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**Author:** Dongen, Marloes van  
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CHAPTER 3

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


M.G.J. van Dongen¹; B.F. Geerts²; S. Bhanot³, E.S. Morgan⁴, M.L. de Kam¹, M. Moerland¹, J.A. Romijn⁴; A.F. Cohen¹, J. Burggraaf¹

1. Centre for Human Drug Research (CHDR), Leiden, The Netherlands
2. Leiden University Medical Center (LUMC), Leiden, The Netherlands
3. Isis Pharmaceuticals Inc, Carlsbad, CA, United States of America
4. Academic Medical Center (AMC), Amsterdam, The Netherlands
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 [6,6-²H₁] 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 β-cell function, individuals can compensate for insulin resistance with appropriate hyperinsulinemia. However, loss of β-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 (cccr) 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 cccr 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 cccr 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 ≥ 30 kg/m²), 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 [6,6-²H₂] 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 CT-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 [6,6-²H₂] 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].
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 [10]. The fat biopsies were well tolerated and none of the participants 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).
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 can be restored by increasing the dose of glucagon [22]. In an animal model it was demonstrated that 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/racee 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 ccgr mRNA is expressed in adipose tissue [17]. As far as we are aware, there are no reports on ccgr mRNA expression in humans. We explored the expression of the ccgr in human adipose tissue, especially since this could be a direct biomarker of pharmacological activity of drugs that affect ccgr 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 ccgr mRNA expression in adipose tissue of patients with t2dm. However, at present ccgr mRNA expression, at least in healthy humans, is not a feasible biomarker for estimation of the amount or function of the ccgr.

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 pharmacodynamic tool with good reproducibility.


**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.

<table>
<thead>
<tr>
<th></th>
<th>Intra-individual variability (%)</th>
<th>Inter-individual variability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic glucose production</td>
<td>10.7</td>
<td>15.4</td>
</tr>
<tr>
<td>Glucose disposal</td>
<td>10.6</td>
<td>16.4</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>12.5</td>
<td>9.7</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th></th>
<th>1 hr before pancreatic clamp</th>
<th>5 min before pancreatic clamp</th>
<th>1 hr after start pancreatic clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>RR-interval (ms)</td>
<td>1065.5 (809 - 1184)</td>
<td>1101.5 (854 - 1198)</td>
<td>1105.0 (862 - 1279)</td>
</tr>
<tr>
<td>LF (ms²)</td>
<td>1259.5 (233 - 7766)</td>
<td>964.50 (280 - 5811)</td>
<td>1639.5 (351 - 3772)</td>
</tr>
<tr>
<td>HF (ms²)</td>
<td>1154.0 (131 - 5957)</td>
<td>1139.0 (119 - 6393)</td>
<td>1055.0 (242 - 9052)</td>
</tr>
<tr>
<td>LF/HF ratio</td>
<td>1.27 (0.28 - 3.35)</td>
<td>1.22 (0.23 - 3.25)</td>
<td>1.18 (0.29 - 4.94)</td>
</tr>
</tbody>
</table>

**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 glucagon versus glucose plots (right panel), based on the mean of observed values. Arrows indicate time course.

**Figure 3** Mean (so) hepatic glucose production during glucagon challenge separated by 6 weeks (n=12).

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**REFERENCES**


CHAPTER 4

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

Submitted for publication

M.G.J. van Dongen¹, M. Moerland¹, M. Derks², M.L. de Kam¹, N.A. Mazer², J.A. Romijn³, A.F. Cohen¹, J. Burggraaf¹

1. Centre for Human Drug Research (CHDR), Leiden, the Netherlands
2. F. Hoffmann-La Roche Ltd, Basel, Switzerland
3. Academic Medical Centre (AMC), Amsterdam, the Netherlands