7) or constipation (n=2) and nausea (n=1). The AEs were mild, transient and did not need intervention. No significant differences between treatments were found for the occurrence of AEs. All 36 subjects finished the study and attended all three occasions.

HORMONES AND GLUCOSE

The effect on average serum glucose concentration of insV treatment with or without added leucine was indistinguishable, and both interventions resulted in 4.7% lower glucose concentrations compared to placebo (p=0.0036, 95% CI: -7.7% to -1.6% for both, Figure 1, Table 4).

There was no difference in postprandial glucose concentrations between placebo and the treatment with unhydrolysed protein (95%CI from -4.8% to 1.5%). The difference in effect on average serum glucose concentrations between the unhydrolysed protein and both insV treatments was 3%. Glucose declined to baseline at approximately 3 hours and was below baseline for the remainder of the observation period.

Average insulin concentrations increased rapidly after intake of all protein containing meal replacements (Figure 2). The strongest insulin increase compared to placebo occurred after insV with added leucine (Table 4: 51.8%; 95% CI: 41.1 to 63.4%, expressed as estimate of the difference over the 4 hour observation period). The increase in insulin after insV treatment without leucine (26.1%) was significantly less than the increase in insulin after unhydrolysed protein (36.0%).

Average glucagon attained peak concentrations at approximately 30 minutes and declined below baseline approximately 1 to 1.5 hours after treatment intake (Figure 3). The increase of glucagon after placebo was minimal. The increase in glucagon after the treatment with unhydrolysed protein and both insV treatments compared to placebo was similar and amounted to approximately 14% (95% CI: 7.5% to 20.2%, Figure 3).

DISCUSSION

Postprandial glucose concentration is increasingly seen as an important determinant for management of HbA1c concentrations [1;23]. A reduction in postprandial glucose of 0.8 mmol/L (about 10%) (due to diet and lifestyle intervention) is related to a reduction in T2DM incidence of 58% [24]. The diabetes prevention program research group showed that lifestyle intervention was significantly more effective than metformin (reduced incidence of 58% vs 31%) [25]. It is reasonable to assume that the combination of an energy-balanced diet and protein hydrolysates may have clinical relevance in opposing the development of insulin resistance by attenuating the postprandial rise in blood glucose concentration.

The aim of this study was to compare the effects of a single, low dose of hydrolysed protein, with or without added leucine, and of unhydrolysed protein on blood concentrations of glucose, insulin and glucagon in T2DM patients. The present data confirm results from earlier studies [12;15;19] that protein hydrolysates can lower postprandial glucose concentrations when taken along with a carbohydrate load. This study also shows that this effect is still present at much lower doses than employed previously. Many studies employed doses of around 0.3-0.4 g/kg hydrolysate [12;15;19;26] which is considerably higher than the 15 g protein (approximately 0.2 g/kg body weight) used in the current study. Furthermore, the effect of the tested hydrolysate on postprandial glucose does not depend on its leucine content, since treatment with either insV or insV+leu reduced postprandial glucose to a similar extend, even though insV+leu induced a larger insulin response. Both insV and

insV+leu outcompeted unhydrolysed protein in terms of glucose lowering. All three active treatments induced a relatively short lasting increase in glucagon concentrations.

There is ample evidence that certain proteins, protein hydrolysates and amino acids stimulate the release of insulin. Indeed, consumption of a combination of proteins and carbohydrates causes a more pronounced insulin response than ingestion of carbohydrates alone [7]. Previous research with insV in patients with T2DM has shown that co-ingestion of this product with carbohydrate augments the insulin response and enhances glucose disposal [12;15;19]. It should be noted that protein hydrolysate was administered on top of the carbohydrate shake that was provided in the placebo group, which induced a higher caloric load in the treatment groups. The fact that the hydrolysates lowered glycemic response even in the face of a higher caloric load further stresses the glucose lowering potential of the interventions.In this study the effect of additional leucine in hydrolysated casein was evaluated in view of contradictory previous findings [15;27]. The data indicate that this mixture induced the largest increase in insulin concentrations (Figure 2). However, the postprandial glucose responses following treatment with insV+leu were similar to treatment with insV without additional leucine. These findings are in line with an earlier postprandial study [15] with the same product. It is unclear why insV+leu induced a greater insulin response, but no additional lowering of plasma glucose, compared to insV. Glucagon responses were measured to assess whether the glucose response to the leucine-induced insulin increase would be offset by increased glucagon concentrations. This appeared not to be the case, since the glucagon responses to insV and insV+leu were similar (Figure 3).

INSUVIDA™ VERSUS UNHYDROLYSED PROTEIN

insV tended to lower glycemic responses compared to intact casein and this is significant over the 0-2 hr period (estimated of the difference -4.3%; 95%CI: -1.1 to -7.4%, Figure 1). These data support the notion that for postprandial glucose management, the added value of a casein hydrolysate as compared to intact casein is especially pronounced in the early post-prandial phase. This is probably due to a difference in rate of appearance of amino acids in the peripheral circulation between whole casein protein and the casein hydrolysate [28]. Casein protein and their respective peptide hydrolysates were emptied from the stomach at similar rates, but the speed of intestinal amino acid absorption was slower for the casein protein solution. Although a minute amount of chromium (0.25 mg) was present in the insV treatments, it is unlikely that this contributed to the difference between the hydrolysate and the unhydrolysed protein as none of the well-designed studies have ever demonstrated any effect of chromium on (post-prandial) glucose regulation in humans [29, 30].

In view of the size of the insulin response to protein-rich treatment, the postprandial glucose lowering effect is moderate [12]. It is well known that glucagon and insulin have opposite effects on (postprandial) glucose concentrations and are closely interlinked. Therefore, it is more important to consider the ratio of insulin to glucagon in evaluating a clinical situation rather than considering the concentrations of either hormone alone.

Plasma glucagon concentrations peaked before a decrease in plasma glucose occurred; respectively at 30 min and 60 min (Figure 1 and 3). Therefore, other factors than glucose concentrations must have been influencing this glucagon response. Proteins and amino acids stimulate both α- and ß cells in the pancreas, thus stimulating both the glucagon and the insulin release [9, 10, 31, 32]. Moreover, ß cells appear to be more responsive to protein than α cells [31]. It has been shown that glucagon release depends on the protein-to-carbohydrate ratio of the meal [33-35]; a higher ratio is associated with an increased glucagon release. Calbet *et al*. found that the glucagon response to protein feeding was linearly related to the plasma amino acid concentration [9]. Thus, lower protein loads would be more appropriate to induce higher Insulin/Glucagon (I/G) ratio, while by increasing the protein load mainly glucagon responses will be affected resulting in lower I/G ratios [31]. Therefore, it appears that the secretion of pancreatic glucagon during protein intake in association with insulin secretion, serves to limit the decline of glucose concentration. This may explain that hypoglycemia has never been observed in this or the previous studies with this hydrolysate.

A single dose of a casein hydrolysate with and without the addition of leucine enhanced the carbohydrate-induced insulin response in T2DM patients, resulting in significantly lower plasma glucose concentrations compared to placebo and intact casein. In addition, the secretion of pancreatic glucagon was increased during all three active treatments associated with increased insulin secretion. This mechanism may have limited the decline of plasma glucose concentrations and may be responsible for the absence of hypoglycemia. Further research is required to determine the long-term clinical benefit of a low dose of insuVida™, with and without additional leucine.

TABLE 1 Composition of individual treatments

	Placebo	Unhydrolysed protein	insuVida™ g/serve	insuVida™ & leucine
Protein: insuVida™ casein hydrolysate	-	-	17.61**	17.61**
Protein: Sodium caseinate (Saneigien)	-	15.0	-	-
L-leucine	-	-	-	5.00
Maltodextrin: Maldex G 120	17.00	17.00	17.00	17.00
Dextrose monohydrate (Meritose 200)	18.47	18.47	18.47	18.47
Vegetable oil (rapeseed)	5.00	5.00	5.00	5.00
Additives (specified below)	8.94	8.94	8.94*	8.94*

*Additives: Vitamin+Mineral premix: 2.85 gram, Gum (Xanthan and Guar) 0.48 and 0.12 gram, Sucralose 0.036 gram, Flavour lemon 0.3 gram, Lecithin: 0.15 gram and * Chromium 0.00025 gram. ** Net protein weight: 15 gram.*

TABLE2 Subject demographics

Gender	9 Females / 27 Males
Age (yrs)	61.5 ± 5.1
BMI (kg/m²)	28.1 ± 3.6
Diagnosis of T2DM (yrs)	7 ± 5
HbA1c (%)*	6.8 ± 0.9
Fasting glucose (mmol/L)	9.6 ± 2.3
Total cholesterol (mmol/L)	4.67 ± 1.02
Triglycerides (mmol/L)	1.57 ± 0.70

** HbA1c glycated hemoglobin*

TABLE 3 Concomitant medication

Oral antidiabetics	Monotreatment	n=15
	Dual treatment	n=18
	Triple treatment	n= 3
Cardiovascular	Lipid Lowering	n=19
	Blood pressure lowering	n=23
	Anticoagulant/antiplatelet	n=10
Central nervous System	Analgesic / Occasional sedative	n=6
GI tract	Antacid	n=4
Endocrine	Thyroid hormone	n=2
Urogenital tract	Prostate hypertrophy	n=2
Pulmonary tract	Anti-allergic	n=1
	Anti-asthma	n=1
Miscellaneous	Eye drops / vitamins / Acetylcysteine / Allopurinol	n=5

TABLE 4 Summary of profile analysis results

Estimate of the difference (%) of postprandial serum glucose levels. Insulin and glucagon levels from 0-4 hours after treatment ingestion with 95% confidence interval and p-value.

	Protein vs. Placebo	insV vs. Placebo	insV + leu vs. Placebo	insV vs. Protein	insV vs. insV + leu	insV + leu vs. Protein
Serum glucose 0-4 hrs (mmol/L)	-1.7% (-4.8%, 1.5%) p=0.2781	-4.7% (-7.7%, -1.6%) p=0.0036	-4.7% (-7.7%, -1.6%) p=0.0038	-3.0% (-6.1%, 0.1%) p=0.0578	0.0% (-3.1%, 3.3%) p=0.9810	-3.0% (-6.1%, 0.1%) p=0.0610
Insulin 0-4hrs (mU/L)	36.0% (26.4%, 46.3%) p=<.0001	26.1% (17.1%, 35.7%) p=<.0001	51.8% (41.1%, 63.4%) p=<.0001	-7.3% (-13.8%, -0.2%) p=0.0430	20.4% (11.9%, 29.6%) p=<.0001	11.7% (3.8%, 20.1%) p=0.0037
Glucagon 0-4 hrs (ng/L)	13.8% (7.7%, 20.2%) p=<.0001	13.5% (7.5%, 19.8%) p=<.0001	13.8% (7.8%, 20.2%) p=<.0001	-0.3% (-5.6%, 5.3%) p=0.9120	0.3% (-5.0%, 5.9%) p=0.9142	-0.0% (-5.3%, 5.6%) p=0.9973

FIGURE 1 LSMS Serum glucose for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV, Circle: insV+leucine. The figure represents the time course of the least square mean of serum glucose (with 95% CI error bars). Significant differences as compared with placebo are indicated for insV and insV+leucine (p<0.003).

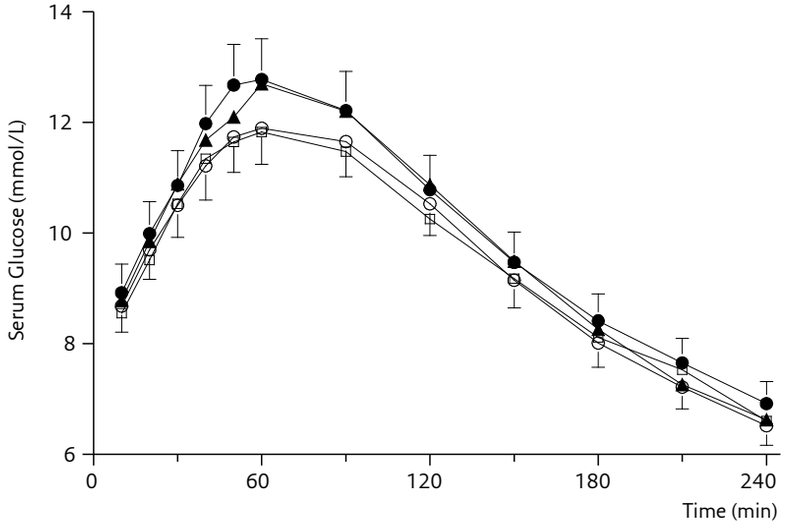


FIGURE 2 LSMS Insulin for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV; Circle: insV + leucine. The figure represents the time course of the least square mean of insulin (with 95% CI error bars). Significant differences as compared with placebo are indicated for all three treatments (p<0.0001).

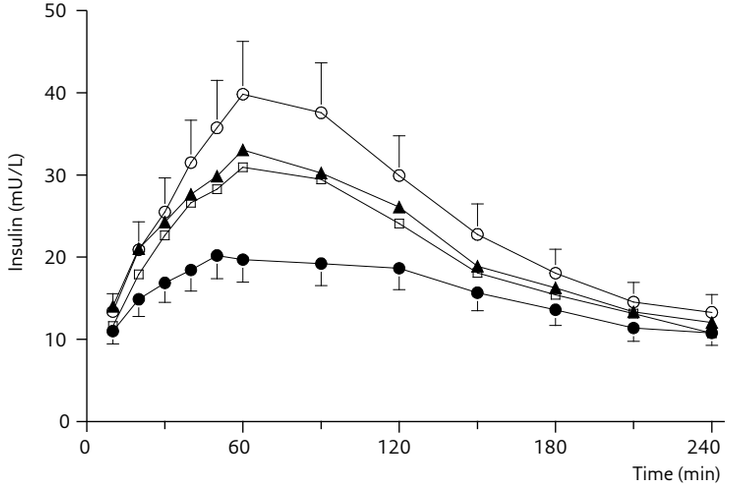
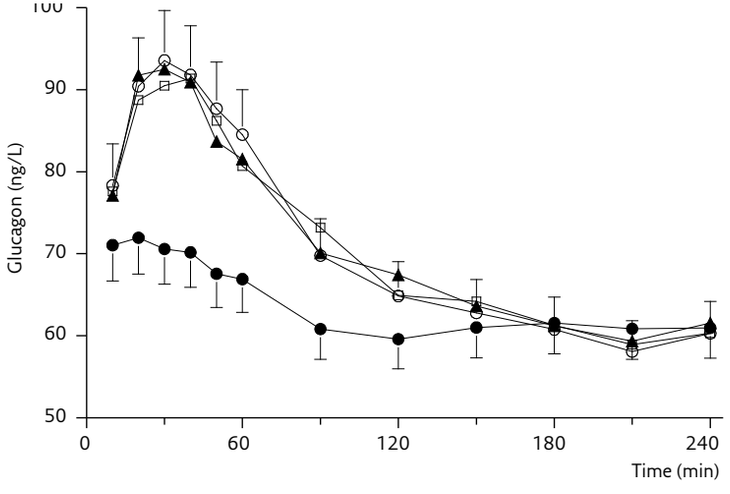


FIGURE 3 LSMS Glucagon for each treatment

Dot: placebo, Triangle: unhydrolysed casein, Square: insV, Circle: insV+leucine. The figure represents the time course of the least square mean of glucagon (with 95% CI error bars). Significant differences as compared with placebo are indicated for all three treatments (p<0.0001).



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CHAPTER 3

Characterization of a standardized glucagon challenge test as a pharmacodynamic tool in pharmacological research

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ABSTRACT

BACKGROUND The aim of this study was to characterize a glucagon challenge test as a tool in diabetes research by assessing the inter- and intra-individual variability, and investigating the activity of the autonomic nervous system (ANS) during the challenge, as this might have an indirect impact on glucose homeostasis.

METHODS The study was performed in 24 healthy volunteers separated in two groups. The first group of 12 volunteers underwent a 5h glucagon challenge during a pancreatic clamp procedure with infusion of [6,6-$^2H_2$] glucose in combination with heart rate variability measurements. In the second group, 12 other healthy volunteers underwent two 6h glucagon challenges separated by 6 weeks, and fat biopsies were taken for analysis of glucagon receptor expression.

RESULTS Serum glucose rose rapidly after glucagon infusion, and reached a plateau at 90 min. The time profiles suggested rapid development of tolerance for glucagon-induced hyperglycemia. During the glucagon challenge intra- and inter individual variabilities for hepatic glucose production, the rate of disappearance of glucose and plasma glucose were approximately 10-15% for all variables. Hyperglucagonemia did not affect heart rate variability. Human adipose tissue had a low, but variable, expression of glucagon receptor MRNA.

CONCLUSIONS This standardized glucagon challenge test has a good reproducibility with only limited variability over 6 weeks. It is a robust tool to explore in detail the contribution of glucagon in normal and altered glucose homeostasis and can also be used to evaluate the effects of drugs antagonizing glucagon action in humans without confounding changes in ANS tone.

INTRODUCTION

The WHO estimated that the number of people in the world suffering from diabetes by 2030 will have surpassed 360 million [1]. Especially the number of type 2 diabetes mellitus (T2DM) patients is expected to rise exponentially [2]. T2DM is a characterized by chronic insulin resistance in liver and muscle, impaired insulin secretion in relation to insulin resistance and relative glucagon excess. In the absence of a defect in ß-cell function, individuals can compensate for insulin resistance with appropriate hyperinsulinemia. However, loss of ß-cell function ultimately leads to postprandial and fasting hyperglycemia that characterizes T2DM. Current treatments focus on reduction of insulin resistance, on stimulation of insulin secretion and/or on insulin treatment. However, these treatments are not successful in restoring glucose metabolism in T2DM patients. Therefore, alternative targets are being investigated to improve glucose homeostasis in T2DM.

Glucagon could be such a target as elevated levels of circulating glucagon in T2DM patients results in increased hepatic glucose output, which contributes to (postprandial) hyperglycemia [3;4]. The glucagon receptor (GCGR) is mainly expressed in liver and in kidney with lesser amounts found in heart, adipose tissue, adrenal glands, pancreas, cerebral cortex and gastrointestinal tract [5]. Considering the role of hyperglucagonemia in the pathophysiology of T2DM, inhibition of GCGR action could represent an innovative target of therapeutic agents for T2DM [6], as attenuation of glucagon action in the liver reduces hepatic glucose output in experimental models and humans [7-9]. Hence, there is a need for reproducible tests that can be used to quantify the contribution of glucagon on glucose homeostasis. Ideally, these tests should be suitable to be used in drug development aimed at interfering with GCGR action in humans.

Two variants of glucagon tests have been explored: bolus administration or infusion of glucagon [7;10]. Bolus administration of glucagon, which is most commonly used to treat insulin-induced hypoglycemia in diabetic patients, has the disadvantage that it only allows exploration of instantaneous effects. Furthermore, as bolus administration of glucagon is commonly performed without inhibition of the release of other (pancreatic) hormones affecting glucose homeostasis, it is not an optimal method to adequately assess the contribution of glucagon per se. The infusion variant with selective hyperglucagonemia and concomitant, fixed insulin concentrations theoretically offers advantages, compared with the bolus test, but these advantages have not been exploited fully, because some important characteristics of this glucagon test variant have not been reported. We therefore investigated the inter- and intra-individual variability, the repeatability over a period of 6 weeks, and the effect of this test on the autonomic nervous system (ANS). This time frame was chosen as most diabetes drugs are to be taken over a longer period and a 6-weeks period allows to characterize the sustainability of the mechanism of action as therapeutic effects are commonly at steady state at this period. Furthermore, we assessed the activity of the ANS during the challenge, as this might have an indirect impact on glucose homeostasis [11;12].

Pre-clinical studies have shown that the glucagon receptor is expressed in mammalian adipose tissue [13]. When the glucagon receptor would also be expressed in human adipose tissue, it could be a direct biomarker of pharmacological activity in humans for drugs that act by reducing the amount of the receptor or modulation of the receptor because adipose tissue can be obtained easily in humans [14]. As a first step we performed human abdominal and dorsal fat tissue biopsies to investigate glucagon receptor MRNA expression.

METHODS

STUDY DESIGN

The study was performed in 24 healthy volunteers divided in two groups of 12 subjects each. The first group of 12 subjects underwent a 5-hr glucagon challenge test in combination with heart rate variability measurements. In the second group, 12 other subjects underwent two 6-hr glucagon challenges separated by 6 weeks. Fat biopsies were taken for analysis of glucagon receptor expression in adipose tissue.

Healthy volunteers of both genders and aged between 18-65 years were included into the study after informed consent was obtained. Exclusion criteria consisted of pregnancy or the intention to become pregnant; known or suspected addiction to alcohol or narcotics; positive test results for Hepatitis B, Hepatitis C or HIV; and any clinically significant abnormality in the routine physical exam (incl. BMI $\geq$ 30 kg/m^2), hematology, biochemistry and urinalysis. All experiments were approved by the institutional ethical commission and performed according to the principles of the International Conference on Harmonization, Good Clinical Practice and the Helsinki Declaration.

THE HYPERGLUCAGONEMIC CHALLENGE DURING PANCREATIC CLAMP CONDITIONS

Subjects were required to continue their normal diet and activities. After an overnight fast of 10h, a catheter was inserted into an antecubital vein of each subject for infusion of the hormones used in the glucagon challenge. The challenge consisted of two phases: a run-in period of 3 hrs to achieve steady state conditions of glucose tracer/trace ratio's, followed by a 3 hr pancreatic clamp. Glucose kinetics were assessed by administration of a priming bolus of 5 mg.kg^{-1} followed by continuous IV infusion of 0.05 mg.kg^{-1}.min^{-1} of [6,6-$^2H_2$] glucose (Cambridge Isotope Laboratories, USA). The pancreatic clamp procedure consisted of simultaneous infusion during 3hrs of somatostatin at 0.1 µg.kg^{-1}.min^{-1}, glucagon at 3 ng.kg^{-1}.min^{-1} and insulin at 4 mU.m^{-2}.min^{-1}. Somatostatin was purchased from UCB Pharma (The Netherlands) and glucagon and insulin were purchased from Novo Nordisk Pharma (The Netherlands). A second catheter was inserted into a contralateral dorsal hand vein for blood sampling. The hand was placed in a heated-box (50°C) to arterialize venous blood for labeled glucose sampling. Venous blood sample were drawn at regular intervals and were used for determination of (non-)labeled glucose, glucagon, insulin, C-peptide and growth hormone concentrations. Glucose, glucagon and insulin samples were collected every 15 min during the last hour of the enrichment episode and every 15 min during the glucagon infusion. Samples for C-peptide and GH were drawn every 30 minutes during the glucagon infusion. For subject safety frequent bedside glucose measurements were made throughout the observation period using a glucometer (Glucocard X-meter GT-1910, Arkray Inc, Japan).

HEART RATE VARIABILITY

In the first 12 healthy volunteers, 5-min ECG-recordings were made in supine position using the CardioPerfect ECG recording system (Cardiocontrol, The Netherlands). Measurements were made 1 hr and immediately before the start of the challenge and 1 hr after the start of the challenge, with the latter time point coinciding with the period of maximal hyperglycemia. Subjects were instructed to remain calm and breath quietly, not allowed to speak and to fall asleep. ECG recordings were scrutinized for artefacts and subsequently analyzed for the time and frequency domain parameters with the software supplied with the device according to the most recent guidelines [15]. The selected read-outs were the power in the low (LF) and high frequency (HF) domain and the ratio of the low over high frequency power (LF/HF).

ADIPOSE TISSUE BIOPSIES

Fat biopsies were taken before the glucagon infusion test, as previously described [14]. In short, subcutaneous fat samples were taken of the dorsal gluteal area and the abdominal wall by suction after local infiltration with lidocaine. Samples were handled under aseptic conditions, snap frozen in liquid nitrogen and subsequently stored at −80° C until analysis.

LABORATORY ANALYSIS

Blood samples for insulin and C-peptide were collected in plain tubes and analyzed using standard validated immune-radio-metric assays (Biosource Europe S.A. Nivelles, Belgium; assay CV 5.9-7.9% for insulin, 2.9-5.4% for C-peptide). Glucagon samples were collected in plain tubes with aprotinin (Trasylol™, Bayer, 500 KIE/50 µl) and analyzed using radioimmuno assay (Linco research, Missouri, USA; assay CV 4.3%). Blood samples for glucose and [6,6-$^2H_2$] glucose were analyzed using a validated gas chromatography with mass spectrometry as detection method as described previously [16]. Glucagon receptor expression in the fat biopsies was determined using a previously validated protocol for successful purification of mRNA from adipose biopsy samples [14]. Glucagon receptor mRNA expression in adipose tissue was quantified using a standard quantitative reverse transcription and polymerase chain reaction (qRT-PCR) as previously described for hepatocytes [17].

STATISTICS

Hepatic glucose production and rate of disappearance of glucose were calculated using the single pool non steady-state Steele's equations (pool fraction: 60%), as adapted for the use of stable isotopes [18]. Glucagon, insulin, hepatic glucose production, C-peptide, and growth hormone (GH) responses are presented as average responses over time. The inter- and intra-individual variability of the test was assessed using the area under the curves for glucagon, insulin, rates of appearance and disappearance of glucose, and plasma glucose concentrations during the glucagon infusion. Literature data suggests that glucagon receptor antagonism using small molecules, monoclonal antibodies or antisense approaches can reduce HGP in the order of 30-70% [7-9]. Based on our data and the literature data, power calculations were performed to calculate the number of subjects that would be needed to detect a 40% difference in hepatic glucose production rate after a 6 wk treatment with 80% power at an alpha level of 0.05 using a 2-sided paired t-test. The time profile of the LF/HF ratio was compared after log-transformation using a mixed model of variance.

Data are presented as mean and standard deviation unless otherwise indicated. All analyses were performed using SAS for Windows version 9.1.3 (SAS Institute, Inc, Cary, NC, USA).

RESULTS

The first group of 12 subjects consisted of 4 females/8 males, age 32 ± 15 (18-61) years, and BMI 24 ± 3 kg/m². The second group of 12 subjects consisted of male subjects, age 44 ± 20 (18-63) years, and BMI 24 ± 3 kg/m².

During the glucagon infusions, short-lasting, transient adverse events such as nausea, dizziness and headache of minor severity occurred as reported previously [19]. The fat biopsies were well tolerated and none of the participants rated the pain/discomfort higher than a vein puncture. During the glucagon challenge tests mean (±SD) growth hormone decreased from 6.7 (± 9.9) to 0.1 (±0.07) mU/L and C-peptide decreased from 0.7 (±0.3) to 0.1 (±0.07) nmol/L, suggesting efficient blockade of endogenous (pancreatic) hormone release. The time profiles of the hormones and glucose were highly similar in both study groups. Therefore, a detailed description is given of results in the first group, except for the comparison between the first and second challenge.

Glucagon levels increased from 56 (±20) ng/L before the challenge to 123 (±27) ng/L within 30 minutes after start of the infusion and remained more or less stable thereafter (Figure 1). Plasma glucose levels rapidly increased from 5.4 (±0.6) mmol/L before the challenge to a maximum of 12.6 (±2.5) mmol/L at 2 hrs during challenge, after which it tended to decrease. Glucose rapidly returned to baseline after stopping the challenge. The glucose enrichment

(tracer-to-tracee ratio) was 3.0 (±0.4) % before the challenge, declined to 2.5 ± 0.3% between 90-150 min after the start of the glucagon infusion, and started to increase thereafter. Insulin concentrations during the challenge (10 mU/L) were slightly lower than baseline levels (13 mU/L) in the first group and at the same concentration during the entire observation period in the second group at the first and second study day (11 mU/L and 12 mU/L, respectively).

The increase in plasma glucose levels was caused by increased hepatic glucose production (HGP, defined as rate of appearance), as the rate of disappearance of glucose remained at a stable level during the entire challenge, and did not differ significantly from the rate of disappearance during the basal period. HGP increased rapidly after the start of the glucagon infusion from 1.00 (±0.56) to 2.85 (±0.65) mmol/min, remained 2-fold higher than basal levels for the first 70 minutes of the glucagon infusion and subsequently showed a gradual decline until the end of the glucagon infusion period (1.49 ± 0.59 mmol/min). Plotting the glucagon concentration vs. HGP suggests that tolerance occurs for the HGP as the increase in glucagon (or maintenance of high glucagon levels) increases the HGP not further (Figure 2). The translation of increased HGP into increased plasma glucose concentrations is characterized by a substantial delay (counter-clockwise hysteresis).

The repeat of glucagon test after 6 weeks in the second group of 12 subjects showed highly similar results compared to the first group of volunteers. The variability in hormone levels and effect measures was estimated by calculating the area on the curves for glucagon, insulin, rates of appearance and disappearance of glucose, and plasma glucose concentrations (Table 1). There was limited inter-test variability of these parameters as also reflected in the highly similar time course of the HGP (Figure 3). Using these data it was calculated that a study with 8 participants would have 80% power to detect a 40% difference in HGP at a 2-sided alpha level of 0.05.

HEART RATE VARIABILITY (HRV)

There was no effect of hyperglucagonemia on heart rate. The heart rate before the challenge was 57 (±7) bpm and during the challenge, when the glucose level was more than 2-fold higher compared to baseline, the heart rate was 55 (±8) BPM. Hyperglucagonemia did not affect any of the HRV parameters, including the LF/HF ratio (Table 2).

ADIPOSE TISSUE GLUCAGON RECEPTOR MRNA EXPRESSION

The quality of RNA obtained from the tissue biopsy was good with sufficient RNA from many of the biopsy samples and no signs of degradation. However, initial QRT-PCR analysis revealed that the expression of glucagon receptor in the purified RNA of the human adipose tissue was extremely low and variable (data not shown).

DISCUSSION

This study demonstrated that a doubling of plasma glucagon concentrations in the presence of constant insulin concentrations induced hyperglycemia throughout the entire challenge period with maximal plasma glucose concentrations of approximately 12.6 mmol/L at 2hrs, in accordance with previous studies [7;20]. Hyperglucagonemia increased plasma glucose levels by stimulation of endogenous glucose production, whereas hyperglucagonemia did not affect the rate of glucose disappearance. The prompt increase in plasma glucose levels within the first 30 min of glucagon infusion was caused by an almost 3-fold increase in the rate of hepatic glucose production, most likely explained by stimulation of glycogenolysis [21]. We demonstrated that the inter- and intra-individual variability of this glucagon test variant is relatively low (10-15%). Literature data suggests that glucagon receptor antagonism using small molecules, monoclonal antibodies or antisense approaches can reduce HGP in the order of 30-70% [7-9]. We therefore performed a power calculation assuming a 40% reduction and showed that repeat challenges can be performed with a low number of subjects to detect differences of this magnitude. It can thus be concluded that the test in the format that we employed is a robust pharmacodynamic tool with good reproducibility.

Our observations suggest that the effect of glucagon on HGP shows features of tolerance. This is not surprising as the GCGR belongs to the G-protein coupled (class B) receptor family for which development of tolerance is common. It has been described previously that continuous stimulation of GCGR by glucagon results in a diminished response over time, and that the response can be restored by increasing the dose of glucagon [22]. In an animal model it was demonstrated that GCGRs are internalized after 30 min of glucagon stimulation [23], which is in line with the time course of the decay in HGP observed in our study. An additional explanation for the observed glucagon tolerance could be that glycogen stores become depleted in the liver. Thirdly, hyperglycemia might directly antagonize the stimulatory action of glucagon on the liver [24], also under our experimental conditions in which hyperglycemia-related hormonal responses were inhibited by somatostatin infusion [22]. It would be interesting to investigate if, and to what extent, tolerance to glucagon also occurs in patients with T2DM with elevated glucagon concentrations, and if differences exist between the effect of glucagon on glycogenolysis and gluconeogenesis. In addition, we found that the translation of increased HGP into plasma glucose levels is delayed. This finding warrants further investigation as this occurred while insulin concentrations were absolutely stable and the disappearance rates of glucose were also rather stable. It might be argued that the observations on glucose disposal are less reliable as it is well known that the outcome of Steele's equation depends on assumptions for instance on volume of distribution. However, also the time course of the tracer/tracee ratio's tended to increase slowly over time, suggesting that the delayed translation of HGP into plasma glucose is a 'real' phenomenon. It is tempting to hypothesize that during the challenge other mechanisms also play a role. These could be, among others, other hormones/incretins involved in the regulation of glucose homeostasis.

Liver, muscle and fat tissue play a major role in glucose metabolism. Glucose metabolism is tightly regulated by interaction of hormonal and nervous signals. Insulin and glucagon regulate glycemic levels both directly and indirectly by influencing sympathetic and parasympathetic branches of the autonomous nervous system [25]. Therefore, we also measured heart rate variability and assessed the effect of hyperglucagonemia and the ensuing hyperglycemia on the components of ANS tone. However, we did not observe an effect on heart rate and heart rate variability during hyperglucagonemia in healthy subjects. This suggests that changes in autonomic nervous system tone do not contribute significantly to the effects of hyperglucagonemia.

In diabetic mice GCGR mRNA is expressed in adipose tissue [17]. As far as we are aware, there are no reports on GCGR mRNA expression in humans. We explored the expression of the GCGR in human adipose tissue, especially since this could be a direct biomarker of pharmacological activity of drugs that affect GCGR action. We found that the expression level of glucagon receptor mRNA in human adipose tissue was extremely low. This discrepancy in mRNA expression between humans and rodents might reflect a currently unknown physiological difference in adipose glucagon metabolism between humans and mice, and/or reflect a consequence of the pathological condition of the diabetic mice. It may be interesting to investigate GCGR mRNA expression in adipose tissue of patients with T2DM. However, at present GCGR mRNA expression, at least in healthy humans, is not a feasible biomarker for estimation of the amount or function of the GCGR.

In conclusion, we demonstrated that the effects of continuous infusion of glucagon on glucose metabolism has low intra- and inter-individual variability and is highly reproducible. The test is a robust tool to further explore the contribution of glucagon in normal and altered disease or drug-induced glucose homeostasis.

TABLE 1 Coefficients of variation for intra- and inter-individual variability (n=12) for hepatic glucose production, glucose disposal and plasma glucose levels during 2 glucagon challenges separated by 6 weeks.

	Intra-individual variability (%)	Inter-individual variability (%)
Hepatic glucose production	10.7	15.4
Glucose disposal	10.6	16.4
Plasma glucose	12.5	9.7

The variability was calculated using the area under the curve for the period of the challenge.

TABLE 2 Median (range) of heart rate variability parameters in the frequency domain (n=12).

	1 hr before pancreatic clamp	5 min before pancreatic clamp	1 hr after start pancreatic clamp
RR-interval (ms)	1065.5 (809 - 1184)	1101.5 (854 - 1198)	1105.0 (862 – 1279)
LF (ms^2)	1259.5 (233 – 7766)	964.50 (280 – 5811)	1639.5 (351 – 3772)
HF (ms^2)	1154.0 (131 – 5957)	1139.0 (119 – 6393)	1055.0 (242 – 9052)
LF/HF ratio	1.27 (0.28 - 3.35)	1.22 (0.23 - 3.25)	1.18 (0.29 - 4.94)

FIGURE 1 Time course of glucagon, plasma glucose, glucose enrichment, hepatic glucose production (rate of appearance) and glucose disposal (rate of disappearance) during the hyperglucagonemic challenge. The start of the challenge was at t=120 min and consisted of simultaneous infusion of somatostatin, glucagon and insulin. Infusion of labeled glucose was at t=0 min.

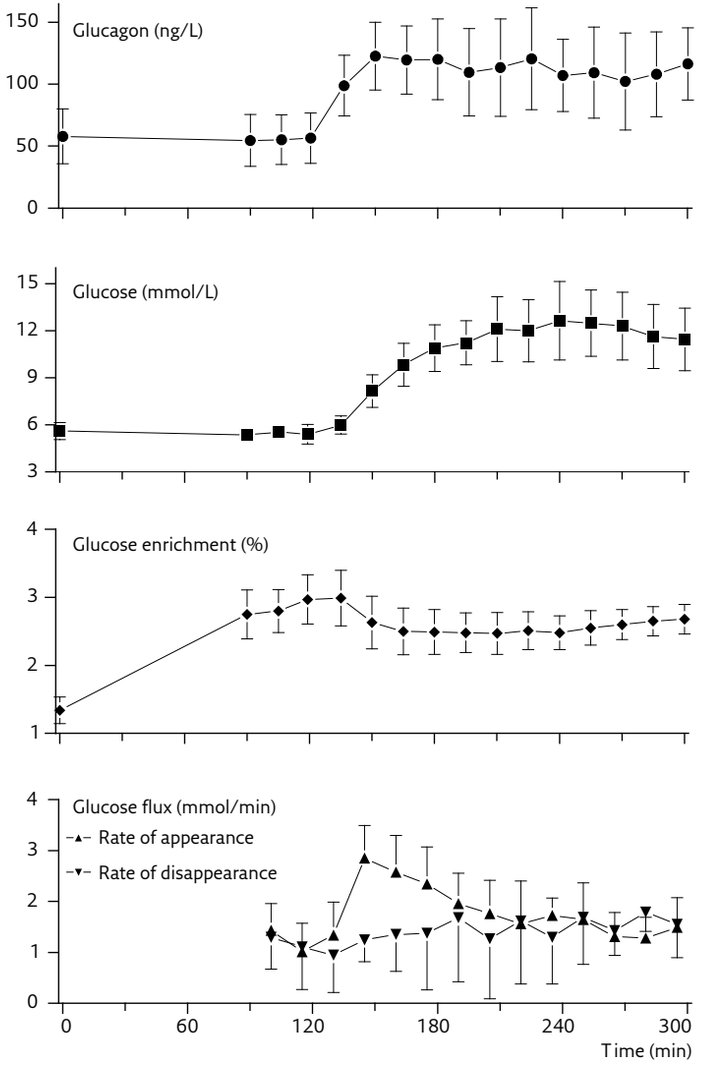


FIGURE 2 Glucagon versus HGP (left panel, indicating tolerance), HGP versus glucose (middle panel, indicating delayed response), and glucagon versus glucose plots (right panel), based on the mean of observed values. Arrows indicate time course.

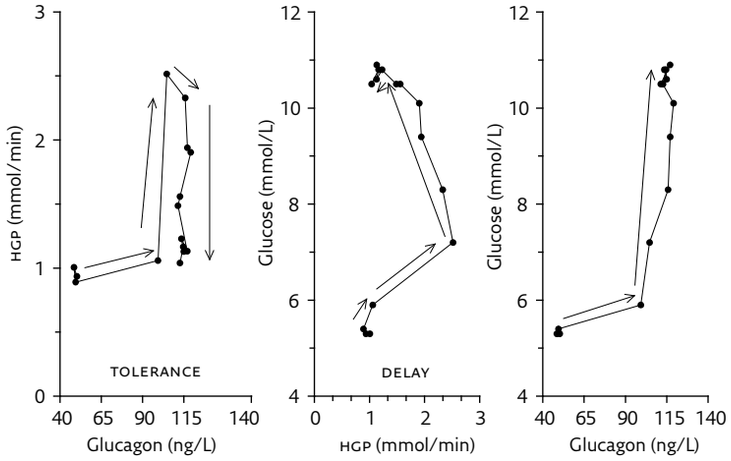
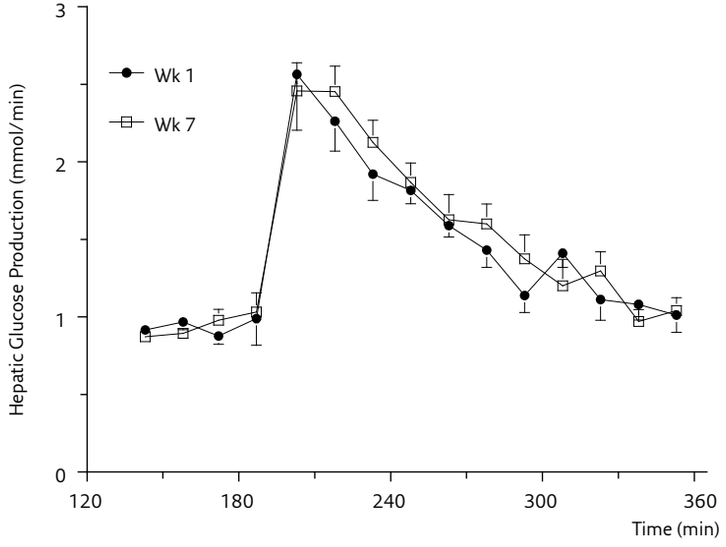


FIGURE 3 Mean (SD) hepatic glucose production during glucagon challenge separated by 6 weeks (n=12).



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CHAPTER 4

Metabolic responses to a glucagon challenge: comparison of healthy subjects and T2DM patients with and without oral antidiabetic drugs

Submitted for publication

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ABSTRACT

BACKGROUND Fasting and postprandial glucagon levels are increased in Type 2 diabetes mellitus (T2DM), potentially contributing to inappropriate hepatic glucose production (HGP). We compared the glucagon response between healthy volunteers and T2DM patients, and investigated the effects of oral antidiabetic drugs on glucagon-induced HGP.

METHODS A hyperglucagonemic challenge was performed in two populations; 36 healthy volunteers (HV) underwent one challenge in the first study, and 22 T2DM patients underwent two challenges in a second randomized crossover study, once when continuing and once when discontinuing oral antidiabetic medication and receiving overnight insulin to achieve normoglycemia.

RESULTS A glucagon challenge induced a comparable increase in glucagon levels in HV and T2DM subjects. The elevated glucagon levels resulted in a stronger increase in glucose levels in T2DM continuing medication and HV than in T2DM subjects on insulin (11.8, 10.1 and 9.3 mmol/L, respectively), which was associated with a stronger stimulation of HGP (1.96, 1.74, and 1.41 mmol/min, respectively). The rate in decline that followed maximal HGP was faster for HV compared to T2DM patients, independent of therapy.

CONCLUSIONS These results demonstrate that HV have a more pronounced increase in HGP upon glucagon infusion and a faster decline in hepatic glucose production than T2DM patients, and exogenous insulin suppresses glucagon-induced hepatic glucose production. This suggests that mitigation of the effects of glucagon in T2DM might be a therapeutic option, by introducing insulin in an earlier stage of the disease and/or developing medication targeting the glucagon receptor.

INTRODUCTION

In healthy subjects, the postprandial rise in glucose levels is limited by a prompt decrease in glucagon and increase in insulin levels. In patients with Type 2 diabetes mellitus (T2DM), plasma glucagon levels are not suppressed normally in response to nutrient ingestion [1], in addition to blunting of the normal postprandial rise in insulin levels [2]. Postprandial plasma glucagon levels remain at, or above, the pre-prandial levels, despite postprandial hyperglycemia. This failure to suppress glucagon secretion results in a substantial impairment in glucose tolerance due to an inappropriate postprandial hepatic glucose release [3]. Obviously, glucagon levels should be considered relative to the prevailing levels of both glucose and insulin, because when a normal insulin profile is reproduced, lack of glucagon suppression has a much smaller effect on postprandial glucose metabolism [4-6]. Nevertheless, in T2DM, elevated postprandial glucagon levels are not suppressed sufficiently and contribute to hyperglycemia [3;7]. Given the increasing recognition of glucagon as a key factor in the pathogenesis of T2DM [8], it is conceivable that novel treatment modalities may emerge including treatments that mitigate glucagon-mediated hepatic glucose output (HGP).

The glucagon challenge test (or hyperglucagonemic clamp test) can be used to assess the effect of glucagon on hepatic glucose production (HGP). This glucagon challenge test has been evaluated as a method to investigate glycogenolysis in healthy volunteers [9;10] and diabetic patients [11;12]. Recently, we reported on the inter- and intra-individual variability, and repeatability of the glucagon challenge in healthy volunteers, and on the effect of the test on the autonomic nervous system [10]. Direct comparison of the response to hyperglucagonemia between healthy volunteers and T2DM patients is lacking, and the effect of oral antidiabetic medication on the glucagon response in T2DM is unknown. Therefore, we assessed the response to hyperglucagonemia in T2DM patients, at the same conditions applied previously in healthy volunteers. The patients underwent the glucagon challenge two times: once after continuation of oral antidiabetic drugs and once after withdrawal of oral medication for two weeks and replacement by an overnight insulin infusion before the challenge. The glucagon response between T2DM patients and healthy volunteers was compared, and the effect of oral antidiabetic drugs on the response was explored.

METHODS

HEALTHY VOLUNTEERS

Glucagon challenge data for healthy volunteers were obtained from a clinical study performed at the Centre for Human Drug Research (CHDR) in Leiden, preceding the clinical study in T2DM patients. Healthy volunteers between 18-65 years were included after obtaining informed consent. Exclusion criteria consisted of pregnancy or the intention to become pregnant, known or suspected addiction to alcohol or narcotics, positive test results for Hepatitis B, Hepatitis C or HIV, and any clinically significant abnormality in the routine physical exam (incl. BMI $\geq$ 30 kg/m^2), hematology, biochemistry and urinalysis.

T2DM PATIENTS

T2DM patients participated in a two-way randomized, cross-over study at CHDR, with the study periods separated by at least one month. All patients used metformin (dose range: 500-3000 mg daily) and 12 patients used a sulfonylurea derivative (glimepiride: n=8; tolbutamide: n=3, glibenclamide: n=1). The patients had never been treated by insulin or other oral antidiabetic

agent(s), and were not taking any medication known to affect glucose metabolism. Furthermore, patients were free of any clinical evidence of cardiovascular, pulmonary, renal, hepatic, or other major organ system disease as determined by history, physical examination, and routine laboratory tests. In one period the subjects continued their oral antidiabetic drugs and in the other period the oral antidiabetics were discontinued for 2 weeks before the challenge. During the latter period, the study day was preceded by an overnight insulin infusion to achieve normoglycemia before the start of the challenge. During the wash-out period, patients reported to the clinical research unit after 1 week for a check including measurement of fasting plasma glucose (FPG) levels. When FPG was > 15 mmol/L during the wash-out period, patients were withdrawn from further participation. Immediately before start of the glucagon challenge, capillary blood glucose was measured as well; if FPG was >12 mmol/L, the glucagon challenge was cancelled. Furthermore, frequent bedside glucose measurements were performed throughout the clinical study period using a glucometer (Glucocard X-meter GT-1910, Arkray Inc, Japan).

The study protocol was approved by the ethics committee of Leiden University and performed according the Dutch Law on Medical Research.

THE HYPERGLUCAGONEMIC CLAMP TEST

Subjects were required to continue their normal diet and activities. After an overnight fast, the hyperglucagonemic clamp test was performed, consisting of two phases: a run-in period of 3 hrs to achieve stable glucose isotope enrichment, and a 3 hr pancreatic clamp study (Figure 1). After a priming dose of 5 $mg.kg^{-1}$ IV, [6,6-$^2H_2$] glucose was infused continuously throughout the study at a rate of 0.05 $mg.kg^{-1}.min^{-1}$ (Cambridge Isotope Laboratories, MA, USA). The pancreatic clamp study consisted of a simultaneous 3hrs infusion of somatostatin (0.1 $\mu g.kg^{-1}.min^{-1}$; UCB, The Netherlands), glucagon (3 ng/kg/min; Novo Nordisk, The Netherlands), and insulin (4 $mU.m^{-2}.min^{-1}$; Novo Nordisk, The Netherlands). A second catheter was inserted into a contra-lateral dorsal hand vein for blood sampling. The hand was placed in a heated-box (50°C) to arterialize venous blood for labeled glucose sampling. Venous blood samples were drawn to determine (non-) labeled glucose, glucagon, insulin, C-peptide and growth hormone concentrations. Samples for isotope analyses, glucose, glucagon and insulin were collected every 15 minutes from 30 minutes before the start of the challenge until the end of the glucagon challenge (t=6 hour). Samples for C-peptide and GH concentrations were drawn before, during and at the end of the glucagon infusion period. The procedures did not differ between healthy subjects and T2DM patients with the exception that when oral treatment was discontinued in T2DM patients, subjects received an overnight insulin infusion before the glucagon challenge to ensure fasting plasma glucose levels between 4.5 and 6.5 mmol/L before start of the challenge. This insulin infusion rate was continued during the glucagon challenge, in addition to the standard 4 $mU.m^{-2}.min^{-1}$ insulin during the pancreatic clamp.

LABORATORY ANALYSIS

Blood samples for insulin and C-peptide were collected in plain tubes and analyzed using standard validated immune-radio-metric assays (Biosource Europe S.A., Belgium; assay CV 5.9-7.9% for insulin, 2.9-5.4% for C-peptide). Glucagon samples were collected in plain tubes with aprotinin (Trasylol™ (500 KIE/50 µl) Bayer, The Netherlands) and analyzed using a radioimmuno assay (Linco research, Missouri, USA; assay CV 4.3%). Blood samples for glucose and [6,6-$^2H_2$] glucose were analyzed using a validated gas chromatography with mass spectrometry as detection method as described previously [13]. All samples were analyzed in batches to reduce assay variability.

STATISTICS

The healthy volunteer study and the T2DM study were designed and conducted as two separate studies. The study in T2DM patients was designed to have 80% power to detect a difference in means of glucose AUC/time of 1.3 mmol with a 2-sided paired t-test and $p<0.05$ in a population of 16 patients. The hepatic glucose production and rate of disappearance of glucose were calculated using the Steele equations for non-steady state conditions as adapted for the use of stable isotopes [14]. The glucagon response between T2DM patients and healthy volunteers was compared, and the effect of oral antidiabetic drugs on the glucagon response was explored in the T2DM patients. Contrasts were analyzed using a mixed model analysis of variance (ANOVA). Insulin was the only parameter that was log transformed. Estimated differences are presented for the overall time profile during the challenge, between 180 and 360 minutes and corrected for pre-values, unless otherwise stated. All analyses were performed using SAS for Windows version 9.1.2 (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Thirty-six healthy volunteers underwent a glucagon challenge; demographic data are presented in Table 1. Short-lasting, transient adverse events such as nausea, dizziness and headache of minor severity occurred during the glucagon infusions. During the glucagon infusions, headache (10 out of 36 subjects, 28%), nausea (5 out of 36 subjects, 14%) and dizziness (5 out of 36 subjects, 14%) were the most common AEs. In the patient study, twenty-two T2DM patients on oral antidiabetic drugs were included; demographic data

are presented in Table 1. All patients used metformin (500-3000 mg daily), 12 subjects in combination with a sulfonylurea derivative (8 subjects glimepiride, 3 subjects tolbutamide, 1 subject glibenclamide). Within the population of T2DM patients, 12 subjects (59%) used statins, and blood pressure lowering agents were used by 6 patients (27%). None of the patients used drugs that have a known or suspected effect on glucose homeostasis such as ß-blockers or diuretics. All concomitant medication was continued throughout the study.

Fifteen of the 22 subjects underwent both clamps (68%), the baseline characteristics were comparable. In seven patients a second challenge was not performed because of nausea and vomiting during the first challenge (n=5), or because glucose levels exceeded 15 mmol/L after one week without oral antidiabetic medication (n=2). During the glucagon infusions, headache (9 out of 22 subjects, 41%), nausea (9 out of 22 subjects, 41%) and vomiting (4 out of 22 subjects, 18%) were the most common AEs. After stopping the challenge these symptoms resolved spontaneously within 5 minutes and without sequelae.

Before the start of the hyperglucagonemic challenge, plasma glucagon levels were well comparable between healthy volunteers and T2DM patients continuing oral medication, but lower in patients discontinuing oral medication and receiving overnight insulin (Table 2 and Figure 2A). After start of the hormone clamp (at t=180 min), glucagon levels increased to approximately 125-145 ng/L within 30-60 minutes in all three studied groups. This plateau in glucagon concentration was maintained during the entire clamp without significant differences between populations or between both study periods for the T2DM patients (Figrue 2A). During the glucagon challenge tests, endogenous release of glucagon and insulin was completely inhibited by somatostatin, as demonstrated by suppression of C-peptide levels (from 0.7 ±0.3 to 0.1 ±0.04 nmol/L for T2DM on oral medication, and from 0.26 ±0.18 to 0.07 ±0.02 nmol/L for T2DM receiving additional insulin).

Before start of the glucagon challenge, insulin concentrations were 10.1 mU/L in healthy volunteers and 11.8 mU/L in T2DM patients on oral medication (reflecting endogenous insulin levels), and 2-fold higher when patients received insulin (reflecting endogenous insulin levels plus additional exogenous insulin to ensure normal fasting plasma glucose levels before start of the challenge, Figure 2B). During the challenge, plasma insulin concentrations remained stable in all groups with an average of 10.6 mU/L in healthy volunteers, 9.2 mU/L in patients on oral medication, and 19.2 mU/L in patients treated with insulin (Table 2).

Plasma glucose levels were stable before the start of the glucagon challenge, but lower when T2DM patients were studied after receiving overnight insulin (Figure 2C). Plasma glucose levels increased during the glucagon challenge to maximal values of 13.5 ±1.9 mmol/L at the end of the challenge when the patients used oral antidiabetics. During the challenge that was performed after the overnight insulin, maximal glucose levels increased to 9.8 ±3.3 mmol/L. These levels were reached earlier; at 2 hrs after start of the challenge. The healthy volunteers had lower baseline glucose levels compared to T2DM patients in both study periods. The glucagon challenge induced a rise in glucose level to a maximal concentration of 11.5 ±1.7 mmol/L in healthy volunteers, which is lower than the patients continuing their standard medication, but higher than the patients receiving overnight insulin.

The increase in plasma glucose levels is explained by an increased HGP (defined as rate of appearance), since the rate of glucose disappearance remained fairly stable during the entire challenge in healthy volunteers and T2DM patients under both conditions (data not shown). HGP increased rapidly after start of the glucagon infusion. HGP increased approximately 142% (from 1.2 ±0.3 to 2.8 ±0.6 mmol/min) in T2DM patients continuing oral antidiabetic drugs, while about the same increase of 149% (from 0.9 ±0.2 to 2.2 ±0.7 mmol/min) was observed when patients received additional insulin (Figure 2D). The increase in HGP in healthy subjects exceeded the rise in T2DM patients, irrespective of the study condition, and amounted to 195% (from 0.9 ±0.2 mmol/min to 2.8 ±1.0 mmol/min) at 25 minutes after challenge start. The rapid rise in HGP was followed by a gradual decline in all three study groups. Although, the rate of HGP decline appeared equal for the T2DM patients in both conditions studied (Figure 2D), HGP level returned to baseline in patients receiving additional insulin before the end of the glucagon challenge, but was still elevated compared to baseline in patients continuing oral antidiabetic drugs. The rate in HGP decline was faster for healthy volunteers compared to T2DM patients, reaching baseline HGP levels at the end of the glucagon challenge.

DISCUSSION

There is accumulating evidence that increased glucagon levels and glucagon action play an important role in the pathophysiology of T2DM. We performed glucagon challenges in T2DM patients to compare the hyperglucagonemic response between patients and healthy volunteers, and to assess the effects of commonly prescribed oral antidiabetic drugs (metformin and sulfonylurea) on glucagon-mediated stimulation of endogenous glucose production. We demonstrated that the initial response to glucagon was stronger in healthy subjects and in diabetic patients on oral medication than in patients on insulin treatment. The gradual decline in HGP following the initial peak markedly differed between T2DM patients and healthy volunteers. The decline in HGP, with an onset already early during the glucagon challenge, was faster in healthy volunteers than in T2DM patients, irrespective of antidiabetic treatment. The exact mechanism for this difference between populations is unknown, but since the decline in HGP is explained by the induction of glucagon tolerance [15;16], the explanation is likely related to that phenomenon.

The rise in HGP following the glucagon challenge resulted in increased glucose levels. The glucose response to hyperglucagonemia in healthy volunteers was not markedly different from the glucose response in T2DM patients on oral antidiabetics, at least not when taking into account the difference in baseline glucose levels between both groups. This is in line with the observed initial increase in HGP upon hyperglucagonemia in both study groups, which was larger than the rise in HGP in diabetic subjects receiving additional insulin to control glucose levels. The maximal glucose response to a hyperglucagonemic challenge appears to be primarily driven by this initial rise in HGP. Differences between healthy volunteers and T2DM patients in subsequent HGP decline do not readily explain the glucose response.

Since the acute stimulatory effect of glucagon on HGP mainly reflects glycogenolysis [17], our data confirm that insulin directly inhibits the glycogenolytic response to hyperglucagonemia [18]. This explanation of a direct inhibition by insulin is also supported by our finding that the glucose disappearance rate was stable during the challenge. Obviously, indirect insulin effects such as the inhibition of lipolysis and proteolysis resulting in decreased release of gluconeogenic precursors or altered neural stimulation of the liver [19], or direct effects of glucose, may have contributed to a certain extent to the observed responses.

Our data suggest that mitigation of the effects of glucagon in T2DM might be a therapeutic option, since in T2DM the increase in HGP that follows upon glucagon stimulation is longer-lasting than in healthy volunteers. An antiglucagon-based therapy could have a beneficial effect on elevated glucose levels as add-on therapy for insulin-based therapies, especially to limit the glycemic effects of elevated postprandial plasma glucagon levels as observed in T2DM [3]. However, it should be stressed that physiological interpretation of the collected data is not straightforward. It should be taken into consideration that baseline glucagon levels were lower in patients received overnight insulin than in healthy volunteers or patients continuing their oral antidiabetic medication. This discrepancy was inevitable since it was aimed to reach comparable normoglycemic levels (i.e. between 4.5 and 6.5 mmol/L) preceding the glucagon challenge in both conditions, and to prevent symptomatic hyperglycemia during the challenge. We suggest that the most valuable exploration of the current data sets would require the development of an integrated glucagon-insulin-glucose biometrical model. Such a model would allow a more sophisticated assessment of the relationship between observed effects, and offer insight into the potential effects of (pharmacological) modulation of one or more components of the integrated model.

Although it is tempting to regard the hyperglucagonemic challenge as a relatively mildly burdening and safe methodological tool in clinical research, it is important to note that hyperglucagonemia is associated with short-lasting, transient adverse events such as nausea, vomiting, dizziness and headache [20]. It is remarkable that these events are not reported in literature [11;12]. In our experience, these effects were minimal in healthy subjects, but incidentally so severe in T2DM patients that procedures had to be interrupted. In the present study, the events were equally divided over study periods and appeared to be unrelated to glucose levels. As methodological tool in T2DM patients, an alternative glucagon challenge should be considered at a more physiological glucagon dose. Rational adaptations of the test procedure would be to gradually administer glucagon, or to limit the duration of hyperglucagonemia.

In conclusion, our results demonstrate that the response to a hyperglucagonemic challenge markedly differs between T2DM patients and healthy volunteers. Healthy volunteers show a greater increase in HGP upon glucagon infusion and also a faster decline in hepatic glucose production than T2DM patients, and exogenous insulin suppresses the glucagon-induced hepatic glucose production. This suggests that mitigation of the effects of glucagon in T2DM might be a therapeutic option, by introducing insulin in an earlier stage of the disease and/or developing medication targeting the glucagon receptor. Further research is needed to elucidate the potential role of glucagon antagonists as a therapeutic agent in T2DM.

TABLE 1 Baseline characteristics healthy volunteers and T2DM patients.

	Healthy (n=36) (± SD; min-max)	T2DM (n=22) avg ± SD (min-max)
Age (yrs)	40.5 ±18.3; (18-64)	56.0 ± 8.7 (29-65)
Gender	36 M	18M / 4F
BMI (kg/m²)	24.0 ±3.1; (17.6-29.9)	26.8 ± 3.2 (22.8-33.5)
Fasting glucose (mmol/L)	5.1 ±0.4; (4.2-5.7)	6.4 ± 1.8 (4.0-10.7)
HbA1c (%)	5.2 ±0.3; (4.6-5.7)	6.4 ± 0.6 (5.2-7.8)
Hip circumference (cm)	92.7 ±6.9; (82-109)	98.6 ± 5.3 (89.1-111)
Waist circumference (cm)	88.0 ±10.5; (70-111)	94.5 ± 8.8 (81-116)

TABLE 2 Statistical analysis results of glucagon, insulin, plasma glucose, hepatic glucose production (HGP) and rate of disappearance (Rd) before and during challenge without correction for pre-value. Presented is the 95% confidence interval of the treatment difference.

				Contrasts (95% CI and p-value)		
	Healthy volunteers	T2DM-oral	T2DM-insulin	HV vs T2DM-oral	HV vs T2DM -insulin	T2DM-oral vs insulin
Pre-challenge						
Glucagon (ng/L)	55.2	58.3	44.3	-10.3 to 4.2 p= 0.4008	3.6 to 18.2 p= 0.0038	10.0 to 17.9 p= <.0001
Insulin* (mU/L)	10.1	11.8	23.8	-29.3 to 3.5 p= 0.1070	-64.9 to -48.5 p=<.0001	-54.8 to -45.5 p=<.0001
Glucose (mmol/L)	5.2	7.2	6.3	-2.4 to -1.5 p=<.0001	-1.5 to -0.6 p=<.0001	0.7 to 1.1 p=<.0001
During challenge						
Glucagon (ng/L)	128.4	139.5	140.6	-23.5 to 1.3 p=0.0777	-24.6 to 0.4 p=0.0568	-5.3 to 3.3 p= 0.6448
Insulin * (mU/L)	10.6	9.2	19.2	-2.4 to 36.0 p= 0.0934	-53.2 to -34.7 p= <.0001	-54.3 to -49.6 p= <.0001
Glucose (mmol/L)	10.1	11.8	9.3	-2.5 to -1.0 (p= <.0001)	0.01 to 1.6 (p= 0.0479)	2.2 to 2.8 p= <.0001
HGP (mmol/min)	1.74	1.96	1.41	-0.4 to -0.06 p=0.0079	0.16 to 0.5 p= 0.0003	0.46 to 0.66 p= <.0001
Rd (mmol/min)	1.28	1.42	1.18	-0.29 to 0.001 p= 0.0512	-0.06 to 0.25 p= 0.2063	0.12 to 0.37 p= 0.0001

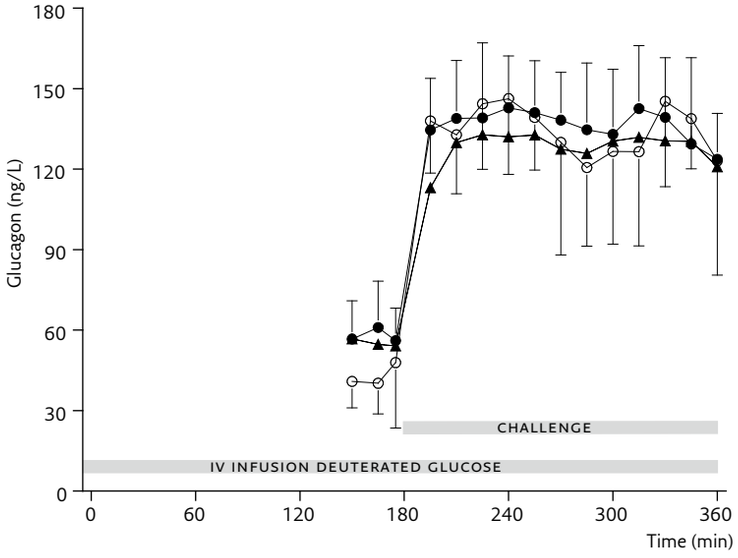
T2DM-oral: Continuation of oral therapy
T2DM-insulin: during insulin therapy (discontinuation of oral therapy)

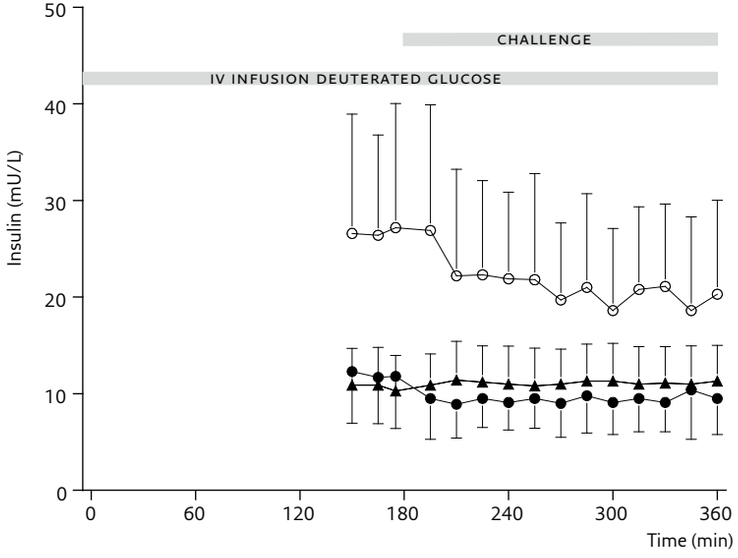
FIGURE 1 Glucagon challenge: infusion of labeled glucose, somatostatin, insulin and glucagon.

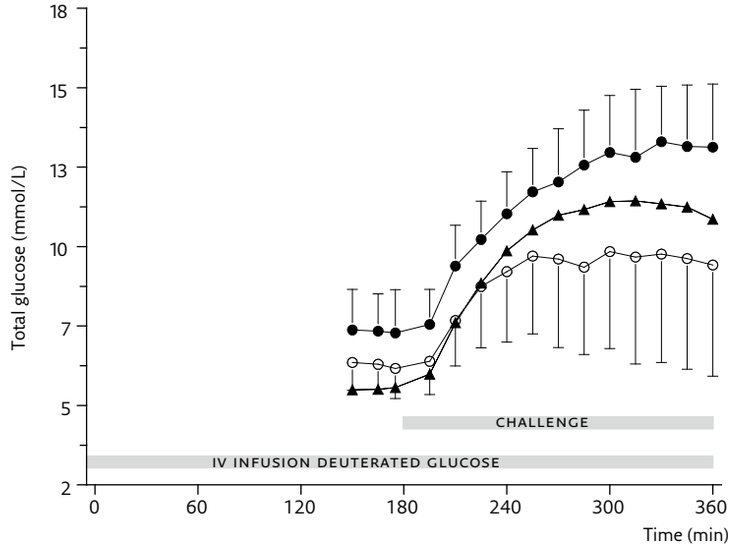
Basal period		Hyperglucagonaemic period
0 min - - - 5 min	5 min - - - 180 min	180min - - - 360 min
[6.6'-2H2] glucose bolus 5 mg.kg^{-1}	[6.6'-2H2] glucose continuous 0.05 mg.kg^{-1} .min^{-1}	
		Somatostatin 0.1 µg.kg^{-1}.min^{-1}
		Glucagon 3 ng.kg^{-1}.min^{-1}
		Insulin 4 mU.m^{-2}.min^{-1}

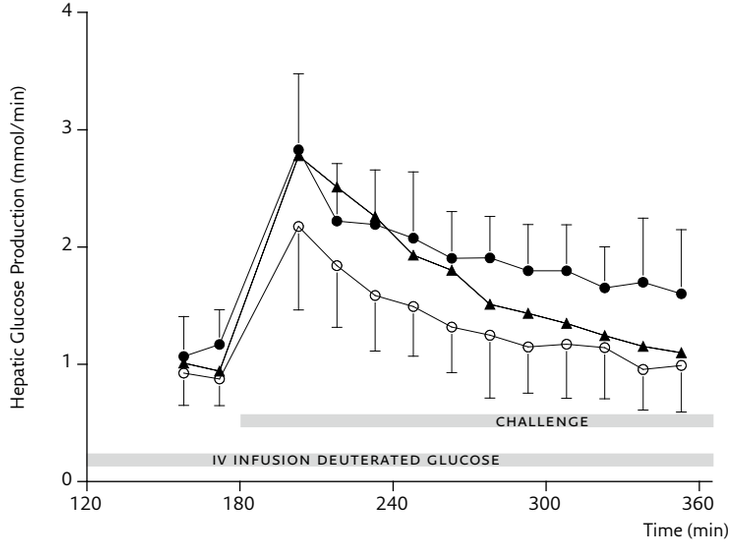
** Patients discontinuing oral medication received an overnight insulin infusion before the glucagon challenge, see Methods. This insulin infusion rate was continued on top of the standard 4 mU.m^{-2}.min^{-1} insulin during the pancreatic clamp.*

FIGURE 2 Glucagon (A), insulin (B), plasma glucose (C), and HGP (D) in healthy volunteers and T2DM patients; averages with SD error bars (T2DM only). Closed circles: continuation of oral medication. Open circles: insulin therapy (discontinuation of oral therapy). Triangles: healthy volunteers.









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CHAPTER 5

First proof of pharmacology in humans of a novel glucagon receptor antisense drug

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ABSTRACT

BACKGROUND Fasting and postprandial hyperglucagonemia in type 2 diabetes mellitus (T2DM) patients cause excessive hepatic glucose production (HGP), suggesting that attenuation of hepatic glucagon action could be a therapeutic strategy for T2DM.

METHODS In this study we evaluated the safety, tolerability, pharmacokinetics and pharmacodynamics in healthy human volunteers of single and multiple doses (50 to 400 mg) ISIS 325568, a 2´-O-MOE antisense (ASO) developed to reduce hepatic glucagon receptor (GCGR) MRNA expression. In the multiple dose cohorts, treatment consisted of 8 doses of ISIS 325568 or placebo over 6-weeks. Drug effects were assessed using serial fasting glucagon measurements and the glycemic response to a glucagon challenge at baseline and at the end of 6-week treatment.

RESULTS ISIS 325568 was not associated with clinically relevant changes. Dose-dependent predominantly mild injection site reactions were the most common side-effect. Active treatment caused a gradual increase in fasting glucagon levels and, compared to placebo, a significantly blunted glucagon-induced increase in plasma glucose AUC (24%, $p<0.0001$) and HGP (13%, $p=0.007$) at the 400 mg/week dose.

CONCLUSION Six weeks treatment with ISIS 325568 in healthy volunteers attenuated glucagon-stimulated HGP and glucose excursions, supporting further evaluation of the GCGR antisense approach in patients with T2DM.

INTRODUCTION

Currently available drug therapy for patients with type 2 diabetes mellitus (T2DM) including the use of insulin is not completely successful in restoring glycemic control. Thus, there remains a need for agents with novel mechanism(s) of action. A possibly feasible approach could be to ameliorate excessive hepatic glucose production. This may be achieved by attenuating glucagon action as it has been shown that in T2DM the glucagon-insulin ratio is disrupted [1] and increased glucagon action is largely responsible for hallmark features of T2DM such as hepatic insulin resistance and increased rates of glucose production [2]. This suggests that targeting glucagon action is a distinct mechanism that may offer therapeutic possibilities (as add-on therapy) for T2DM patients currently uncontrolled with oral anti-diabetic agents. The effect of glucagon are mediated by binding to and activating the glucagon receptor (GCGR), which is mainly expressed in the liver and kidney, with lower expression levels in cardiac, adipose and other tissues [3;4]. Pharmacological antagonism of glucagon action has been investigated non-clinically as a potential therapeutic approach

for T2DM. Both peptide antagonists and monoclonal antibodies against GCGR attenuate hyperglycemia in animal models [5;6]. In addition, antagonism of the GCGR in humans suggests that the concept may be worthwhile to pursue [7], but issues relating to pharmacokinetics, selectivity, cross species differences and lack of sustained effects after non-competitive blockade have thus far hindered the development of clinically applicable therapies [8]. An alternative approach could be to use specific antisense oligonucleotides (ASOs) to the GCGR. ISIS 325568 is such an ASO targeting GCGR MRNA and was designed to function through an RNASE H-dependent antisense mechanism [9]. RNASE H efficiently induces antisense-mediated cleavage of MRNA and is present in most mammalian cells. Antisense-mediated reduction of target MRNA levels by this mechanism results in subsequent reduction in target protein levels [10]. Pharmacology studies with antisense drugs with dedicated (physicochemical) properties have shown that it is possible to achieve tissue-selective inhibition. This was also shown for species-specific GCGR antisense drugs including ISIS 325568. It is possible to achieve selective inhibition of hepatic and adipose tissue GCGR expression, resulting in normalization of blood glucose levels in diabetic animal models without development of hypoglycemia [11;12]. In addition, GCGR antisense therapy increased levels of active GLP-1 levels and improved pancreatic ß-cell function [12]. This suggests that specific liver- and fat-directed GCGR antisense therapy could offer a novel therapeutic approach in T2DM. Therefore, a study was performed for the first time in human subjects to evaluate the effects of the specific GCGR antisense drug ISIS 325568 with the aim to obtain proof of pharmacology in humans while also assessing its tolerability and pharmacokinetics.

METHODS

The study was conducted according to the principles of the Declaration of Helsinki, the Guideline for Good Clinical Practice, and the pertaining Dutch law. The study protocol was approved by the Central Committee on Research involving Human Subjects of the Netherlands. All subjects gave written informed consent before any study-related procedure was performed.

EXPERIMENTAL DRUG – ISIS 325568

ISIS 325568 is the 19 sodium salt of a 3´→ 5´ phosphorothioate oligonucleotide 20-mer in which each of the 19 internucleotide linkages is an O-O-linked phosphorothioate diester. There are two 2´-O-(2-methoxyethyl) (MOE) modified ribonucleosides at the 3´and 5´termini flanking sixteen 2´-deoxyribonucleosides. The sequence of ISIS 325568 is 5´- $G^{Me}CA^{Me}CTTTGTGGTC^{Me}$ $C^{Me}CAAGG^{Me}C$- 3´ where the underlined bases are 2´-O-MOE riboses and the

cytosine nucleotides are methylated. As such, it is a second-generation antisense phosphorothioate oligonucleotide. In cynomolgus monkeys, which show almost 100% GCGR receptor homology at the ISIS 325568 binding site, ISIS 325568 was broadly distributed after subcutaneous (sc) injection, mainly to kidney and liver. Full-length ISIS 325568 is cleared from monkey tissues with a half-life of approximately 11 days in kidney and 13 days in liver. In 13-week mouse and monkey studies, ASO dose-dependently reduced liver GCGR MRNA expression to a maximum of 75-90% and resulted in 4- to 6-fold increases over baseline in plasma glucagon and active GLP-1 levels. Despite the substantial inhibition of GCGR expression, decreases in serum glucose levels below normal were not observed. Apart from the well described common systemic toxicities and side effects (for example, local inflammation resulting in injection site reactions) noted for this generation of ASOs at doses and exposures that exceed the intended clinical dose level and regimen no adverse findings were noted ([13] and data on file). Importantly, no alterations in renal function were observed in monkeys treated with 60 mg/kg/week.

STUDY POPULATION

The study was performed in 58 male volunteers who were considered healthy after medical screening. The subjects were between 18 and 65 years of age with a body mass index (BMI) below 30 kg/m², a fasting plasma glucose concentration below 6.4 mmol/L and Hb1Ac below 6.3%. Main exclusion criteria were clinically significant abnormalities in medical history or physical examination, and abnormalities on laboratory examination.

PROCEDURES

The study consisted of two parts. In the first part single sc doses of ISIS 325568 were administered to 16 volunteers using a double blind design. The doses were 50, 100, 200 and 400 mg with a randomization ratio active:placebo of 3:1. The 50, 100 and 200 mg doses were given as a single sc injection in the abdominal region, and the 400 mg dose was administered as 2 sc injections (200 mg each; one abdominal and one upper arm). Twelve samples for drug assay were taken from the time just prior to injection to approximately 72 hours after injection for pharmacokinetic assessment of the drug. A 24-hour urine sample was also collected from each subject. Also, measurements of vital signs, ECG-parameters, routine laboratory, coagulation and complement factors were performed, and adverse events were recorded. A final follow-up assessment took place at approximately 30 days after dosing.

In the second part of the study 4 sequential cohorts were given 8 doses of study drug over 6 weeks. The doses administered were 50, 100, 200 and 400 mg with a randomization ratio active:placebo of 3:1 for the 50 mg cohort (4 subjects) and 2:1 in the other cohorts (12 subjects in each cohort). In all cohorts, samples for fasting glucagon levels were taken before treatment up to 6 weeks after study drug administration. One week before dosing initiations, subjects in 100, 200 and 400 mg dose cohorts underwent a pre-treatment glucagon challenge. The glucagon challenge was performed as previously described [7;14]. The procedure started with a run-in infusion with [6,6-$^2H_2$] glucose (Cambridge Isotope Laboratories, MA, USA), consisting of a priming dose of 5 mg.kg^{-1} and a 3 hrs continuous infusion of 0.05 mg.kg^{-1}.min^{-1}. Subsequently, simultaneous infusions of glucagon (3 ng.kg^{-1}.min^{-1}), somatostatin (0.1 µg.kg^{-1}.min^{-1}) and insulin (4 mU.m^{-2}.min^{-1}) were given for 3 hrs, while the infusion of [6,6-$^2H_2$] glucose was continued. Samples for (labeled) glucose, insulin and glucagon were taken regularly.

One week later, the subjects were admitted to the clinical center and study drug was given on day 1, 3, and 5 by one hour IV infusions. Thereafter, the subjects received the subsequent 5 sc doses at weekly intervals starting 3 days after the final IV dose. The site of injection alternated between the abdomen and upper arm. One week after the final sc dose a second glucagon challenge was administered. Thereafter, follow-up assessments were done for 9 weeks. Physical examinations, measurement of vital signs, collection of blood specimens for clinical chemistry, hematology, coagulation, and complement tests, collection of urine for urinalysis, ECG measurement, and collection of blood and urine for drug assay were performed regularly throughout the trial. Full plasma pharmacokinetic profiles were obtained following the first intravenous infusion and following the final sc injection. Additional plasma samples were collected to measure trough and peak levels of the subsequent IV infusions. Samples for drug assay were taken just prior to the first, third, fifth and final sc dose. Twenty-four hour urine samples for drug assay were collected following the first and last dose.

DRUG ASSAY

Plasma samples were assayed for ISIS 325568 using a validated hybridization ELISA [15]. The analysis quantifies the parent compound in plasma with <6% cross-reactivity to oligonucleotide with one base removed (19mer), and no cross-reactivity to the n-2 metabolites of ISIS 325568 (18mer) and endonuclease shortened oligonucleotides. The calibration range of the assay was 0.5 to 50 ng/mL for ISIS 325568, with the low end defining the lower limit of quantitation.

Urine samples were analyzed after a 2-step solid phase extraction. Analysis was accomplished by capillary gel electrophoresis (CGE) with UV detection (260 nm) using a method similar to that previously reported [16]. The linear quantification range was from 0.2 to 20.0 µg/mL, with the lower limit representing the lower limit for quantitation. Metabolite concentrations and total

oligonucleotide concentrations were quantified using calibrators and normalized to the relative extinction coefficients of the putative metabolites and that of the parent compound.

LABORATORY TESTS

Samples for routine clinical chemistry, hematology, and coagulation were analyzed using routine methodology. Insulin was measured with an immunoradiometric assay (Biosource Europe S.A.; interassay coefficient of variation: 6.1-6.5 %), glucagon was measured with a radioimmunoassay Kit (LINCO research, Missouri, USA; CV 7.3-13.5 %), and active GLP-1 was measured using the Meso Scale Discovery assay by Pacific Biometrics Inc (Seattle, Washington, USA). The assays were performed in batches to reduce assay variability.

Glucose, labeled [6,6-$^2H_2$] glucose concentrations and tracer/tracee ratios were measured using a validated GC/MS assay at the laboratory for endocrinology laboratory of LUMC [17].

For exploratory purposes, IFNα, IL-6, MCP-1, and MIP-1α were measured with ELISA kits that are validated for in vitro diagnostic use, but had low detection limits and reasonable variability. Complement C5a was measured using human C5a ELISA kits (HyCult Biotechnology, Uden, The Netherlands).The placebo and pre-dose data of all subjects in this study showed a mean (SD) C5a concentration of 267 (437) ng/mL. Factor Bb was measured using Quidel Bb Plus Enzyme Immunoassay Kit (Quidel, San Diego, CA, USA). The placebo and pre-dose data of all subjects in this study showed a mean (SD) Bb value of 0.88 (0.22) µg/mL.

STATISTICAL ANALYSIS

The safety and tolerability data analysis was performed on all subjects that were dosed and was descriptive. Pharmacokinetic parameters were obtained from plasma ISIS 325568 concentration-time profiles following single sc injection (first study part), single dose 1-hr iv infusion (first dose second part) and the final sc injection (last dose second part of the study). In addition, urinary excretion of ISIS 325568 and its oligonucleotide metabolites were measured in 0-24 hr collections following both single and multiple dose administration. Plasma pharmacokinetics for ISIS 325568 were analyzed using a non-compartmental method with WinNonLin Professional Version 5.01 software (Pharsight Corp., Mountain View, CA), and included peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}), area under the plasma concentration-time curve extrapolated to infinity (AUC_∞), terminal half-life, and bioavailability (%F). These pharmacokinetic parameters and the urinary excretion values were summarized using descriptive statistics. Dose normalized AUC and C_{max} values (normalized to the 200 mg dose) were compared using ANOVA (analysis of variance) to test dose-linearity of exposure.

The analysis on the pharmacodynamic effects of ISIS 325568 was performed on subjects that received all doses and completed the pre- and post-treatment glucagon challenge. The analysis concerned the hepatic glucose production, the systemic glucose disposal, the glucose AUC obtained during the challenges and the difference in fasting glucagon and GLP-1 concentrations during the treatment. Hepatic glucose production was calculated using the Steele equations for non-steady state conditions as adapted for the use of stable isotopes using a pool fraction of 65% [18]. The data was first checked for normality with a Shapiro-Wilk test. Two analyses were performed. The first analysis consisted of a comparison within each dosing group (week 1 versus week 7) with an ANOVA. Results are reported with the estimated differences along with the corresponding 95% confidence interval and p values.

The second analysis consisted of the comparison of active treatment vs. placebo. This was analyzed with a mixed model analysis of variance with treatment, week and time as fixed factors and all the interactions, and subject, subject by week and subject by time as random factors. Results are given as the least squares mean estimates, the estimated difference with the corresponding 95% confidence interval, and the p-value. The data of the 12 placebo-treated subjects were pooled.

The analysis for the change from baseline for glucagon was analyzed with an ANCOVA with treatment and time as fixed factor, subject, subject by time and subject by treatment as random factors and pre-dose value as covariate. Results are given as the least squares mean estimates, the estimated difference with the corresponding 95% confidence interval, and the p-value. While it is difficult to draw firm conclusions on the PK-PD relationship from this study, a correlation of plasma trough concentration (C_{min}) and the PD end-points was attempted. Steady-state levels of ISIS 325568 were achieved by approximately 4-weeks in this study, with no significant difference between the 4 and 6 week C_{min} levels. Therefore, the average plasma C_{min} values for weeks 4-6 were averaged and their relationship with the plasma glucagon levels and the reduction in glucose AUC during the glucagon challenge was explored. All statistical analyses were done using SAS software (version 9.1.3; Cary, NC).

RESULTS

BASELINE CHARACTERISTICS

Sixteen male subjects were dosed in the single dose part of the study and had an age range of 18 to 63 years and a body mass index (BMI) between 19 and 30 kg/m^2, and all subjects completed the study. In the multiple dose part of the study a total of 43 male subjects were dosed, ranging in age from 18 to 64 years (mean 39 yrs). The BMI ranged from 18.6 to 29.7 kg/m^2 (mean 23 kg/m^2) and the

baseline glycated hemoglobin levels ranged from 27 to 42 mmol/mol (mean 33 mmol/mol). During the entire study four subjects were withdrawn; two subjects for non-treatment related intercurrent diseases (anemia, epididymitis), one subject for personal reasons and one subject because of an unsuccessful pre-dose glucagon challenge. The subjects were replaced and the replacements received the same treatment, except for the subject participating in the 50 mg dose group of the multiple dose part of the study who developed anemia.

SAFETY

There were no clinically significant changes in vital signs, ECG recordings and routine laboratory parameters in the single and multiple dose part of the study. In addition, there were no dose-dependent trends or differences in these parameters between placebo-treated and ISIS 325568-treated volunteers. Specifically, renal function parameters such as serum creatinine and urinary excretion of ß2-microglobulin (data not shown) were not different between the treatment groups. There was a dose-dependent and transient prolongation of APTT at 1 hr post IV infusion (maximum average increase of 27 sec in the 400 mg dose group) which had returned to baseline at 3 hrs post infusion. Complement (C5a and Bb), and serum cytokines (IFN-α, IL-6, MIP-1α, MCP-1) showed that there was no indication for meaningful changes at any dose (data not shown).

The most commonly observed treatment-related AE were predominantly mild injection site reactions (ISRs) that were observed in the multiple dose cohorts, particularly at doses exceeding 100 mg. Injection site reactions occurred as erythema at the sc injection site with or without itch and/or minimal swelling and resolved spontaneously, although in 6 subjects injection site reactions persisted as a small hyperpigmented area. Local lymphadenopathy or other signs of symptoms were not observed.

After IV dosing with 400 mg ISIS 325568, transient feelings of malaise and tiredness, nausea, and headache were observed in 7 of the 8 subjects and was accompanied by fever (max temperature 39.3°C) in 4 subjects. These symptoms were not observed upon any of the sc administrations.

PHARMACOKINETICS

The drug plasma concentration-time curves followed a poly-exponential pattern (Table 1). Distribution half-life was 1-2 hrs following termination of the IV infusion. This rapid distribution phase was followed by at least one much slower elimination phase. Apparent terminal elimination half-lives following the final sc dose administered in the multiple dose cohorts ranged from 14 to 21 days, consistent with slow drug elimination from tissue. Plasma trough levels were stable after the third sc dose suggesting that steady-state levels were

reached. C_{max} and AUC increased in a dose-dependent and linear fashion over the dose range studied (50 mg to 400 mg), both after the IV and sc single dose administration. Upon repeated dosing, C_{max} or AUC did not increase compared to first dose which is consistent with the rapid uptake of the ASO into tissues of distribution and confirms a lack of accumulation in plasma. ISIS 325568 was well absorbed after sc administration with absolute bioavailability ranging between 72-101%. While AUCs were similar following sc injection and IV administration, C_{max} following sc administration was approximately 4-fold lower and reached later. Urinary excretion was low, with less than 5% of the administered dose excreted over 24 hr following sc injection. After IV administration of 400 mg approximately 10% of the dose was excreted in urine over 24 hr.

PHARMACODYNAMIC EVALUATION

Fasting plasma glucagon levels increased dose-dependently after multiple doses of ISIS 325568 (Figure 1). The increase in glucagon levels for the integrated response (entire time profile over treatment period) between placebo (60.2 ng/mL) and active treatment was significant for the 400mg dose (73.9 ng/mL) and amounted to 23% (p=0.017). The baseline corrected average change in fasting plasma glucagon compared to placebo increased for the 400 mg dose only. The change in glucagon concentrations at this dose was 25 ng/ml (p=0.007), while the glucose levels for both treatments were similar (placebo vs. 400 mg treatment: 5.1 vs.5.2 mmol/L before dosing and 5.1 and 4.9 mmol/L after dosing (NS), respectively).

Treatment with GCGR antisense also affected HGP under conditions of selective hyperglucagonemia and basal insulin concentrations. Here it was shown that after initiation of the glucagon infusion, plasma glucagon levels rapidly increased and reached a steady-state concentration that was about 2-fold higher than the pre-infusion concentration in all cohorts. During the pre-treatment glucagon challenges, hepatic glucose production (HGP) increased rapidly upon initiation of the glucagon infusion from 1.0 to 2.5 mmol/min (Figure 2). HGP remained 2-fold higher than basal levels for the first 90 minutes of the glucagon infusion and subsequently showed a gradual decline by the end of the glucagon infusion period. The rate of glucose disposal was more or less constant during the glucagon infusion. The glucagon infusion resulted in a rapid elevation of plasma glucose levels from a basal level of 5.5 mmol/L to a steady-state level of 12 mmol/L (Figure 3). This effect was consistent between all the treatment groups. After treatment with ISIS 325568, the glucagon profile was highly similar, but the increase in HGP was blunted, while the rate of disappearance was unaffected (Figure 2 and Table 2). As a consequence, the increase in plasma glucose levels during the hyperglucagonemic period were blunted after the 200 and 400 mg dosing regimen (Figure 3). The effect assessed by the glucose AUC values during the 3-hour challenge was 6% (ns) in the 200 mg group

and 24% (p<0.0001) in the 400 mg group. Variability and lack of consistency in the GLP-1 assay did not allow interpretation of this parameter.

DRUG CONCENTRATION AND PD RESPONSE RELATIONSHIP

The relationship between changes in plasma glucagon levels and the reduction in glucose AUC during the glucagon challenge, by dose group, plotted against the average trough concentrations of ISIS 325568 obtained over 6 weeks of treatment is shown in Figure 4. The C_{min} values correlated with the change in both pharmacodynamic end-points. The changes in plasma glucose and glucagon levels in the 400 mg group occurred at average PK trough concentrations of approximately 13 ng/mL and higher. Pre- and post-treatment changes in plasma glucose and glucagon levels were minimal and not statistically significant at the lower doses (100 and 200 mg), which correlated with lower average PK trough concentrations.

DISCUSSION

This study investigated the effects and pharmacokinetics of ISIS 325568, a second-generation antisense phosphorothioate oligonucleotide. As there was no clinical experience with ISIS 325568, the dose range chosen for this study which was approximately 0.6 to 6 mg/kg body weight was based on the pre-clinical data of ISIS 325568, and clinical experience with other 2´-MOE-modified ASOs that were demonstrated to be well-tolerated in healthy volunteers at doses up to 600 mg and treatment durations of 12 weeks [13]. Six weeks of treatment with ISIS 325568 caused a significant inhibition of the glucagon-mediated increase in hepatic glucose production.

This study extends the safety findings of 2´-MOE-modified ASOs as no clinically relevant changes in vital signs, ECG, clinical chemistry or urinalysis occurred after weekly doses up to 400mg. Particularly, fasting hypoglycemia was not observed and ISIS 325568 was not associated with untoward renal effects. The latter finding is important as one of the metabolic routes of ASOs is uptake by proximal tubular cells, followed by sequestration and break-down in lysozomes. Apparently, accumulation of ISIS 325568 in the proximal tubules in humans does not affect renal function, which is consistent with the findings in animals. The flu-like symptoms, which possibly reflect a mild systemic inflammatory reaction, were observed at the 400 mg IV dose only and not after sc administration of the same dose. These findings are consistent with previous observations with this class of ASOs [13;19]. Local injection site reactions occurred frequently at weekly doses ≥ 100 mg with an incidence of ~60%. The incidence of injection site reactions appears to be lower with the more recent antisense compounds (data on file ISIS Pharmaceuticals Inc., USA).

The pharmacokinetics of ISIS 325568 showed an almost complete bioavailability after sc dosing with terminal elimination half-lives in order of 14-21 days, consistent with slow drug elimination from tissue. C_{max} and AUC increased in a dose-dependent and linear fashion over the dose range studied (50 mg to 400 mg), both after the IV and sc single dose administration. However, consistent with the rapid uptake of the ASO into tissues, C_{max} or AUC did not increase upon repeated dosing. Urinary excretion over 24 hr amounted to only 5% of the administered dose, with a trend for higher excretion (10%) after IV administration of the 400 mg dose. The latter observation suggests a higher unbound fraction in plasma available for filtration.

This study showed that 6 weeks of treatment with ISIS 325568 translated into a significant inhibition of the glucagon-mediated increase in hepatic glucose production. This effect was observed in the 400 mg dose cohort and amounted to approximately 15-20 % inhibition in HGP. As in non-human primates ~60% knock-down is associated with a 30% reduction in HGP, it seems that the level of knock-down in humans after treatment with the 400 mg dosage regimen was in the same order of magnitude. Proof of pharmacology in humans was further demonstrated by the gradual increase in fasting glucagon levels, a validated biomarker for this target based on preclinical studies. The increase was 23% (NS) in the 200 mg dose group and 41% (p=0.007) for the 400 mg treatment. The most likely explanation of the elevated basal glucagon levels is a feedback of the pancreas as a response to diminished hepatic GCGR receptor availability. However, it can also be a direct effect as pre-clinical studies with GCGR ASOs showed activation of pancreatic alpha cells (evidenced by increased plasma concentrations of active GLP-1). As a result of the increase in active GLP-1 induced by GCGR ASO, islet insulin content is increased, insulin secretion is preserved and glucose tolerance is improved [12]. Importantly, it is unlikely that hypoglycemia resulted in elevated glucagon levels as none of the volunteers had low glucose levels throughout the entire observation period.

Although it is recognized that it is difficult to draw firm conclusions on the PK-PD relationship from this study, it appeared that average trough plasma concentration of ISIS 325568 correlated with the change in both the pharmacodynamic end-points (plasma glucagon levels as well as reduction in glucose AUC during the glucagon challenge). Importantly, plasma trough concentration are reflective of tissue drug concentration and we found that the changes in plasma glucose and glucagon changes in the 400 mg group occurred at average PK trough concentrations of approximately 13 ng/mL and higher. These levels are predictive of liver drug concentrations of 80-100 µg/g liver tissue, which have been shown to produce pharmacology in preclinical models.
In conclusion, 6 weeks treatment with GCGR ASO in healthy volunteers resulted in the first demonstration of pharmacology in humans. These results support further evaluation of ASO-based therapies against the glucagon receptor in patients with type 2 diabetes mellitus.

TABLE 1 Mean ± standard deviation of pharmacokinetic parameters for ISIS 325568 after the initial 1-hour intravenous infusion (A) and final sc administration (B) in the multiple dose part of the study.

A (IV)	**50mg (n=3)**	**100mg (n=10)**	**200 mg (n=8)**	**400 mg (n=8)**
C_{max} (µg/mL)	8.2 ± 0.6	14±1.6	30 ± 7.0	54 ± 18
$AUC_{0\text{-}inf}$ (µg*hr/mL)	16 ± 2.0	33 ± 4.5	66 ±9.3	121 ± 14
Cl (L/hr)	3.1 ± 0.4	3.0 ± 0.4	3.1 ±0.4	3.3 ± 0.4
Vss (L)	6.5 ± 0.9	6.7 ± 1.0	7.5 ± 1.0	9.1 ± 1.2
T½_distribution (hr)	2.0 ± 0.04	2.2 ± 0.3	2.5 ±0.3	2.7 ± 0.3
T½_final (hr)	8.2 ± 0.2	7.8 ± 1.2	6.4±0.7	6.4 ± 2.1
B (SC)	**50mg (n=2)**	**100mg (n=8)**	**200 mg (n=8)**	**400 mg (n=8)**
C_{max} (µg/mL)	1.9	3.1±0.9	7.0 ± 1.6	9.2 ± 2.2
T_{max} (hr)	4.0	3.4 ± 2.0	2.6 ± 1.0	2.4 ± 0.6
$AUC_{0\text{-}24h}$ (µg*hr/mL)	15	23 ± 3.9	51 ± 6.6	77 ± 15
$AUC_{0\text{-}168h}$ (µg*hr/mL)	16	25 ± 4.3	56 ± 6.5	88 ± 19
% F	101	76 ± 11	86 ± 13	72 ± 13
T½_distribution (hr)	2.3	3.3 ± 1.1	3.4 ± 1.1	4.4 ± 0.9
T½_final (days)	14	21 ± 8.9	19 ± 3.4	18 ± 11

C_{max}: maximal concentration; $AUC_{0\text{-}inf/0\text{-}24h/0\text{-}168h}$: Area Under the Curve for t= 0 min to infinity, 24 hrs, 168 hrs; Cl: Clearance; Vss: volume of distribution; T ½_distribution/_final: half-life associated with distribution/ final phase; % F: percentage bioavailability.

TABLE 2 Mean (SD) values in hepatic glucose production (HGP), glucose rate of disappearance (Rd), and time-corrected area under the curve for glucose (AUC/T) during the hyperglucagonemic challenge before (pre) and after (post) treatment for 8 weeks with different dose of ISIS 325568 or placebo. The point estimate of the difference (along with the corresponding 95% confidence interval (95% CI) and p-value are reported.

		HGP (µmol/ min)	**Difference (95% CI) p-value**	**Estimated difference (post-pre) vs placebo (post-pre)**	**Rd (µmol/ min)**	**Difference (95% CI) p-value**	**Estimated difference (post-pre) vs placebo (post-pre)**	**Glucose AUC/T (mmol/L)**	**Difference (95% CI) p-value**	**Estimated difference (post-pre) vs placebo (post-pre)**
Placebo (n=12)	Pre	1525 ± 246.0			1169 ± 251.0			9.6 ± 1.20		
	Post	1587 ± 275.2	62 (-81 / 205) 0.36		1173 ± 178.5	4 (-115 / 122) 0.95		9.8 ± 1.28	0.2 (-1.0 / 1.3) 0.75	
100mg ISIS 325568 (n=8)	Pre	1628 ± 202.0			1318 ± 166.1			10.0 ± 1.73		
	Post	1593 ± 268.2	-35 (-244 / 174) 0.70	-129 (-319 / 72) 0.20	1304 ± 217.1	-14 (-298 / 269) 0.91	-7 (-227/ 213) 0.95	9.9 ± 1.79	0.1 (-0.9 / 1.1) 0.8571	-0.2 (-1.4 / 1.0) 0.75
200mg ISIS 325568 (n=8)	Pre	1838 ± 164.3			1232 ± 205.9			11.2 ± 0.80		
	Post	1792 ± 259.5	-45 (-231 / 140) 0.58	-96 (-296 / 103) 0.33	1264 ± 254.1	32 (-181 / 245) 0.73	21 (-196 / 238) 0.85	10.5 ± 0.93	-0.7 (-1.6 / 0.2) 0.11	-0.9 (-2.1 / 0.3) 0.12
400mg ISIS 325568 (n=8)	Pre	1774 ± 293.0			1224 ± 254.9			10.0 ± 1.11		
	Post	1539 ± 261.2	-235 (-313 / -157) 0.0002	-282 (-482 / -82) < 0.01	1204 ±175.1	-20 (-157 / 117) 0.74	-32 (-250 / 185) 0.76	7.6 ± 1.09	-2.6 (-3.4 / -1.7) 0.0002	-2.7 (-3.9 / -1.6) <.0001

FIGURE 1 Time course of plasma glucagon levels (mean and SD) during multiple dose treatment with ISIS 325568. Intravenous doses are indicated by the dashed arrows and solid arrows indicate the weekly sc doses. For clarity the SD bars are given for the placebo and 400 mg dose regimen only.

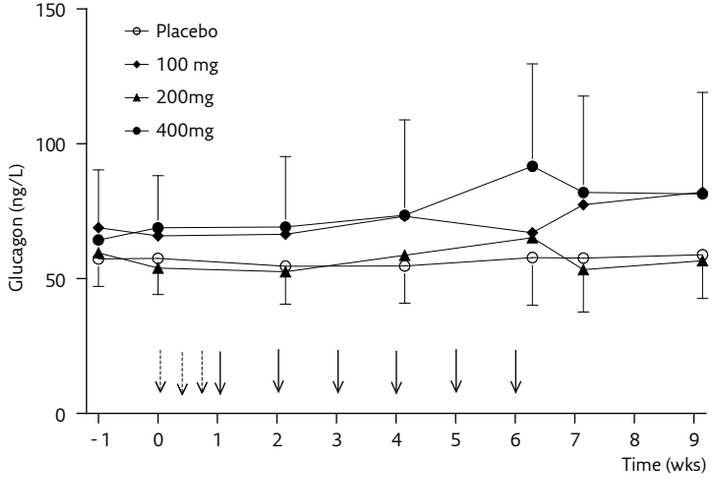


FIGURE 2 Time course of hepatic glucose production (HGP) and rate of glucose disposal (Rd) before and after 8 weeks treatment with placebo (left panel; n=13) or 400 mg ISIS 325568 (right panel; n=8).

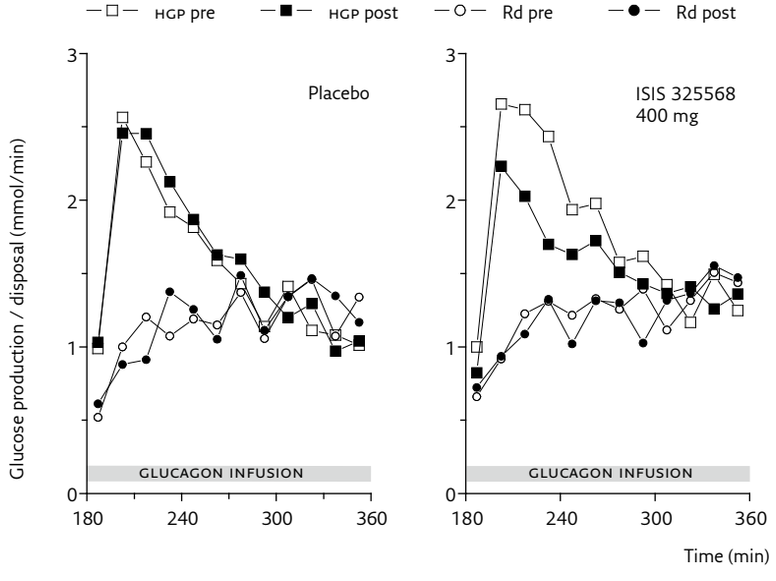


FIGURE 3 Mean glucose values during glucagon challenge for the different dose groups before (closed circles) and after 8 weeks treatment (open squares) with ISIS 325568.

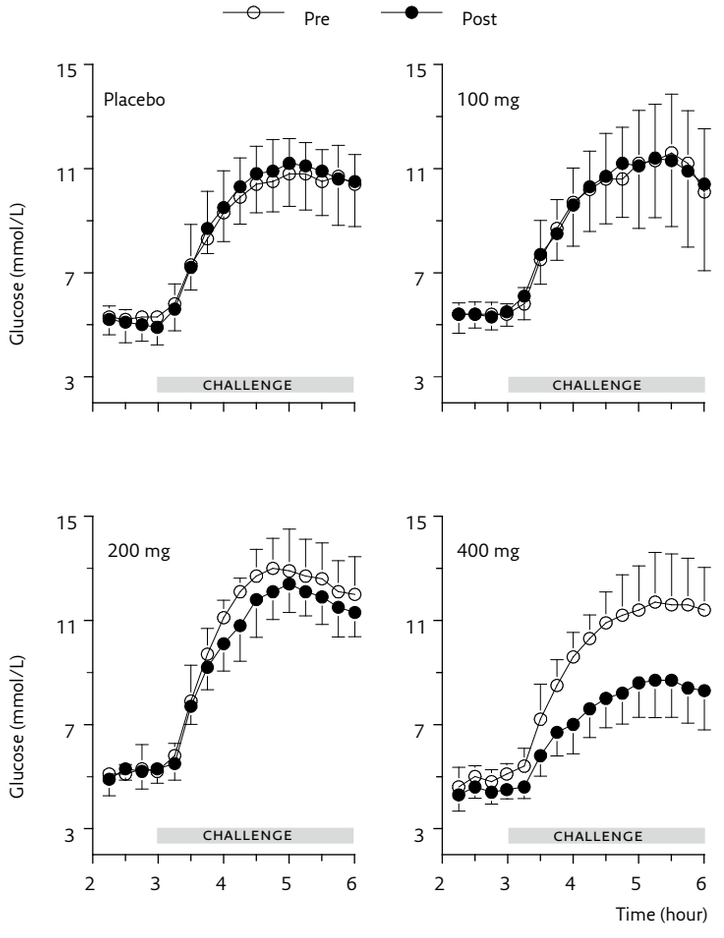
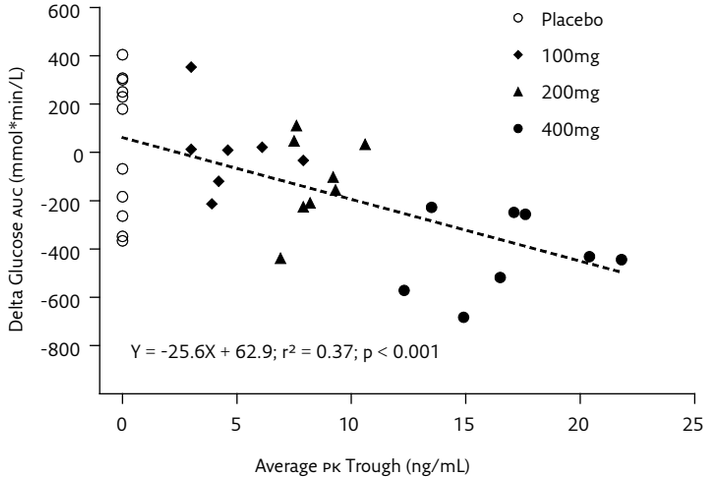
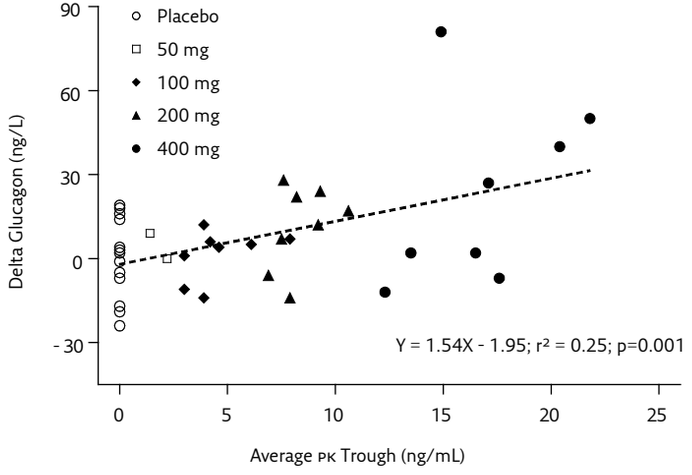


FIGURE 4 Plots of steady state trough concentration of ISIS 325568 after 6 weeks of treatment versus the change in glucose AUC during the glucagon challenge (A) and change in glucagon (B).





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CHAPTER 6

Modeling the effect of a glucagon challenge on glucose homeostasis in humans

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ABSTRACT

Modeling glucose metabolism is challenging because of the complex interactions between the hormones involved and the simultaneous effect on glucose in the system. This is particularly true for models incorporating the contribution of glucagon to glucose homeostasis; in fact models incorporating modulation the glucagon receptor have not been described yet. Therefore we have developed a semi-mechanistic model to describe the glucagon-insulin-glucose homeostasis in 36 healthy subjects during a glucagon challenge. Briefly, this challenge consisted of a 3 hrs infusion of somatostatin to block endogenous release of insulin and glucagon with simultaneous infusion of high dose glucagon and physiological dose insulin. The model captured glucagon and glucose dynamics, including the amplifying effects of glucagon on hepatic glucose production with the magnitude of the effect being dependent on the prevailing insulin concentration. Glucagon production was inhibited by elevated glucose concentrations. We observed a rapid increase of the glucose concentration in response to glucagon followed by a slow decrease in the HGP, which is consistent with the internalization of glucagon receptors (GCGR) upon stimulation by glucagon, which was captured by incorporating an effect compartment. The model also captured the effects of glucose on insulin production and the insulin-independent and insulin-dependent effect on glucose elimination.

This model, based on glucagon challenge data, could contribute to a better understanding of pathophysiology of diabetes mellitus. It describes the general trend and can therefore serve as a basis for drug development. Furthermore, an extension of this model could probably be incorporated in the recently developed automated, bihormonal, bionic pancreas for type 1 diabetes mellitus patients.

INTRODUCTION

In recent years, an increased interest has re-emerged in the role of the glucagon receptor (GCGR) in diabetes and its utility as a therapeutic target [1;2]. In healthy subjects the endocrine pancreas regulates glucose production and metabolism by a synchronized reciprocal release of insulin and glucagon in response to changes in blood glucose levels, free fatty acids (FFA), amino acids, incretin hormones (such as GLP-1 and GIP), among many other signals. In type 2 diabetes mellitus (T2DM), there is evidence for a dysregulation of glucose-insulin-glucagon interaction [3-5]. Elevated fasting and postprandial glucagon concentrations in T2DM patients could suggest a combination of dysregulated glucagon secretion and insulin resistance. The most important consequence of chronic hyperglucagonemia seems to be related to increased glucose production (gluconeogenesis but not glucogenolysis) aggravating hyperglycemia in diabetic patients [6]. Understanding the contribution of glucagon on the glucose metabolism is important for (patho)physiological insight and may also be important in guiding drug development for compounds targeting glucagon.

Glucose metabolism is challenging because of the complex interactions between the hormones involved and the simultaneous effect on glucose in the system. More than 3 decades ago Bergman *et al.* [7] developed the first model for glucose regulation. With help of this model the estimation of glucose tissue uptake sensitivity to insulin levels hypothesis was corroborated in clinical experiments, the biphasic insulin secretion profile was defined and the relationship between insulin and glucose concentrations as a possible mechanism of the disease was suggested.

Basic models in animals and *in vitro* have helped to understand underlying mechanisms and to explore new possible therapeutic targets for diabetes [8-10]. Farhy *et al.* [9] integrated glucagon into the physiological model, creating an explanation of the system from an intercellular level, suggesting high glucagon concentrations as lack of inhibition by glucagon counter regulation in ß-cell deficiency.

Several models based on clinical endpoints in healthy subjects and/or T2DM patients have been developed to describe and better understand the relationship between glucose and insulin [11-17]. PK/PD models have quickly evolved from minimalistic models describing glucose plasma concentrations to complex system pharmacology models that take multiple interactions into account [13;16-19]. Based on earlier models we can describe the general trends between insulin and glucose very well, and find that additional regulators in glucose homeostasis are required to improve our understanding of drug action in healthy volunteers, but more important in diabetes patients. For instance, since 2008 mathematical models of glucagon secretion and glucagon counter regulation based on *in vitro* studies were published [9]. Recently, a semi-mechanistic, integrated glucose-insulin-glucagon model was developed to assess the effects of individualized glucokinase activator on glycemic response [20-22].

However, models incorporating contribution of glucagon to glucose homeostasis while modulating the glucagon receptor, have not been described yet. Therefore we developed a semi-mechanistic model to describe the glucagon-insulin-glucose homeostasis in healthy subjects during a glucagon challenge. Briefly, this challenge consisted of a 3 hrs simultaneous infusion of high glucagon concentrations (supra-physiological) and insulin, while somatostatin was given to block endogenous release of these hormones. The model was subsequently used to predict the influence of the amount of hepatic glucagon receptors in the glucose plasma availability and to perform simulations with hypothetical scenarios for altered glucagon conditions, such as a glucagon challenge.

METHODS

SUBJECTS

Thirty-six healthy male volunteers participated in the clinical study, which was conducted at a single center (CHDR). Their mean (±SD) age was 40.5 ±18.3 years, weight 78.9 ±10.3 kg, body surface area 1.98 ± 0.156 m^2 and body mass index (BMI) 24.0 ±3.1 kg/m^2. Mean fasting glucose and glycosylated hemoglobin A1c baseline values were 5.1 ±0.4 mmol/L and 5.2 ±0.3%, respectively. The protocol was approved by the Central Committee on Research involving Human Subjects of the Netherlands (CCMO). Written consent was obtained from all participants included in the study. The study has already been published [23].

STUDY DESIGN

Subjects were required to continue their normal diet and activities. After an overnight fast, the hyperglucagonemic clamp test was performed, consisting of two phases: a run-in period of 3 hrs to achieve stable glucose isotope enrichment and a 3 hr pancreatic clamp study (Figure 1). After a priming dose of 5 $mg.kg^{-1}$, [6,6-$^2H_2$] glucose was infused continuously throughout the study at a rate of 0.05 $mg.kg^{-1}.min^{-1}$ (Cambridge Isotope Laboratories, MA, USA). The pancreatic clamp study consisted of a simultaneous 3 hrs infusion of somatostatin (0.1 $\mu g.kg^{-1}.min^{-1}$; UCB, The Netherlands), glucagon (3 $ng.kg^{-1}.min^{-1}$; Novo Nordisk, The Netherlands) and insulin (4 $mU.m^{-2}.min^{-1}$; Novo Nordisk, The Netherlands). A second catheter was inserted into a contra-lateral dorsal hand vein for blood sampling. The hand was placed in a heated-box (50°C) to arterialize venous blood for labeled glucose sampling. Venous blood samples were drawn to determine (non-) labeled glucose, glucagon, and insulin concentrations. Samples for isotope analyses, glucose, glucagon and insulin were collected every 15 minutes from 30 minutes before the start of the challenge until the end of the glucagon challenge (t=360 min). After the challenge capillary bedside (finger prick) glucose was measured for informative purposes in 16 subjects at 420, 450 and 480 min.

LABORATORY ANALYSIS

Blood samples for insulin were collected in plain tubes and analyzed using standard validated immune-radio-metric assays (Biosource Europe S.A., Belgium; assay CV 5.9-7.9% for insulin). Glucagon samples were collected in plain tubes with aprotinin (Trasylol™ (500 KIE/50 µl) Bayer, The Netherlands) and analyzed using a radioimmunoassay (Linco research, Missouri, USA; assay CV 4.3%). Blood samples for glucose and [6,6-$^2H_2$] glucose were analyzed using a validated gas chromatography with mass spectrometry as detection method as described previously [24]. All samples were analyzed in batches to reduce assay variability.

MODELING GLUCAGON CHALLENGE

INHIBITION

Somatostatin infusion during the glucagon challenge, which inhibits the endogenous glucagon and insulin secretion, was modeled by an inhibitory function $I_i(t)$ equal to 1, except during the inhibition, when they take on a lower – but still non-negative – value.
The inhibitory functions $I_1(t)$ and $I_3(t)$ in, respectively, equations {4} and {6}, are given by:

$$I_i(t) = 1 - I_{i,max} \times H(t; t_{begin}, t_{end}, \alpha_i), (i = 1,3) \quad \{1\}$$

Where I_{max} denotes the maximal inhibition (i.e., $0 < I_{max} \leq 1$). The function $H(t; t_{start}, t_{end}, \alpha_i)$ denotes a *smooth step function* defined by: {2}

$$H(t; t_{begin}, t_{end}, \alpha_i) = \begin{cases} 0 & \text{for } 0 \leq t \leq t_{begin} \\ 1 - e^{-\alpha_i (t - t_{begin})} & \text{for } t_{begin} < t < t_{end} \\ (1 - e^{-\alpha_i (t_{end} - t_{begin})}) \cdot e^{-\alpha_i (t - t_{end})} & \text{for } t_{end} \leq t < \infty \end{cases}$$

Here $\alpha_i > 0$ is the rate at which the inhibition reaches its maximum value $\approx I_{max}$. The times t_{begin} and t_{end} denote when the inhibitions begin and end.

INFUSIONS

The functions $Q_1(t)$, $Q_2(t)$ and $Q_3(t)$ which model the infusions in, respectively, equations {4}, {5}, and {6}, are given by:

$$Q_i(t) = Q_{i,max} \times \text{Step}(t, T_{begin}, T_{end}), (i = 1, 2, 3) \quad \{3\}$$

Where Step $(t; T_{begin}, T_{end})$, is a simple step-function, which is equal to 1 for $T_{begin} < t < T_{end}$ and 0 elsewhere.

STRUCTURAL MODEL DEVELOPMENT

Using population approach nonlinear mixed effects modeling, glucose-, insulin- and glucagon data from a glucagon challenge in healthy volunteers were simultaneously analyzed. First, the somatostatin effect was modeled as an on-off effect. However, as this led to numerical difficulties in the algorithms, this

was expanded to an inhibitory function (Equation 1). The first-order conditional estimation method with interaction (FOCE I) was used. The log-likelihood ratio test was used to discriminate between hierarchical models, based on the objective function value (OFV), where $p<0.05$ [decrease OFV of at least 3.84 points for one degree of freedom, Chi-square (χ^2) distribution] was considered to be statistically significant. Various goodness-of-fit plots such as: observations vs. population predictions, observations vs. individual predictions, weighted residuals vs. time, (conditional)weighted residuals vs. observed concentrations, histogram and/or QQ plots of the post hoc individual estimates of ETAS (when ETAS were available), population and/or individual predictions vs. observed individual or population concentrations; were considered for diagnostic purposes. Covariate screening was performed by graphical analysis and analysis of the correlation of the post hoc individual estimates of ETA for each parameter versus the covariate values. Three different error types were tested (additional, proportional and both). A model was considered acceptable if the conditional weighted residuals with $\eta-\varepsilon$ interaction (CWRESI) was between -2 and 2. Whenever a new parameter was introduced or the robustness of the model was to be evaluated, the mean population predictions plotted against the observations was performed. The compartmental semi-mechanistic final model structure that best described the observations, including the feedback mechanisms, is illustrated in Figure 2.

GLUCAGON (A_1 IN NANOGRAM)

Glucagon dynamics was described by a turnover equation (Equation 4). The production term (k_{in1}) is taken inversely proportional to the amount of glucose (A_2), as glucagon secretion by α-cells in the pancreas is inhibited by plasma glucose [25;26].

$$\frac{dA_1}{dt} = \frac{k_{in1}}{A_2} \cdot I_1(t) - k_{out1} A_1 + Q_1(t) \quad \{4\}$$

Glucagon elimination was modeled by a first order reaction term with a rate constant k_{out1}, and glucagon infusion was modeled by a function $Q_1(t)$.

PLASMA GLUCOSE (A_2 IN GRAMS)

Glucose dynamics was described by a turnover equation with hepatic glucose production (HGP) and insulin-dependent elimination:

$$\frac{dA_2}{dt} = k_{in2} \frac{A_1}{A_4} - k_{out2} A_2 - k_{out2D} A_2 \cdot A_3 + Q_2(t) \quad \{5\}$$

Glucagon is a potent stimulus of hepatic glucose production, with the magnitude of the effect being dependent on the prevailing insulin concentration;

increasing insulin levels dampen the glucose production [27]. It is known that continuous glucagon infusion results in glucagon receptor internalization (A_4). Intestinal incretin hormones GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) were not included in our model, because all subjects were in a fasting state. Incretin hormones are only released during absorption of orally taken meals and then stimulate pancreatic ß-cells to secrete insulin [28]. Furthermore, the stimulatory effect of autonomic nervous system on hepatic glucose production has not been incorporated in our model, because our previous study showed that changes in the autonomic nervous system tone do not contribute significantly to the effects of the glucagon challenge [23].

Elimination of plasma glucose, which consists of glucose uptake by the liver and muscles is modeled by an insulin independent term ($k_{out2}A_2$) representing e.g. brain glucose consumption and an insulin dependent term ($k_{out2D}A_2 \cdot A_3$) The direct inhibition (negative feedback) of plasma glucose level (A_2) on the liver (HGP) via GLUT-1 transporter is incorporated in the equation as well. Labeled glucose infusion was modeled by a function $Q_2(t)$. Labeled glucose infusion was assumed to have the same disposition properties as total plasma glucose [23;24], therefore no difference in glucose has been made. Labeled glucose and enrichment measurements were not considered in the model.

INSULIN (A_3 IN MILLIUNITS)

Insulin dynamics was described by a turnover equation. Since insulin secretion by ß-cells in the pancreas is stimulated by plasma glucose, the production term is taken proportional to the amount of glucose A_2.
This results in the equation:

$$\frac{dA_3}{dt} = k_{in3} A_2 \cdot I_3(t) - k_{out3} A_3 + Q_3(t) \quad \{6\}$$

Insulin elimination was modeled by a first order elimination process with rate constant k_{out3}, and insulin infusion was modeled by a function $Q_3(t)$, according to equation 3.

INTERNALIZATION OF GLUCAGON RECEPTORS-EFFECT COMPARTMENT (A_4-DIMENSIONLESS)

We observed a rapid increase of the glucose concentration in response to glucagon followed by a slow decrease in the HGP, which is consistent with the internalization of glucagon receptors (GCGR) upon stimulation by glucagon [23;29;30]. Krilov *et al*. [29] have previously shown that, upon 30 minutes of glucagon stimulation, GCGR are internalized in vivo. This internalization of the GCGR was captured by incorporating an effect compartment (A_4). With this we also incorporated a delayed negative effect of hepatic glucose production into

the model (Equation 5). The rate constant of removal from the effect compartment characterized the effect delay.

$$\frac{dA_4}{dt} = k_{in4} A_2 - k_{out4} A_4 \quad \{7\}$$

In the equations 4-7 constitute a coupled system of feedback models: the Glucagon-Glucose system interacting with the Glucose-Insulin system through the effect of hepatic glucose production.

DATA ANALYSIS AND MODEL EVALUATION

The software used for the analysis included: PROMASYS V7.1 (PROMASYS BV, Leiden, The Netherlands) for the database storing and exporting, NONMEM V7.2.0 (Icon Development Solutions, Ellicott City, MD, USA) for the numerical estimations for the established equations [ADVAN 13; tolerance 8], GNU Fortran (GCC) 4.6.0 as compiler and R V2.13.1 (R Foundation for Statistical Computing, Vienna, Austria) for the predictions (simulations), database editing, system solution and graphics elaboration, and MATLAB R2013a (MathWorks, Natick, MA, USA) for simulations.

RESULTS

A semi-mechanistic model simultaneously describing glucagon, plasma glucose, insulin and glucagon receptor internalization, during a hyperglucagonemic challenge was developed. The study cohort investigated in this study covers a broad range of individual data for the model. For each of the 36 subjects, in total 16x glucagon-, 16x glucose- and 16x insulin concentrations before and during the glucagon challenge were measured, and were used for the model. The initial values at steady state for the three compartments were obtained from the first observations (before the challenge was started), the volumes of distribution were obtained from the literature and used as initial estimates [13] and limits were given to values that would be physiological. Also, inter- and intra-individual variability (IIV) was considered but could not be identified given the data.

MODEL EVALUATION / SIMULATIONS

Fitting the model to the data resulted in the following baseline values for glucagon, glucose, insulin and effect compartment:

$$BL_1 = 1170 \text{ ng}, BL_2 = 4.20 \text{ g}, BL_3 = 17.7 \text{ mU}, BL_4 = 4.63 \times 10^6 \quad \{8\}$$

and volumes of distribution of the Glucagon-compartment, the Glucose compartment and the Insulin compartment:

$$V_1 = 21.1 \text{ L}, V_2 = 4.44 \text{ L}, V_3 = 1.53 \text{ L respectively.} \quad \{9\}$$

This results in the following baseline concentrations for the glucagon, plasma glucose and insulin:

$$C_1 = 55.45 \text{ ng/L}, C_2 = 0.946 \text{ g/L}, C_3 = 11.6 \text{ mU/L respectively.} \quad \{10\}$$

The baseline values were used as initial values: $A_i(0) = BL_i$.

Fitting the model to the data yields parameter estimates listed in Table 1, where the values for k_{in1}, k_{in2}, k_{in3} and k_{in4} are computed from the estimated values for k_{out1}, k_{out2}, k_{out2d}, k_{out3} and k_{out4}, and the estimates of the baseline amounts BL_i (i = 1, ...,3) obtained from (Equation 8).

Parameters involved in the glucagon challenge and the infusions are listed in Table 2.

In computing the infusion rates Q_1 and Q_3 in Table 2, we have assumed that the average weight is 80 kg and the average body surface area 1.984 m^2.

Four representative individuals were selected from the whole dataset and are presented in Figure 4. The medians of the simulated individual concentration-time profiles are plotted over the observed profiles of glucose, glucagon and insulin during baseline and during challenge. Individual diagnostic plots for the evaluation of the quality of the model fit, illustrate that the model described the data. During challenge, glucagon data show a decreasing trend, which is captured by the model. As inter- and intra-individual variability could not be identified, the height of the glucagon concentrations could not be captured on an individual level. For plasma glucose concentration during challenge, the increase in glucose levels over time is well captured by the model. There seems to be a slight decreasing trend in glucose concentrations at the end of the challenge, which is better fitted by including the effect compartment (A_4). Even though the fitting is not completely optimal towards the end of the challenge, because it does not describe in some individuals the diminished response to glucagon (glucagon desensitization), inter-individual variability could better explain this phenomenon.

Regarding the recovery phase of plasma glucose levels after challenge, only assumptions were made based on the minor finger prick glucose data points. No laboratory data were collected in the recovery phase.

During challenge a standard insulin dose was infused, and endogenous insulin production was inhibited by somatostatin, therefore insulin concentrations are almost stable during challenge.

However, the predicted insulin concentrations exhibit a peak at the start of the challenge and a drop immediately at the stop of the infusion. These are

artifacts caused by the step function, involving instantaneous onset and termination, while the inhibition of insulin release by somatostatin takes time as does the release of endogenous insulin upon cessation of somatostatin. We decided to not further model this artifact. Firstly, the exact nature of the simultaneously occurring increase in insulin (by the infusion) and the decline in endogenous insulin (by somatostatin) is not very well known. Secondly, the additional parameters required to describe a gradual increase would cause over-parameterization of the model.

In addition to the four representative individuals, Figure 5 shows the diagnostic plots of the entire dataset. It clearly shows that the general trend of all glucagon and glucose data is well captured by the model. The insulin data is also well captured with exception for the peaks at the start and end of the challenge, for reasons mentioned before.

Concentration population predictions vs. conditional weighted residuals graphs for glucagon, glucose and insulin are shown in Figure 6. The glucagon and glucose observations are randomly spread around the identity line and most observations lie within the acceptance criterion. The insulin conditional weighted residuals over time plots show a clear structural bias; the high predicted concentration range is overpredicted (peaks at the start and end of challenge), which is corrected for towards the lower predicted concentration range that, as a result, ends in underpredicted values. The steady state conditions and calculations are supplied in Appendix A.

DISCUSSION

This glucagon receptor (GCGR) modeling approach was a practical tool to describe the glucagon-insulin-glucose homeostasis in healthy subjects during a glucagon challenge [23]. Although this model represents a simplification of complex physiology, the main counter-regulatory elements in the glucose regulation system (glucagon and insulin) were used to describe glucose's profile in a semi-mechanistic way. Application to clinical data showed that the model is able to describe the levels of insulin, glucagon and glucose before and during the glucagon challenge.

Somatostatin was infused during the glucagon challenge to suppress the endogenous hormone production. The model assumed a maximal inhibition (I_{max}) of 1, representing 100% inhibition. Although there is consensus in the literature on the inhibiting effect of somatostatin on endogenous hormone production, the extent of maximal inhibition has not been irrefutably demonstrated. The measured individual glucagon profiles (Figure 4) show a decreasing trend in the glucagon concentrations during the constant glucagon infusion period. The glucagon pattern could be explained by a small reduction of endogenous production on top of the glucagon infusion or the increasing plasma glucose concentration that have an inhibiting effect on the remaining endogenous glucagon excretion.

For the subjects in this study, glucagon levels at baseline were higher when plasma glucose levels were lower. This could be explained by the glucose-dependent regulation of glucagon secretion in the α-cell, as reported in animal studies [26;31]. At low glucose concentrations, the moderate activity of K_{ATP} channels situates the α-cell membrane potential in a range that allows the opening of Na^+ and Ca^{2+}-channels triggering the exocytosis of glucagon granules and vice versa.

The amount of plasma glucose concentrations in this model (Equation 5) is highly dependent on glucagon (A_1) because during the glucagon challenge a supraphysiologic concentration of glucagon was reached. The effect of insulin infusion results in glucose uptake by fat and muscles and a reduction of the supply of gluconeogenic precursors reaching the liver. The data of the study of Sindelar *et al*. [32] strongly suggest that the liver responds directly, rapidly, and sensitively to the plasma insulin levels by a reduction in HGP. The slight decreasing trend in glucose concentrations at the end of the challenge has not been completely captured by the effect compartment (internalization of glucagon receptors). No data were collected for the recovery phase after stopping the glucagon challenge. Therefore, no firm conclusions can be drawn regarding the recovery phase, only assumptions based on the minor finger prick glucose data points.

Insulin and glucagon are fundamental components of the regulator mechanisms that control glucose homeostasis. These counter-regulatory hormones help to keep the blood glucose level within optimum limits. In this work, the insulin, glucagon and glucose interactions following a glucagon challenge have been described in a single model. The adequate descriptive performance of this model has been confirmed by the diagnostic plots per subject and for the total population.

This model, based on glucagon challenge data, could contribute to a better understanding of pathophysiology of diabetes mellitus. It describes the general trend and can therefore serve as a basis for drug development. Furthermore, an extension of this model could probably be incorporated in the recently developed automated, bihormonal, bionic pancreas for type 1 diabetes mellitus patients [33].

APPENDIX A

STEADY STATE AMOUNTS

In the absence of inhibitions and infusions, i.e., when $I_i(t) = 0$ for $i = 1, 3$ and $Q_i(t) = 0$ for $i = 1, 2, 3$ we denote the steady state amounts of the four compounds by $A_{n,ss}$ (n= 1,...,4).

We denote the quotients of k_{in} and k_{out} for each of the four equations by:

$$R_1 = \frac{k_{in1}}{k_{out1}}\ ,\ R_2 = \frac{k_{in2}}{k_{out2}}\ ,\ R_3 = \frac{k_{in3}}{k_{out3}}\ ,\ R_4 = \frac{k_{in4}}{k_{out4}} \qquad \{A.1\}$$

With this notation, $A_{1,ss}$, $A_{3,ss}$ and $A_{4,ss}$ can readily be expressed in terms of $A_{2,ss}$:

$$A_{1,ss} = \frac{R_1}{A_{2,ss}}\ ,\ A_{3,ss} = R_3 A_{2,ss}\ ,\ A_{4,ss} = R_4 A_{2,ss} \qquad \{A.2\}$$

When we substitute these expressions into the right hand side of Equation 5 for dA_2/dt, we obtain an equation for $x = A_{2,ss}$ alone:

$$F(x) \overset{\text{def}}{=} \frac{R_1 \cdot R_2}{R_4} - x^3 - \gamma R_3 \cdot x^4 = 0\ ,\ \gamma = \frac{k_{out2D}}{k_{out2}} \qquad \{A.3\}$$

The function $F(x)$ is seen to have a unique zero: $x = A_{2,ss} = 4.20$. For the parameter values of Table 1, we obtain:

$$R_1 = 4914,\ R_2 = 2.16 \times 10^5,\ R_3 = 4.21,\ R_4 = 1.10 \times 10^6,\ \gamma = 0.650 \qquad \{A.4\}$$

Using these values in the expressions of Equation 5, we obtain for the steady state amounts

$$A_{1,ss} = 1170 \text{ ng},\ A_{2,ss} = 4.20 \text{ g},\ A_{3,ss} = 17.7 \text{ mU},\ A_{4,ss} = 4.63 \times 10^6 \qquad \{A.5\}$$

In the light of the estimated volumes, given in Equation 9, the corresponding steady state concentrations are:

$$C_{1,ss} = 55.45 \text{ ng/L},\ C_{2,ss} = 0.946 \text{ g/L},\ C_{3,ss} = 11.6 \text{ mU/L} \qquad \{A.6\}$$

TABLE 1 Parameter estimates for glucagon, plasma glucose, insulin, glucagon receptor internalization and volume of distribution. Parameters with an asterix denote calculated parameters (not directly estimated by NONMEM).

Parameter	Estimate	Unit
k_{in1}	559*	$ng \cdot g \cdot min^{-1}$
k_{out1}	0.114	min^{-1}
k_{in2}	275*	$g \cdot (ng \cdot min)^{-1}$
k_{out2}	0.00127	$(mU \cdot min)^{-1}$
k_{out2D}	8.26×10^{-4}	min^{-1}
k_{in3}	2.09*	$mU \cdot (g \cdot min)^{-1}$
k_{out3}	0.496	min^{-1}
k_{in4}	655*	$(g \cdot min)^{-1}$
k_{out4}	5.94×10^{-4}	min^{-1}

TABLE 2 Parameter values involved in glucagon challenge and infusions.

Parameter	Estimate	Unit
α_1	7.00×10^{-4}	min^{-1}
α_3	1.18×10^{-1}	min^{-1}
Q_1*	240	$ng \cdot min^{-1}$
Q_2(5 min bolus)*	0.001	$g \cdot kg^{-1}$
Q_2*	0.00001	$g \cdot kg^{-1} \cdot min^{-1}$
Q_3*	7.936	$mU \cdot min^{-1}$

** fixed parameters*

FIGURE 1 Glucagon challenge; infusion of somatostatin, glucagon, insulin and labeled glucose.

Basal period		Hyperglucagonaemic period
0 min - - - 5 min	5 min - - - 180 min	180min - - - 360 min
[6.6'-2H2] glucose bolus 5 $mg.kg^{-1}$	[6.6'-2H2] glucose continuous 0.05 $mg.kg^{-1}.min^{-1}$	
		Somatostatin 0.1 $\mu g.kg^{-1}.min^{-1}$
		Glucagon 3 $ng.kg^{-1}.min^{-1}$
		Insulin 4 $mU.m^{-2}.min^{-1}$

FIGURE 2 Schematic representation of the model. Full arrows indicate flows, broken arrows indicate control mechanisms, dotted arrows indicate infusion of somatostatin, glucagon, insulin and labeled glucose.

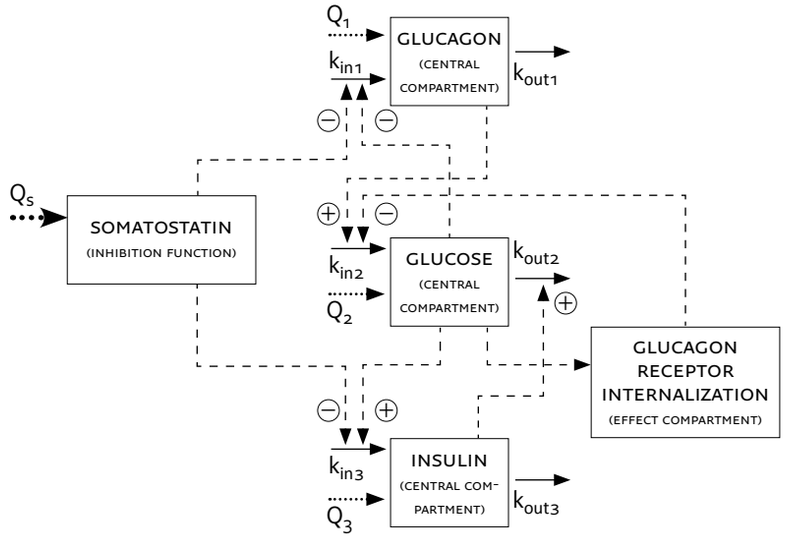


FIGURE 3 The inhibition functions $I_1(t)$, with $\alpha = 0.0007$, and $I_3(t)$, with $\alpha = 0.118$, for $t_{begin} = 5$ and $t_{end} = 180$ min.

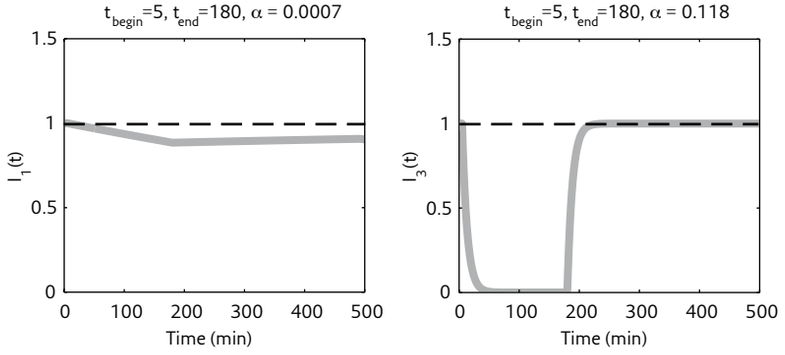


FIGURE 4 Representative profiles for glucagon, glucose and insulin of four typical healthy subjects. Observations from the original data set are plotted as points. The lines show the average predicted values of the model. The vertical dashed lines represent the time of start and stop of the glucagon challenge test.

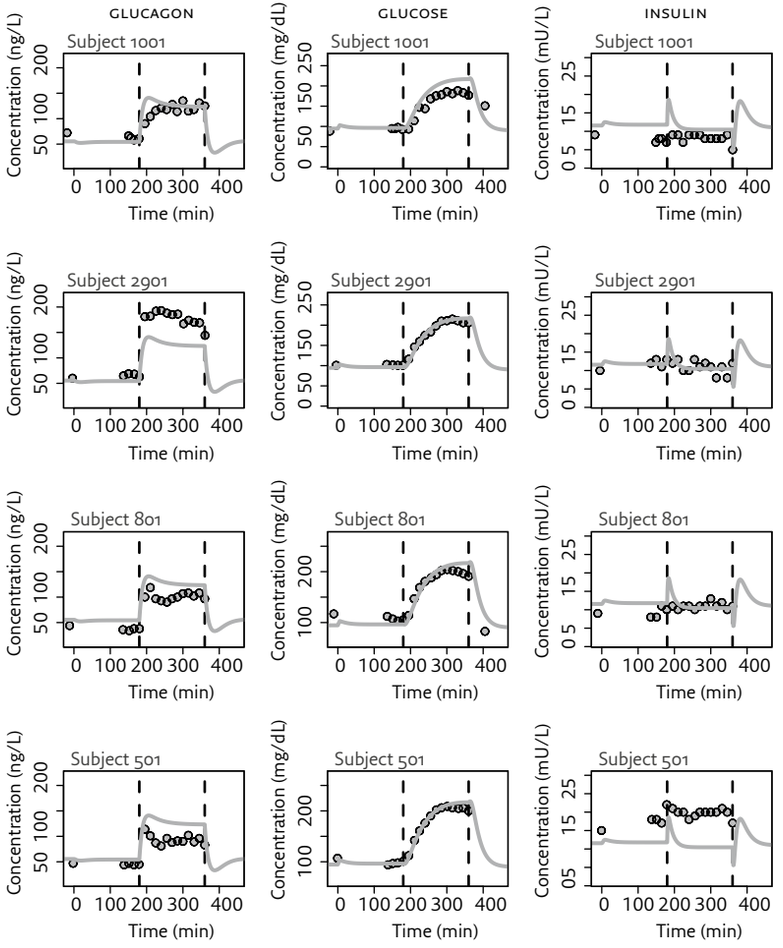


FIGURE 5 Diagnostic plots representing the mean population prediction (black) over time for glucagon, plasma glucose and insulin, with the observations (circles).The vertical dashed lines represent the time of start and stop of the glucagon challenge test.

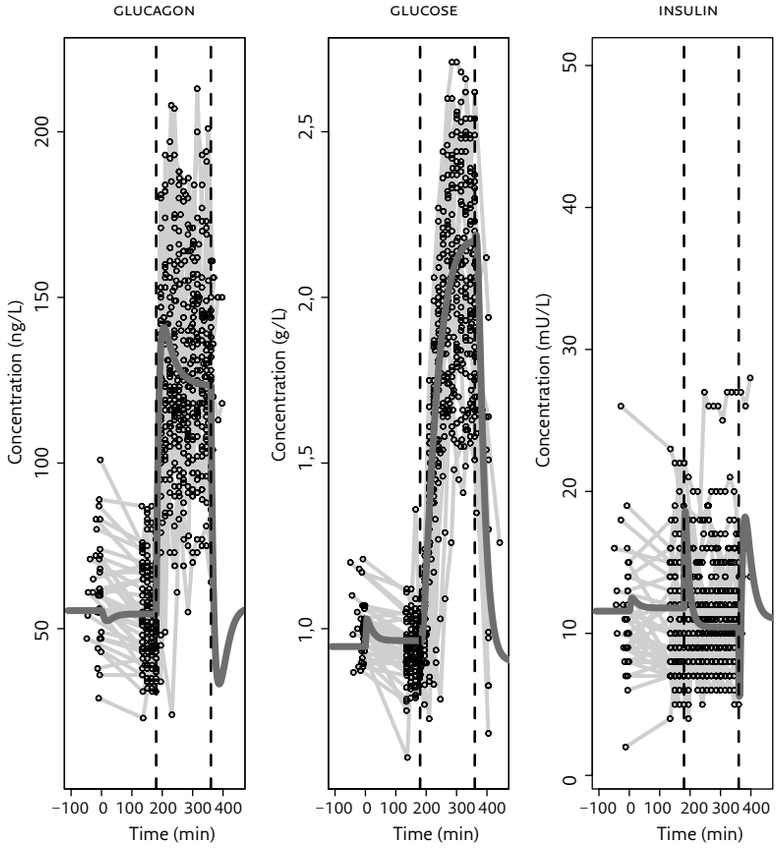
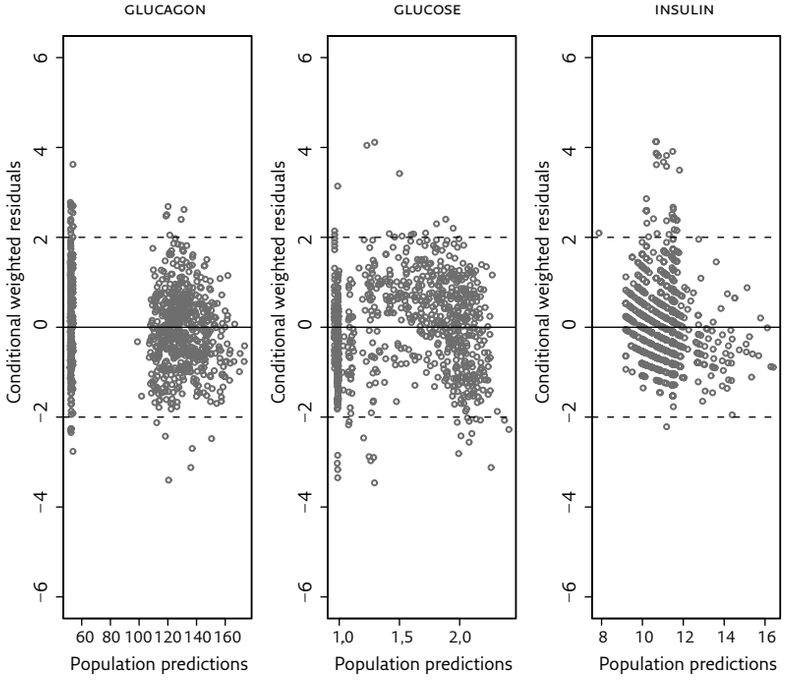


FIGURE 6 Glucose, glucagon and insulin conditional weighted residuals graphed versus predicted population concentrations. Data points are plotted as circles. The horizontal line is the identity line at zero and the dotted line represents the acceptance criterion.



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CHAPTER 7

Summary and general discussion

In this thesis, a variety of human studies are described that were designed to investigate the role of glucagon in glucose homeostasis in healthy volunteers and type 2 diabetes (T2DM) patients.

In non-diabetic subjects glucagon is released into the bloodstream when circulating glucose level is low. The main physiological role of glucagon is to stimulate hepatic glucose output, thereby leading to increases in glycemia. This provides the major counterregulatory mechanism for insulin in maintaining glucose homeostasis in vivo. Compared with healthy subjects, diabetic patients have abnormal secretion of not only insulin but also glucagon [1-3]. Hyperglucagonemia and altered insulin-to-glucagon ratios play important roles in initiating and maintaining pathological hyperglycemic states. These observations have initiated a renewed interest in the role of glucagon as modulating glucagon (receptor) action may be a potential target for the therapeutic treatment of diabetes.

Glucagon is one of the therapeutic targets of the currently available incretin-based therapies. A decade ago, before the introduction of incretins, only insulin-based therapies were available: biguanides, sulfonylurea and thiazolidinediones. Incretins, GLP-1 analogs and DDP-IV inhibitors, stimulate insulin release from the pancreatic ß-cells as well as suppress glucagon release from the pancreatic α-cells. Drugs that target the glucagon receptor (GCGR) are in development, but small molecules were not successful so far due to limited drug selectivity, cross-species differences and lack of sustained effects after non-competitive blockade. Isis pharmaceuticals Inc. (Carlsbad, USA) developed a second-generation antisense phosphorothioate oligonucleotide (ASO) glucagon receptor antagonist (ISIS 325568). The compound was developed as a potential treatment for T2DM patients. Glucagon receptor knock-out mice have hyperglucagonemia and α-cell hyperplasia, but their glucose tolerance is improved and they develop only a mild fasting hypoglycemia [4]. These results are consistent with the pre-clinical experiments with antisense oligonucleotides for the glucagon receptor. Diabetic db/db mice treated with these oligonucleotides had lower glucose, triglyceride and free fatty acids blood levels, as well as improved glucose tolerance, and they developed hyperglucagonemia without apparent effects on α-cell size or number [5]. This approach is also accompanied by an increase in GLP-1 and insulin levels in Zucker diabetic fatty rats and db/db and ob/ob mice [6].

AIM OF THIS THESIS

This thesis aimed to gain further insight in the role of glucagon and the glucagon receptor antagonist ISIS 325568 on serum glucose levels in healthy volunteers and T2DM patients, in order to explore whether a glucagon receptor antagonist may be a useful therapeutic intervention in the treatment of T2DM.

The specific control of glucagon secretion by pharmacological modulation is complex since several components of the α-cell stimulus-secretion coupling are also present in ß-cells. Therefore, we also further explored the glucagon challenge test, described by Petersen *et al*.[7], as a tool for investigating the effect of a novel GCGR-antagonist.

CHAPTER 1 comprises a general introduction. This chapter starts with a general description of glucose homeostasis, pathophysiology of T2DM and current treatment options. Pancreatic α-cells and glucagon secretion are fundamental components of the regulatory mechanisms that control glucose homeostasis.

CHAPTER 2 studies the effects of a single protein hydrolysate meal replacement (insuVida™) on postprandial serum glucose, insulin and glucagon levels in T2DM patients. We concluded that a single dose of a casein hydrolysate with and without the addition of leucine enhanced the carbohydrate-induced insulin response in T2DM patients, resulting in significantly lower plasma glucose concentrations compared to placebo and intact casein. However, besides insulin, the secretion of pancreatic glucagon was increased as well during the use of all casein-rich meal replacers. This mechanism may have limited the decline of plasma glucose concentrations and may be responsible for the absence of hypoglycemia. Further research is required to determine the long-term clinical benefit of a low dose of insuVida™, with and without additional leucine. This chapter also highlights that investigating only one hormone involved in glucose homeostasis (e.g. insulin) as was done in all previous studies with the meal replacement product is a too limited approach. Characterization of interventions that are developed to improve disturbed glucose homeostasis should be more comprehensive.

The aim of the study in CHAPTER 3 was to characterize a glucagon challenge test as a tool in diabetes research, by assessing the inter- and intra-individual variabilities of the glucagon challenge test and by investigating the activity of the autonomic nervous system (ANS) during this challenge, as this might have an indirect impact on glucose homeostasis. Hepatic glucose production was assessed using stable isotope glucose. Furthermore, we determined whether human adipose tissue expresses glucagon receptor MRNA. The latter may be a direct biomarker of pharmacological activity of drugs that affect GCGR action. The results of this study in 24 healthy volunteers demonstrated that serum glucose concentrations rose rapidly after glucagon infusion, and reached a plateau at 90 min. The time profiles suggested rapid development of tolerance for glucagon-induced hyperglycemia. During the glucagon challenge intra- and inter individual variabilities for hepatic glucose production, the rate of disappearance of glucose and plasma glucose were approximately 10-15% for all variables. Hyperglucagonemia did not affect heart rate variability. In contrast to GCGR MRNA expression in diabetic mice, we found that the expression level of glucagon receptor MRNA in human adipose tissue was extremely low, and does not qualify as an appropriate biomarker for GCGR knock-down in humans.

From this study it was concluded that the standardized 6-hr glucagon challenge test has a good reproducibility with only limited variability over 6 weeks. It is a robust tool to explore in detail the contribution of glucagon in normal and altered glucose homeostasis and can also be used to evaluate the effects of drugs antagonizing glucagon action in humans without confounding changes in ANS tone.

Based on this study the glucagon challenge was expected to be a robust tool for glucagon-focused research in diabetes. In CHAPTER 4 the effects of a similar glucagon challenge as described in chapter 3 were explored in T2DM patients and compared with the glucagon response in healthy volunteers. In the patients, the influence of oral antidiabetic drugs on the response to hyperglucagonemia was investigated by using a cross-over study design, with and without oral antidiabetic drugs. Our results demonstrate that the response to a hyperglucagonemic challenge markedly differs between T2DM patients and healthy volunteers. Healthy volunteers show a greater increase in HGP upon glucagon infusion and also a faster decline in hepatic glucose production than T2DM patients. Exogenous insulin suppresses the glucagon-induced hepatic glucose production. Furthermore, glucose levels were lower during glucagon challenge when T2DM patients stopped their oral medication for two weeks and receiving higher insulin doses than patients continuing their oral antidiabetics. These findings suggested that attenuation of hepatic glucagon action, by introducing insulin in an earlier stage of the disease and/or developing medication targeting the glucagon receptor, could be a promising therapeutic strategy for T2DM.

In CHAPTER 5 we investigated whether the antisense GCGR-antagonist ISIS 325568 has a glucose lowering effect in humans in vivo. This study was performed using a double-blind, placebo-controlled, dose-escalation design, and evaluated the, tolerability, pharmacokinetics and pharmacodynamics of single and multiple dose administrations of ISIS 325568 at 4 dose levels in healthy subjects. ISIS 325568 is a second-generation antisense phosphorothioate oligonucleotide and was designed to function through an RNase H-dependent terminating mechanism. In the multiple dose cohorts of this study, at each dose level, 8 subjects received 8 doses over 6-weeks (3 IV doses in Week 1 followed by 5 weekly sc doses) and underwent a glucagon challenge procedure (glucagon infusion that doubled both plasma glucagon and glucose levels) at baseline and at the end of 6-week treatment. This study showed that ISIS 325568 did not cause clinically significant changes in vital signs, ECG, hepatic or renal function and no hypoglycemia was observed. The drug was generally well-tolerated, with dose-dependent local injection site erythema as the most common adverse event. All findings were consistent with the adverse event profile for this class of antisense oligonucleotides [8]. Glucagon infusion prior to ASO treatment caused a rapid 2-fold elevation of plasma glucose levels and HGP, which was significantly blunted by the antisense compound at the 400 mg/week dose. No changes in plasma glucose AUC or HGP were observed in the placebo group. Plasma trough drug concentrations after Week 4 reached an apparent steady-state and displayed PK-PD relationship consistent with preclinical data. Importantly, this study provides the first proof of pharmacology with GCGR ASO in humans, as reflected by a reduction in glucagon-induced glucose excursion. These results support further evaluation of ASO-based therapies against the glucagon receptor in patients with T2DM.

In CHAPTER 6 a semi-mechanistic model simultaneously describing glucagon, plasma glucose, insulin and glucagon receptor internalization was build using data from our glucagon challenge study in healthy volunteers (Chapter 3). Although this model represents a simplification of complex physiology, the main counter-regulatory elements in the glucose regulation system (glucagon and insulin) were used to describe glucose's profile in a semi-mechanistic way. Application to clinical data showed that the model is able to describe the levels of insulin, glucagon and glucose before and during the glucagon challenge. This model further elucidated the potential role of glucagon in the pathophysiology of diabetes mellitus. It describes a general trend and can therefore serve as a basis for drug development.

In conclusion, the studies described in this thesis show that:

- The effect of meal replacers containing protein hydrolysate on plasma glucose lowering is limited in T2DM patients due to a collective increase of both insulin and glucagon levels;
- The glucagon challenge test has limited variability in healthy volunteers and no confounding changes in ANS tone;
- T2DM patients respond profoundly different to a glucagon challenge test compared to healthy volunteers;
- The response to a glucagon challenge test in T2DM subjects is influenced by the type of therapy (oral antidiabetics or IV insulin);
- Proof of pharmacology in humans of the antisense glucagon receptor antagonist can be demonstrated by using a well-characterized glucagon challenge test;
- It is possible to design a semi-mechanistic model which simultaneously describes glucagon, plasma glucose, insulin and glucagon receptor internalization using data from glucagon challenges in healthy volunteers. This model could contribute to a better understanding of pathophysiology of diabetes mellitus.

Based on these studies, absolute or relative glucagon excess seemed to be critical in the development and/or maintenance of hyperglycemia in diabetes by increasing hepatic glucose output. Strategies targeted glucagon could be suitable for the improvement of glucose levels.

FUTURE PERSPECTIVES

The studies in this thesis provide more insight in the role of glucagon in glucose homeostasis in healthy subjects and T2DM patients. It seems that the ideal treatment for T2DM might be multi-faceted and should include more than control of blood glucose levels by insulin-related therapy only. Nowadays, mechanisms of action, benefits, and risks of traditional and newer agents for the treatment of T2DM are discussed to better enable the pharmacist and doctor to recommend the best combinations of agents for individual patients. Targeting the glucagon receptor could be a potential therapeutic option for the treatment of T2DM. Additional studies into the cause of the inflammatory (skin) reactions induced by antisense GCGR-antagonist (ISIS 325568) deserve further attention. It would be interesting and worthwhile to further refine the glucose homeostasis model that we developed for instance by including the data of T2DM patients and the effects of GCGR knock-down into the model. Furthermore, if an oral glucose tolerance test (OGTT) could be incorporated in the model, the incretin effect could be implemented to analyze the relationship between glucose regulation and GIP and GLP-1 and glucagon. Then this model could be used for the GLP-analogs and DDP-IV inhibitor drugs as well.

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CHAPTER 8

Nederlandse samenvatting

In dit proefschrift worden diverse klinische studies beschreven die zijn ontworpen om de rol van glucagon in glucose homeostase te onderzoeken bij gezonde vrijwilligers en type 2 diabetes patiënten (T2DM).

Bij mensen zonder diabetes mellitus vindt glucagon secretie plaats bij een lage glucose concentratie in het bloed. De belangrijkste fysiologische rol van glucagon is het stimuleren van de hepatische glucoseproductie, wat leidt tot stijging van plasma glucose. De werking is tegengesteld aan de werking van insuline en verschaft samen insuline een belangrijke rol in het handhaven van de glucose homeostase in vivo. Vergeleken met gezonde proefpersonen, hebben patiënten met diabetes een abnormale secretie van zowel insuline als glucagon [1-3]. Hyperglucagonemie en veranderde insuline-glucagon ratio's spelen een belangrijke rol in het initiëren en onderhouden van pathologische hyperglycemische situaties bij diabeten. Deze waarnemingen hebben geleid tot een hernieuwde belangstelling voor de rol van glucagon en in het bijzonder glucagon (receptor) als potentieel doelwit voor de behandeling van diabetes. Glucagon is een van de therapeutische doelen van de incretine-gebaseerde therapieën. Een decennium geleden, vóór de invoering van behandeling met incretinen, waren er alleen insuline gebaseerde therapieën beschikbaar: biguaniden, sulfonylureumderivaten en thiazolidinedionen. Incretinen, GLP-1 analogen en DDP-IV remmers, stimuleren de insulineproductie en -secretie door de ß-cellen en remmen de glucagonproductie door de α-cellen in de pancreas. Geneesmiddelen die zich richten op de glucagon receptor (GCGR) zijn in ontwikkeling, maar small molecules zijn tot op heden niet succesvol gebleken vanwege de beperkte selectiviteit, verschillen tussen proefdiersoorten en de mens en het ontbreken van langdurige effecten na niet-competitieve blokkade. Isis Pharmaceuticals Inc (Carlsbad, USA) ontwikkelde een tweede generatie antisense oligonucleotide (ASO) glucagon receptor antagonist (ISIS 325568). Deze ASO werd ontwikkeld als een mogelijke behandeling voor T2DM. In GCGR knock-out muizen (muizen zonder glucagon receptor) was er sprake van hyperglucagonemie en α-cel hyperplasie, maar een verbeterde glucosetolerantie en de muizen ontwikkelden slechts een milde nuchtere hypoglycemie [4]. Deze resultaten zijn consistent met de preklinische experimenten met antisense oligonucleotiden tegen de glucagon receptor. Diabetische db/db-muizen behandeld met deze oligonucleotiden hadden lagere glucose, triglyceriden en vrije vetzuren in het bloed, een verbeterde glucosetolerantie en ontwikkelden hyperglucagonemie zonder duidelijke effecten op de grootte en het aantal α-cellen in de pancreas [5]. In Zucker diabetische obese (ZDF) ratten en db/db en ob/ob-muizen was er een toename van GLP-1 en insulinespiegels [6].

DOEL VAN DIT PROEFSCHRIFT

In dit proefschrift wordt de nadruk gelegd op de rol van glucagon op de glucose regulatie bij gezonde vrijwilligers en T2DM patiënten en onderzocht of een (antisense) glucagon receptor antagonist een nuttige therapeutische interventie kan zijn in de behandeling van T2DM. Het is complex om de glucagon secretie farmacologisch te beinvloeden aangezien verschillende onderdelen die de secretie van de α-cel stimuleren ook aanwezig zijn in ß- cellen. Om die reden hebben we de glucagon challenge test, beschreven door Petersen *et al*. [7], verder onderzocht en als instrument gebruikt voor de klinische studie met een nieuwe GCGR-antagonist.

HOOFDSTUK 1 bestaat uit een algemene inleiding. Dit hoofdstuk begint met een algemene beschrijving van de glucose homeostase, pathofysiologie van T2DM en de huidige behandelingsmogelijkheden. De α-cellen van de pancreas en glucagon secretie zijn fundamentele componenten in de glucose regulatie.

HOOFDSTUK 2 bestudeert de effecten van een maaltijdvervanger bestaande uit een enkel eiwit hydrolysaat (insuVida™) op postprandiale plasma glucose, insuline en glucagon niveaus bij T2DM. Wij concludeerden dat een eenmalige inname van een caseïne hydrolysaat met en zonder toevoeging van leucine de koolhydraat-geïnduceerde insulinerespons in T2DM patiënten verbeterde. En dit resulteerde in significant lagere plasma glucose concentraties vergeleken met placebo en intact caseïne. Echter, naast insuline secretie door de pancreas, steeg ook de glucagon concentratie tijdens het gebruik van alle caseïne-rijke maaltijdvervangers. Dit mechanisme kan de daling in plasma glucose concentratie beperken en kan verantwoordelijk zijn voor de afwezigheid van hypoglycemieën. Verder onderzoek is nodig om de lange-termijn klinische voordelen van een lage dosis insuVida™, met en zonder extra leucine te bepalen. Dit hoofdstuk benadrukt tevens dat het onderzoeken van slechts één hormoon dat betrokken is bij de glucose homeostase (in dit geval insuline), zoals werd gedaan in alle voorgaande studies bij de maaltijd vervangende producten, een te beperkte benadering is. Het is belangrijk dat interventies die gericht zijn om een gestoorde glucose-homeostase te verbeteren, volledig moeten worden gekarakteriseerd.

Het doel van de studie in HOOFDSTUK 3 was om een glucagon provocatietest te bestuderen als instrument voor diabetes onderzoek, door beoordeling van de inter-en intra-individuele variabiliteit van de glucagon provocatietest, en door onderzoek naar de activiteit van het autonome zenuwstelsel (ANS) tijdens deze test aangezien dit een indirect effect op de glucose homeostase zou kunnen hebben. De glucagon challenge bestaat uit infusies met suprafysiologische dosering glucagon, insuline, en somatostatine, om de endogene productie van glucagon en insuline te onderdrukken. De hepatische glucoseproductie werd bepaald met behulp van infusie van stabiele isotopen van glucose. Verder hebben we onderzocht of bij mensen glucagon receptor MRNA tot expressie komt in vetweefsel, aangezien dit dan een rechtstreekse biomarker zou kunnen zijn voor het farmacologisch effect van geneesmiddelen die de glucagon receptor beïnvloeden. De resultaten van deze studie in 24 gezonde vrijwilligers toonden een snelle stijging in plasma glucose concentratie na

glucagon infusie, en bereikte een plateau op 90 min. De tijd profielen suggereren dat er sprake is van een snelle tolerantie voor glucagon-geïnduceerde hyperglycemie. Tijdens de glucagon provocatietest was de intra- en inter-individuele variabiliteit voor hepatische glucoseproductie, de snelheid van verdwijnen van glucose en plasma glucose concentratie, ongeveer 10-15% voor alle variabelen. Hyperglucagonemie had geen invloed op de hartslag-variabiliteit. In tegenstelling tot GCGR mRNA expressie in diabetische muizen, vonden we dat de expressie van glucagon receptor mRNA in het menselijk vetweefsel extreem laag was en niet kan dienen als een geschikte biomarker voor de aanwezigheid van GCGR in de mens. Uit deze studie werd geconcludeerd dat de gestandaardiseerde 6-uur durende glucagon challenge test een goede reproduceerbaarheid heeft met slechts beperkte variabiliteit gedurende 6 weken. Het is een krachtige manier om in detail de bijdrage van glucagon bij normale en veranderde glucose homeostase te bestuderen en kan ook worden gebruikt om de effecten van geneesmiddelen met een antagonerende werking op glucagon te onderzoeken zonder invloed van veranderingen in autonome zenuwstelsel.

Op basis van deze studie werd de een robuust hulpmiddel voor glucagon gericht onderzoek in diabetes. In HOOFDSTUK 4 werden de effecten van een soortgelijke glucagon provocatietest zoals beschreven in hoofdstuk 3 onderzocht, maar dan in T2DM patiënten en vergeleken met de glucagon respons bij gezonde vrijwilligers. Bij de patienten groep werd door middel van een cross-over studie ontwerp, met of zonder orale antidiabetica, de invloed van verschillende orale antidiabetica tijdens de glucagon provocatietest onderzocht. Onze resultaten tonen aan dat de reactie op een hyperglucagonemische provocatietest sterk verschilt tussen T2DM patiënten en gezonde vrijwilligers. Gezonde vrijwilligers laten een sterkere stijging van de HGP zien ten gevolge van het glucagon infuus en ook een snellere daling van de hepatische glucoseproductie dan T2DM patiënten. Exogene insuline onderdrukt de glucagon-geïnduceerde hepatische glucoseproductie. Bovendien waren de glucosespiegels lager tijdens glucagon provocatietest bij T2DM patiënten die twee weken hun orale medicatie hadden gestopt en een hogere dosis exogeen insuline kregen tijdens de test dan de patientengroep met voortzetting van hun orale antidiabetica. Deze bevindingen suggereren dat een afname van het effect van glucagon op de lever, door toediening van insuline in een eerder stadium van de ziekte en/of de ontwikkeling van geneesmiddelen gericht op de glucagon receptor, een veelbelovende therapeutische strategie kan zijn voor T2DM.

In HOOFDSTUK 5 onderzochten we of de antisense GCGR-antagonist, ISIS 325568, een bloedglucose verlagend effect heeft bij de mens. Dit onderzoek werd uitgevoerd met behulp van een dubbel-blind, placebo-gecontroleerde dosis-escalatie studie en evalueerde de verdraagbaarheid, farmacokinetische en farmacodynamische eigenschappen van enkelvoudige en meervoudige

doseringen met ISIS 325568 bij gezonde vrijwilligers in 4 verschillende dosisniveau's. ISIS 325568 is een tweede-generatie antisense fosforothioaat oligonucleotide en is ontworpen om te werken door gebruik te maken van het RNase H-mechanisme. In de cohorten met meerdere doseringen ontvingen acht proefpersonen 8 doseringen gedurende 6 weken (drie IV doseringen in week 1, gevolgd door vijf wekelijkse subcutane doseringen) en een glucagon provocatietest bij aanvang en aan het einde van 6 weken behandeling. Deze studie toonde aan dat ISIS 325568 geen klinisch significante veranderingen in de vitale functies, ECG, lever- of nierfunctie veroorzaakte en er werd geen hypoglykemie waargenomen. Het geneesmiddel werd over het algemeen goed verdragen, met een dosis-afhankelijke lokaal erytheem op de injectieplaats als meest voorkomende bijwerking. Alle bevindingen waren consistent met het bijwerkingenprofiel voor deze klasse van antisense oligonucleotiden [8]. Glucagon provocatietest veroorzaakte een snelle 2-voudige verhoging van plasma glucose concentraties en HGP, dit effect was aanzienlijk verminderd na 6 weken behandeling met GCGR ASO in dosering 400 mg/week. Er werden geen veranderingen in plasma glucose AUC of HGP waargenomen in de placebogroep. De dalspiegels van het geneesmiddel in het bloed veranderde na 4 weken doseren niet meer omdat er kennelijk een evenwichts-concentratie (steady-state) was bereikt en de PK-PD relatie was consistent met de preklinische gegevens. Deze studie met GCGR ASO heeft het eerste bewijs van werkzaamheid bij mensen geleverd. Deze resultaten ondersteunen verdere evaluatie van ASO-gebaseerde therapieën tegen de glucagon receptor bij patiënten met type 2 diabetes mellitus. In HOOFDSTUK 6 wordt een semi-mechanistisch model met glucagon, plasma glucose, insuline en glucagon receptor internalisatie gebouwd met behulp van gegevens uit onze glucagon provocatietest studie in gezonde vrijwilligers (hoofdstuk 3). Hoewel in dit model de complexe fysiologie van glucose sterk is vereenvoudigd, werden de belangrijkste contra-regulerende elementen in het glucose regelsysteem (glucagon en insuline) gebruikt om het glucose profiel te beschrijven. Toepassing op klinische gegevens lieten zien dat het model in staat is de niveaus van insuline, glucagon en glucose te beschrijven voor en tijdens de glucagon provocatietest. Door toepassing van dit model wordt de potentiële rol van glucagon in de pathofysiologie van diabetes mellitus verder verduidelijkt. Het beschrijft een algemene trend en kan dus dienen als een basis voor de ontwikkeling van geneesmiddelen.

Concluderend, de studies in dit proefschrift tonen aan dat:

» Het effect van maaltijdvervangers met eiwithydrolysaat op verlaging van de plasma glucose beperkt is in T2DM patiënten als gevolg van een gezamenlijke verhoging van zowel insuline als glucagon;
» De glucagon provocatietest een beperkte variabiliteit heeft in gezonde vrijwilligers en niet wordt beinvloed door veranderingen in autonome zenuwstelsel;

» T2DM patiënten reageren totaal anders op een glucagon challenge test in vergelijking met gezonde vrijwilligers;
» De reactie op een glucagon challenge test in T2DM patiënten wordt beïnvloed door het type behandeling (orale antidiabetica of insuline IV);
» De werkzaamheid van antisense glucagon receptor antagonist in mensen is bewezen en kan worden aangetoond met behulp van een goed gekarakteriseerde glucagon provocatietest;
» Het is mogelijk om een semi-mechanistisch model te ontwerpen dat gelijktijdig glucagon, plasma glucose, insuline en glucagon receptor internalisatie beschrijft, op basis van de resultaten van de glucagon provocatietest bij gezonde vrijwilligers. Dit model kan bijdragen tot een beter begrip van de pathofysiologie van diabetes mellitus.

Een absoluut of relatief glucagon overschot is een cruciale oorzaak voor het ontwikkelen en onderhouden van hyperglycemie bij patiënten met diabetes mellitus door het stimulerende effect op de hepatische glucose output. Strategieën gericht tegen glucagon kunnen geschikt zijn voor het verbeteren van glucose regulatie.

VOORUITZICHTEN

De onderzoeken beschreven in dit proefschrift bieden meer inzicht in de rol van glucagon in glucose homeostase bij gezonde proefpersonen en T2DM patienten. Het lijkt dat de optimale behandeling voor T2DM veelzijdig is en niet alleen insuline-gerichte therapie moet bevatten voor een evenwichtige glucose regulatie. Vandaag de dag worden de werkingsmechanismen, voordelen en risico's van traditionele en nieuwere middelen voor de behandeling van T2DM besproken om de apotheker en arts beter in staat te stellen de beste behandelcombinaties voor individuele patiënten voor te schrijven. Therapieën gericht op de glucagon receptor zouden een mogelijke aanvullende behandeling zijn voor T2DM patiënten in de toekomst. Aanvullend onderzoek naar de oorzaak van het ontstekingsreacties (huid) geïnduceerd door antisense GCGR-antagonist (ISIS 325568) verdienen nog aandacht. In de nabije toekomst zou het nog interessant en nuttig zijn om het door ons beschreven glucose homeostase model verder te verfijnen door de gegevens van T2DM patiënten en de effecten van GCGR antagonisten te verwerken in het model.

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CURRICULUM VITAE

Marloes van Dongen (1982; Woerden, the Netherlands) completed secondary education in 2000 (Atheneum) at R.S.G Broklede, Breukelen, The Netherlands. Subsequently, she attended medical school at Leiden University Medical Center (LUMC) from which she graduated as Doctor of Medicine (MD) in 2006. During her study she performed extra-curricular internships at the Department of Anatomy at Tygerberg hospital in Cape Town, South Africa (2004) and at the Department of Obstetrics and Gynaecology at Diakonessenhuis in Paramaribo, Surinam (2006).

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