Glucagon and Liver Fat are Downregulated in Response to Very Low-calorie Diet in Patients with Obesity and Type-2 Diabetes

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ABSTRACT

Background and Study Aims In patients with obesity and type-2 diabetes, short-time very low-calorie diet may ameliorate hyperglycemia and hepatic steatosis. Whether this also implies the glucose-regulating hormone glucagon remains to be elucidated. This study investigated the effects of a very low-calorie diet on plasma levels of glucagon and liver fat in obese patients with type-2 diabetes.

Patients and Methods Ten obese patients with type-2 diabetes, 6 men and 4 women, were included. At baseline, fasting plasma glucagon, insulin and glucose were determined, and liver fat and stiffness evaluated by transient elastography. The subjects were then prescribed a very low-calorie diet of maximum 800 kcal/day for 7 weeks and reexamined after 7 weeks and 12 months.

Results At baseline, BMI was 42 ± 4 kg/m^2 and fasting glucose 10.6 ± 3.4 mmol/l. All patients had hepatic steatosis. Plasma glucagon was strongly related to liver fat ($r^2 = 0.52$, $p = 0.018$). After 7 weeks of very low-calorie diet, plasma glucagon was significantly decreased by nearly 30 % ($p = 0.004$) along with reductions of BMI ($p < 0.0001$), glucose ($p = 0.02$), insulin ($p = 0.03$), liver fat ($p = 0.007$) and liver stiffness ($p = 0.05$). At 12 months follow-up, both glucagon and liver fat increased and were not different to basal levels, despite persistent reductions of BMI ($p < 0.002$) and glucose ($p = 0.008$).

Conclusion In obese type-2 diabetic subjects, plasma glucagon and liver fat are correlated and similarly affected by a very low-calorie diet, supporting a role of hepatic steatosis in glucagon metabolism.

Introduction

In obese patients who develop diabetes mellitus type 2, insulin resistance manifested by reduced *in vivo* glucose uptake in skeletal muscle and adipose tissue is of major pathophysiologic importance [1]. The results from studies on patients with both diabetes type 2 and non-alcoholic fatty liver disease (NAFLD) have shown that hepatic steatosis further aggravates the development of insulin resistance [2, 3]. Several pathophysiological mechanisms are involved in liver fat accumulation including augmented flux of adipose tissue- derived fatty acids and adipokines, increased hepatic *de novo* lipogenesis and reduced hepatic mitochondrial oxidation. These factors all contribute to decreased hepatic insulin sensitivity and along with altered metabolism of hepatokines, proteins secreted by the liver affecting metabolic function in non-hepatic tissues, peripheral insulin resistance may ensue [4]. It should however be noted that, based on genetic studies, fatty liver may under certain conditions not worsen markers of insulin resistance or result in increased risk of cardiovascular disease [5].

In this context, aberrations in the metabolism of glucagon, an insulin-antagonistic hormone produced by pancreatic alpha-cells, could also be of importance. Glucagon is a potent stimulator of hepatic glucose production [6] and increased levels of plasma glucagon are a common finding in patients with type-2 diabetes [7]. Interestingly, patients with both NAFLD and diabetes mellitus type 2

have higher plasma levels of glucagon than diabetic patients without NAFLD [8, 9], in turn suggesting a role of hepatic steatosis in the regulation of glucagon metabolism.

Weight reduction by very low-calorie diets for up to 12 weeks in obese patients with type-2 diabetes has been shown to normalize glucose control including restoration of beta-cell function and reduction of hepatic glucose production [10–13]. These short-term effects on glucose metabolism were associated with a decrease in hepatic as well as pancreatic lipid stores, whereas the ability to maintain a normal glucose control in the long term was related to recovery of the first phase insulin response [13, 14]. However, in none of these studies, the effects of energy restriction and consequent weight reduction on glucagon metabolism in relation to the observed metabolic effects were elucidated.

Therefore, the aim of the present study was to investigate the short- and long-term effects of a 7 week very low-calorie diet on plasma levels of glucagon, glucose and insulin along with liver fat content and stiffness in 10 obese patients with type-2 diabetes mellitus.

Materials and Methods

This is a sub-study of a prospective and observational investigation on the impact of low-calorie diet followed by a weight maintenance program on long-term glucose control in patients with obesity and diabetes mellitus. Inclusion criteria are men and women age 18–65 years, diagnosis of type-2 diabetes mellitus, BMI > 30 kg/m^2, HbA1c > 52 mmol/mol and stable weight ± 5 % for at least 12 weeks prior to screening. The study is registered at clinicaltrials.gov (NCT02498990). The study was approved by the regional Committee on Ethics at Karolinska Institutet in Stockholm, Sweden (2015/628-31). Patients were given oral and written information before they gave written consent to participate in the study. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Baseline investigation

Thirteen patients were screened for this study and underwent baseline investigation procedures after an overnight fast. One patient did not fulfil the criteria for entering the diet phase (HbA1c > 52 mmol/mol) and one patient withdraw from further participation in the study. One patient started the low-calorie diet phase but experienced gastrointestinal side effects and was taken out from the study after 3 weeks of diet. Consequently, 10 patients participated throughout the study and were included in the result evaluation. In these patients, diabetes duration was between 1 and 26 years, with a mean ± SD of 8.5 ± 8.0 years. Seven patients were treated with metformin, 6 patients were on treatment with a GLP-1 analogue (liraglutide 1.8 mg daily in all patients), 4 patients were on insulin treatment (total daily dosage 78 ± 69 units), 3 patients were on SGLT2-inhibition medication whereas 2 patients had no antidiabetic medical treatment at all. Weight was measured to the nearest 0.5 kg with a digital scale (TANITA model no TBF-305) and height (measured by a fixed tape measure at a wall) was measured to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by square of height (m^2). Blood pressure was measured in the supine position with a cuff of appropriate size after a 15 min rest.

Body composition including body fat (kg) and visceral fat (kg) content was measured with dual-energy X-ray absorptiometry (DEXA) using a GE-Lunar iDXA (GE Healthcare, Madison, WI, USA) and provided software (enCORE version 14.10.022), as described [15].

Using transient elastography (FibroScan®, Echosens, France), Continuous Attenuation Parameter (CAP) and liver stiffness was recorded. The Continuous Attenuation Parameter (CAP), measured in dB/m, was recorded to estimate hepatic steatosis [16, 17]. We used an approximated cutoff for steatosis grade ≥ 1 at 280 dB/m using the XL-probe in all cases although higher cutoffs values have been reported in a recent publication [18]. Liver stiffness was expressed in kPa, and examinations were performed according to the European Association for the Study of the Liver (EASL) guidelines [19]. For being considered a valid examination, at least 10 valid measurements were registered, the ratio of valid to the total number of measurements exceeded 60 % and the interquartile range (IQR) was less than 30 % of the median value (i. e. IQR/median ≤ 30 %) [20]. All patients were obese, why all measurements were performed with a 3.5 mHz XL-probe. Significant fibrosis (F ≥ 2) was defined as elastography values ≥ 6.4 kPa, and cirrhosis ≥ 16.0 kPa [21].

A venous blood sample was taken for the measurement of serum insulin and plasma glucose, alanine aminotransferase (ALT), glucagon and HbA1c at the routine chemistry laboratory at Karolinska University Hospital. P-glucose was analysed by Cobas 8000, Roche Diagnostics Scandinavia AB. Determination of serum insulin concentration was done by COBAS 8000, an immunochemical, non-competitive sandwich method (Elecsys Insulin reagent kit, catalogue No 12017547 122; Roche Diagnostics Scandinavia AB). HBA1c was assayed by chromatographic separation with HPLC (Variant II Turbo, Bio-Rad, KEMI-E03) using the Variant II Turbo HBA1C Kit – 2.0 (Bio-Rad Cat. No. 270-2455EX). Glucagon in plasma was quantified by a competitive radioimmunoassay method (EURO DIAGNOSTICA, catalogue nr RB310) with a CV of 8 %.

Very Low-calorie diet

One week after screening, the patients started seven weeks of low-calorie diet. They were prescribed a meal replacement formula diet, (Modifast® low calorie diet, Impolin AB, Täby, Sweden) of maximum 800 kcal/day. Each portion contained 25 % energy from proteins, 50–53 % from carbohydrates and 18–23 % from fat depending on the flavor of the formula. Except for this, all formula diets contained fibers, vitamines and minerals to cover daily needs. All 10 patients included in the analysis followed the very low-calorie diet for 7 weeks without any serious side effects. During this phase, visits took place once a week and at each visit, the patient saw a physician, a nurse and a dietician for individual counseling. Body weight and blood pressure were measured at each visit and in case of symptoms due to hypotension such as dizziness, medication for hypertension was modified accordingly. Patients were instructed to perform daily self-monitoring of fasting and post-prandial plasma glucose. Except for metformin in three patients, all anti-diabetic medication including insulin was withdrawn before week 7. No case of severe hypoglycemia was recorded. At week 4, body weight, blood pressure and transient elastography were performed. At week 7, all investigations, which were performed at screening, were repeated. In one of the patients, there was insufficient quality of

the transient elastography examination at week 4 and 7. Consequently, statistic evaluations of values for liver CAP and stiffness at these timepoints are based on 9 patients.

Twelve months follow up

The very low-calorie diet phase was followed by 2 weeks of gradual introduction of normal diet of eventually 1500–2000 kcal/day, i. e. 600 kcal/day deficit, based on present body weight and sex. The patients were then followed for 10 months including clinical visits every third month for the measure of body weight, blood pressure and evaluation of glucose control from self-monitoring and HbA1c. During this phase, antidiabetic medication was gradually reintroduced with a fasting plasma glucose target of <7 mmol/l. At month 12, all investigations performed at screening and at week 7 were reperformed except for body composition by DEXA. In one of the patients, there was insufficient quality of the transient elastography examination. Consequently, statistic evaluations of values for liver CAP and stiffness at week 52 are based on 9 patients. At 12 months, the glucagon value for one patient (138 pmol/l) fell outside of the mean + 2 times the SD range and was thus excluded from statistical evaluation. After 12 months, all patients who were initially on insulin treatment were again treated with insulin at a mean total dosage of 79 ± 67 units, 9 patients were treated with metformin, 8 patients were on a GLP-1 agonist, 5 patients were on treatment with an SGLT2-inhibitor and 1 patient did not take any antidiabetic medication at all. At each visit, the patients had individual counseling by both a physician and a dietician and were advised to increase physical activity.

Statistical analysis

Values are expressed as mean ± standard deviation (text), mean and 95 % confidence interval (▶ **Table 1**). Statistical analyses were made using JMP, version 14, and included Student´s paired and unpaired t-test and single regression analysis.

Results

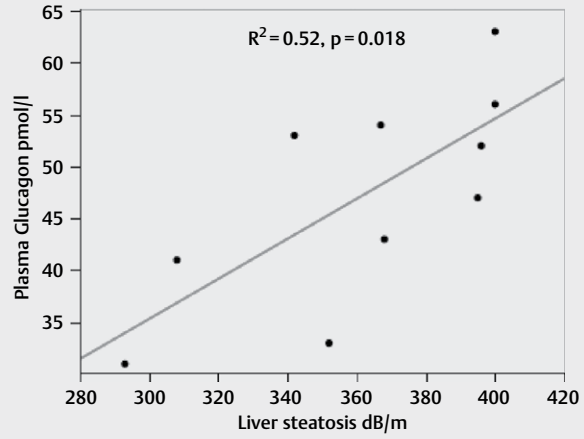
In ▶ **Table 1**, basal clinical parameters are shown. All subjects had a BMI above 35 kg/m^2 and were on average classified as having morbid obesity (> 40 kg/m^2). The mean glucose and HbA1c levels were elevated, whereas blood pressure showed normal values. As expected, the mean CAP value was high and according to the reference value (> 280 dB/m), all patients were classified as having hepatic steatosis. Also, mean liver elastography value was increased and in 6 patients indicative of suspect liver fibrosis (> 6.4 kPa) despite normal mean values for ALT.

As shown in ▶ **Fig. 1**, there was a strong correlation between glucagon and CAP values and based on the r^2-value, this suggests that about 50 % of the variation in plasma glucagon could be attributed to the degree of hepatic steatosis. In contrast, we found no correlations between plasma glucagon and BMI ($r^2 = 0.09$, $p = 0.31$), body fat content ($r^2 = 0.00$, $p = 0.92$), visceral fat content ($r^2 = 0.18$, $p = 0.08$), liver stiffness ($r^2 = 0.13$, $p = 0.23$), plasma glucose ($r^2 = 0.04$, $p = 0.50$) or plasma insulin ($r^2 = 0.15$, $p = 0.10$). In insulin-treated patients there was no correlation between basal plasma glucagon and total insulin dose ($r^2 = 0.06$, $p = 0.60$). Moreover, there was no difference in plasma glucagon between patients on GLP-1 agonist

▶ **Table 1** Clinical, anthropometric and metabolic data.

	Basal values	Week 4	Week 7	Week 52
Male/female (n)	6/4			
Age (years)	53 (47 – 59)			
Insulin treatment (n)	4	0	0	4
Metformin treatment (n)	7	5	3	9
GLP-1 agonist treatment (n)	6	0	0	8
SGLT2-inhibition treatment (n)	3	0	0	5
Body weight (kg)	125 (113 – 138)	116 (104 – 127) * * *	110 (99 – 120) * * *	114 (101 – 127) * * *
BMI (kg/m2)	42 (39 – 45)	39 (36 – 42) * * *	37 (34 – 40) * * *	38 (35 – 42) * * *
Body fat (kg)	58 (50 – 66)	–	47 (40 – 53) * * *	–
Visceral fat (kg)	4.3 (3.5 – 5.1)	–	2.8 (2.3 – 3.3) * * *	–
Liver fat (dB/m)	362 (335 – 390)	294 (262 – 327) * * *	287 (254 – 320) * * *	326 (271 – 381)
Liver stiffness (kPa)	14 (5 – 23)	8.4 (5.3 – 12) * *	7.4 (5.0 – 9.8) *	11 (3.7 – 19)
Systolic blood pressure (mm Hg)	138 (129 – 147)	126 (118 – 133) * * *	130 (119 – 140)	126 (117 – 134) * * *
Diastolic blood pressure (mm Hg)	79 (70 – 88)	77 (72 – 81)	78 (74 – 82)	78 (72 – 84)
P-ALT (mmol/l)	0.57 (0.31 – 0.83)	–	0.53 (0.38 – 0.68)	0.40 (0.30 – 0.50)
fP-Glukos (mmol/l)	10.6 (8.3 – 13)	–	7.5 (6.3 – 8.7) * *	7.3 (6.4 – 8.3) * * *
fS-Insulin all subjects (mmol/l)	42 (14 – 71)	–	14 (7.9 – 20) * *	35 (7.3 – 64)
fS-Insulin insulin treatment (mmol/l)	67 (– 23 – 158)	–	15 (– 3.5 – 34)	66 (– 14 – 146)
fS-Insulin non-insulin treatment	26 (17 – 35)	–	13 (7 – 18) * *	15 (9 – 21) * *
HbA1c (mmol/mol)	67 (57 – 76)	–	53 (46 – 59) * * *	52 (43 – 61) * * *
fP-Glucagon (pmol/l)	47 (40 – 55)	–	34 (28 – 41) * * *	47 (37 – 56)

Values are mean and 95 % confidence intervals. All p-values are compared to basal. * = p = 0.05, * * = p < 0.05, * * * = p < 0.01.



▶ **Fig. 1** Relationship between basal plasma glucagon and liver fat using single regression analysis.

treatment, 49 ± 12, as compared to non-GLP-1 agonist treated patients, 45 ± 9, p = 0.62.

The metabolic effects of weight reduction by 7 weeks of very low-calorie diet were then investigated. As shown in ▶ **Table 1**, body weight was markedly reduced already after 4 weeks of diet and a maximum decline of about 15 kg was found at week 7 corresponding to a decrease in BMI of 5.2 kg/m^2. The body weight reduction was associated with a substantial and significant decrease in liver steatosis by almost 70 dB/m at week 4, which remained essentially at the same level at week 7 along with clear reductions in both body and visceral fat. Liver stiffness was also reduced by the diet at both week 4 and 7 at a statistical borderline level. After 12 months, body weight and consequently BMI were still clearly reduced as compared to basal values and not significantly different to values at 7 weeks (p = 0.15), whereas values for CAP and liver stiffness did not differ from basal findings. At week 7 and month 12, there were normal values for CAP in 4 and 3 patients, respectively, and normal liver stiffness values in 4 and 3 patients, respectively.

In ▶ **Table 1**, the impact of very low-calorie diet on plasma levels of glucagon and glucose and serum insulin are shown. The diet had a marked effect on plasma levels of glucagon, which declined by almost 30 %, 47 ± 10 compared to 34 ± 9 pmol/l at week 7. This effect seemed more pronounced in non-insulin (35 %, p = 0.02) than in insulin (19 %, p = 0.15) treated patients although the number of patients was too small to make a reliable sub-group analysis. At month 12 the glucagon levels were again increased and not different from basal values. The same pattern was found for insulin, whereas plasma glucose at month 12 remained reduced at the same level as in week 7. Plasma levels of ALT were not significantly different at week 7 and month 12, as compared to basal values. There were no significant correlations between changes in plasma glucagon from baseline to week 7 and 52 and the corresponding changes in any of the metabolic or anthropometric variables analysed (p = 0.07–0.50). No correlation was found between glucagon and CAP values at 7 weeks of low-calorie diet (r^2 = 0.07, p = 0.48) whereas a correlation of borderline significance was found at 12 months (r^2 = 0.42, p = 0.06).

Discussion and Conclusions

The present study evaluated the effect of a low-calorie diet on plasma glucagon levels in obese type-2 diabetic subjects. A clear reduction of fasting glucagon during energy restriction was found accompanied by a marked decrease in body weight. All 10 patients in the basal state were diagnostic of liver steatosis based on CAP-values by transient elastography [18], and along with the decline in plasma glucagon, liver fat was significantly reduced and in 4 patients showed normal levels after 7 weeks of hypocaloric diet. Given the suggested role of hepatic steatosis for plasma levels of glucagon, this parallel reduction of both liver fat and glucagon is not surprising. Hepatic steatosis is common in obese type-2 diabetic patients [22, 23] and it has been repeatedly shown that liver fat is reduced as a result of weight reduction by low-calorie diets [10, 11, 13]. Recent studies suggest that the increased levels of glucagon observed in diabetes mellitus type 2 primarily are not due to reduced effects of glucose or insulin on the pancreatic alpha-cells in repressing glucagon. Instead, based on studies in patients with NAFLD, there seems to be a relation between obesity and consequent liver steatosis on the one hand, rather than the diabetic state, and glucagon on the other due to a steatosis-mediated resistance in glucagon action on the liver resulting in compensatory hyperglucagonemia [24]. Interestingly, in the basal state, we also found a strong correlation between fasting plasma glucagon and hepatic steatosis.

The underlying mechanisms for this liver-mediated regulation of glucagon is unclear but it has been hypothesized that it is due to a disturbed feed-back loop between the liver and alpha-cells resulting in steatosis-induced increased levels of circulating amino acids, which in turn have a stimulatory effect on the alpha-cell [25]. We did not measure amino acids levels in the present study. Nevertheless, we used a low-calorie diet (maximum 800 kcal/day) that is clearly hypocaloric and induces increased levels of urine ketone bodies [12]. Thus, it is tempting to speculate that the hypocaloric diet along with the decline in liver fat also resulted in a transient decrease of circulating amino acids that was reversed in the post-diet phase. This suggests that both amelioration of hepatic steatosis as well as the hypocaloric diet per se could have contributed to the effects on glucagon metabolism. This would also be in accordance with the post-diet findings at the 12 months follow-up, where plasma glucagon as well as liver steatosis were not different to basal values despite a consistent reduction of 11 kg body weight.

As expected, the low-calorie diet also had marked effects on glucose and insulin levels resulting in near-normal levels of fasting plasma glucose (about 7 mmol/l). This is line with previous studies [10–13] showing that the pathology of obesity-related diabetes mellitus type 2 in large can be eliminated by short-term weight reduction by energy restriction. However, in contrast to the above-mentioned effects on hepatic steatosis and glucagon, glucose at 12 months remained markedly reduced and not different to the diet-related levels. This also speaks in favor of the notion that glucagon regulation is not primarily influenced by the diabetic state but rather may be a result of liver fat accumulation.

We also measured the effects of low-calorie diet on liver stiffness evaluated with transient elastography. As for liver fat, a decrease in liver stiffness at 4 and 7 weeks of energy restriction was found. Whether liver stiffness also could be of importance for gluca-

gon metabolism remains to be elucidated. However, to the best of our knowledge, this is the first study to demonstrate that a low-calorie diet induced weight reduction also has positive short-term effects on liver elasticity, a finding that in turn may have important clinical implications for the treatment of patients with non-alcoholic fatty liver disease and increased liver stiffness [20].

The present study has limitations. First, the number of patients was relatively small which may increase the risk of false-positive results. Nevertheless, the study was sufficiently powered to demonstrate significant effects on body weight and glucose of the same magnitude that has been seen in previous studies with a similar number of patients [10, 11]. In the present study, no control group was included. Consequently, the results cannot be generalized to patients without obesity or diabetes or to interventions for weight reduction other than hypocaloric diets. The study included both insulin- and noninsulin as well as GLP-1 and non-GLP-1 treated patients which could have an impact on the results. We did not measure insulin resistance which is shown to be highly correlated with hyperglucagonemia [26]. Finally, we did not measure liver fat by the golden standard technique, MRI [27]. However, a good correlation between CAP and MRI-based liver fat content has been demonstrated [28].

The present study showed a marked reduction of fasting plasma glucagon as well as hepatic steatosis and stiffness upon a low-calorie diet in obese men and women with diabetes mellitus type 2. The glucagon levels returned to the basal state in the post-diet 12 month follow up along with a regain in liver fat but despite persistent reductions in body weight and fasting glucose. Moreover, a strong relation was found between plasma levels of glucagon and liver fat. Altogether, these results support the notion that liver steatosis, but not the diabetic state, is a primary regulator of glucagon metabolism in obesity-associated type-2 diabetes.

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Conflict of Interest

None of the authors have potential conflicts of interest with regard to this manuscript.

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