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Effect of the GLP-1 Receptor Agonist Lixisenatide on Counter-Regulatory Responses to Hypoglycemia in Subjects with Insulin-Treated Type 2 Diabetes

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ABSTRACT

OBJECTIVE: Counter-regulatory responses are critical to prevent hypoglycemia in subjects with type 2 diabetes. This is particularly important in insulin-treated patients. This study explored the effect of the GLP-1 receptor agonist lixisenatide on the hormonal counter-regulatory responses to insulin-induced hypoglycemia when added to basal insulin therapy in type 2 diabetes.

METHODS: The study was a single-center, double-blind, randomized, placebo-controlled crossover study involving 18 subjects with type 2 diabetes (11 males) with mean age 55 yrs, diabetes duration 12 yrs, HbA1c 7.7%, fasting blood glucose 9.7 mmol/l and body mass index 33 kg/m² who were treated with basal insulin (mean duration 7 yrs and daily dose 39U/day) and metformin (mean daily dose 2.1g). Subjects received lixisenatide or placebo for six weeks in random order with four week washout in-between. After six weeks treatment, subjects underwent a two-step hyperinsulinemic hypoglycemic clamp at 3.5 mmol/l and 2.8 mmol/l.

RESULTS: After six weeks treatment, HbA1c and FBG were lower after lixisenatide than after placebo. Glucagon and epinephrine levels at the hypoglycemic level of 3.5 mmol/l were significantly lower during lixisenatide than during placebo whereas glucagon and epinephrine levels at 2.8 mmol/l did not differ between the subjects, whereas cortisol, pancreatic polypeptide (PP) and norepinephrine levels did not differ significantly between the treatments.

CONCLUSIONS: Glucagon and epinephrine levels are reduced by lixisenatide at 3.5 mmol/l but their counter-regulatory response to deep hypoglycemia at 2.8 mmol/l glucose are sustained during treatment with lixisenatide in combination with basal insulin.

INTRODUCTION

In people with type 2 diabetes, the glucose-sensing of the islet α -cells is impaired, which leads to glucagon hypersecretion at normal and elevated glucose levels and impaired glucagon counter-regulation during hypoglycemia (1-3). Reducing the hyperglucagonemia is therefore an important target for the treatment of diabetes (4). Hyperglucagonemia is targeted by glucagon-like peptide-1 (GLP-1) and, consequently, the GLP-1 receptor agonists (1,5-8). However, preserving a functional glucose counter-regulation is critically important for glucose-lowering treatment to prevent hypoglycemia (9,10). It has earlier been shown in healthy volunteers (11,12) and in subjects with type 2 diabetes treated with oral agents (13) that GLP-1 or GLP-1 receptor agonists do not compromise glucagon counter-regulation during hypoglycemia. However, since a recent development is that GLP-1 receptor agonists are used also in combination with insulin therapy (14-18), it is of importance to determine the hormonal counter-regulation during hypoglycemia also under this condition. We therefore explored whether the GLP-1 receptor agonist lixisenatide affects the glucagon, norepinephrine, epinephrine, cortisol and pancreatic polypeptide (PP) responses to insulin-induced hypoglycemia when added to basal insulin in subjects with type 2 diabetes. Lixisenatide is a newly developed GLP-1 receptor agonist which is based on the structure of exendin-4 (19). It improves glycemia with low risk for hypoglycemia both in monotherapy, in association with metformin and in combination with insulin (20-23).

METHODS

Study population and study design

The study was a single-center, double-blind, placebo controlled, cross-over study in a total of 18 patients with type 2 diabetes treated with basal insulin plus metformin. The study was undertaken according to Good Clinical Practice, approved by the Ethic Committee in Lund, Sweden, and registered at the clinicaltrial.gov (NCT02020629) and the European Clinical Trials (EudraCT 2012-004959-36) databases. All subjects gave written consent to participate before the study and the study was monitored by an external monitor.

Fig. 1 illustrates the design of the study. Each patient attended a screening visit where the inclusion/exclusion criteria were assessed. The study population consisted of male and female (non-fertile or of child-bearing potential using a medically approved birth control method) patients with type 2 diabetes treated with basal insulin (insulin detemir, insulin glargine or NPH insulin) plus metformin with HbA1c in between 7-10% (53-83 mmol/mol), aged >18 years. Exclusion criteria were treatment with antihyperglycemic agents apart from basal insulin and metformin, type 1 diabetes (including LADA), pregnancy or lactation, a history of any secondary forms of diabetes, acute infections which may affect blood glucose control within 4 weeks prior to visit 1, a history of recent (<2 weeks) recurrent or severe hypoglycemic episodes or hypoglycemia unawareness, donation of one unit (500 ml) or more of blood, significant blood loss equaling to at least one unit of blood within the past 2 weeks or a blood transfusion within the past 8 weeks, treatment with growth hormone and oral or parenteral corticosteroid (> 7 consecutive days of treatment) within 8 weeks prior to visit 1 and thereafter during the whole

study period, use of other investigational drugs within 30 days prior to visit 1, abnormal laboratory findings at the time of screening, including amylase and/or lipase ≥ 3 times the upper limit of the normal laboratory range and P-calcitonin ≥ 20 pg/ml (5.9 pmol/L), personal or immediate family history of medullary thyroid cancer (MTC) or genetic condition that predisposes to MTC (e.g. multiple endocrine neoplasia syndromes), history of unexplained pancreatitis, chronic pancreatitis, pancreatectomy, stomach/gastric surgery, allergic reaction to any GLP-1 receptor agonist, or clinically relevant history of gastrointestinal disease associated with prolonged nausea and vomiting, cardiovascular, hepatic, neurological, or endocrine disease, active malignant tumor or other major systemic disease or patients with short life expectancy.

Eligible patients were randomized at a second visit and completed two treatment periods, receiving a different blinded study medication during each period (lixisenatide or placebo, in random order) in addition to their continued basal insulin and metformin treatment. Baseline visits were performed prior to each of the two treatment periods, at which the patient was assessed clinically and the blinded study medication was dispensed for six weeks treatment. After six weeks, a two-steå hyperinsulinemic hypoglycemic clamp was performed. The blinded study medication was then discontinued and a 4-week washout period before the next treatment period was started and after the second six week treatment period, the clamp study was repeated. Patients therefore received two treatments (lixisenatide and placebo) in a randomized order. Lixisenatide was given at 10µg daily subcutaneously within one hour before breakfast for two weeks, followed by the maintenance dose of 20 µg once daily. In patients

with HbA1c <7.5% (<57 mmol/l), the insulin dose was reduced by 20% at the time of study start in order to limit the risk of hypoglycemia. After 1 week the dose could be increased again at investigator's discretion according to fasting blood glucose levels. If hypoglycemia (cut-off definition 3.1 mmol/l) was confirmed during the treatment period, patients were requested to contact study center for discussion regarding possible adjustment of the insulin dose. All randomized patients completed the study.

Clamp procedure

Hyperinsulinemic hypoglycemic clamps were performed after six week treatment with lixisenatide or placebo. Patients arrived at the study site in the morning after an overnight fast (from 10 pm the preceding evening no food or drinks, except water, was allowed). Blinded study medication (lixisenatide or placebo) and the usual morning regular dose of metformin were taken 15 minutes before the start of the clamp procedure. The regular basal insulin was given the evening before the clamp and those patients who were on twice doses daily did not receive the basal insulin in the morning before the clamp. During the clamp procedure, patients received a primed 15 min infusion of insulin (Actrapid^R; Novo Nordisk, Bagsvaerd, Denmark) followed by a constant rate of insulin infusion to reduce blood glucose concentrations to 3.5 mmol/l and then to further reduce blood glucose levels to 2.8 mmol/l. The 15 min insulin priming infusion was 1.3 IU/m² if fasting glucose was ≤5 mmol/l, 3.9 IU/m² if fasting glucose was 5-7.5 mmol/l, 6.5 IU/m² if fasting glucose was 7.5-10 mmol/l, 9.1 IU/m² if fasting glucose was 10-15 mmol/l and 13 IU/m² if fasting glucose was 15 mmol/l. The following constant infusion rate for the various fasting glucose levels was 3, 9, 18, 21 and 30 IU/m²/h, respectively.

The two target glucose levels were maintained for 30 min. Glucose (200 mg/ml) was infused at a variable rate (dependent on bedside blood glucose monitored every 5 min) that resulted in the desired glucose concentration. The insulin infusion was then stopped, and a standardized meal was given, consisting of chicken, potatoes, sauce and green peas with 442 kcal (13% from fat, 36% from carbohydrate and 51% from protein). Samples were taken at prespecified time points throughout the clamp test for hormonal assays.

Adverse events

Adverse events were sought by non-directive questioning of the patient at each visit during the study. Adverse events were also detected when they were mentioned by the patient during or between visits or through physical examination, laboratory test, or other assessments. All adverse events, including hypoglycemia, were recorded. Hypoglycemia was defined as either symptomatic hypoglycemia, confirmed hypoglycemia (blood glucose concentrations ≤ 3.1 mmol/l), or hypoglycemia episode that requires assistance for control.

Laboratory measurements

Cortisol, epinephrine, norepinephrine, HbA1c, FPG, and safety laboratory assessments were measured by the Department of Clinical Chemistry (Skåne University Hospital, Malmö, Sweden). Samples for glucagon and pancreatic polypeptide (PP) were taken in chilled tubes containing EDTA (7.4mmol/l) and aprotinin (500KIU/ml; Novo Nordisk) and immediately centrifuged at 4°C; plasma was frozen at -20°C until analysis. Glucagon and PP were analyzed at the Department of Clinical Sciences Lund (Lund University). Glucagon concentrations were analyzed with an ELISA

from Merckodia (Uppsala, Sweden) (no 10-1271-01). Glucagon was measured using the recently developed sandwich ELISA, using monoclonal antibodies against both to the C- and N-terminal regions of glucagon, which has been shown to have higher specificity and reliability than previously used methods (24). The assay is specific for pancreatic glucagon and shows 4.4% cross-reactivity with oxyntomodulin and 0.8% cross-reactivity with glicentin but with no other peptide. It has a detection limit of 1 pmol/l and intraassay CV is 3.3-5.1% and interassay CV is 7.3-9.4% at various concentrations ranging from low to high. PP was determined with an ELISA from Merck Millipore (Billerica, MA, USA) (no EZHPP40K). The assay is specific for human PP and shows no cross-reactivity with neuropeptide Y, peptide YY or any other gut hormone. It has a sensitivity of 12.3 pg/ml and CV for intraassay is 3.3-5.0% and CV for interassay analysis is 4.4-9.8% at various concentrations ranging from low to high. Samples for determination of norepinephrine and epinephrine were taken in ice-chilled sodium-heparin tubes and their concentrations were determined by HPLC. Samples for determination of cortisol were taken in sodium-lithium-heparin tubes and its concentrations were determined with the BeckmanCoulter Access immunoassay system (Fullerton, CA).

Data analysis and statistics

During the hypoglycaemic clamp step, the change in concentration of analytes were calculated from time 60 to time 120 min (=step 1 hypoglycemia at 3.5 mmol/l glucose), from time 120 to time 180 min (=step 2 hypoglycemia at 2.8 mmol/l) and from time 60 to 180 min (=steps 1 and 2 together). Analytes during the clamp were also evaluated by calculating the area under the curve (AUC) with the trapezoid method. Between-treatment differences in measured variables were estimated with paired *t* test. There was no difference in responses to placebo or

lixisenatide depending on the order sequence of treatment. Therefore, in the statistical analysis all sequences with lixisenatide and placebo, respectively, were analysed together. A P-value of <0.05 was considered significant.

RESULTS

Subjects

A total of 21 subjects were screened and 18 patients (11 males, 7 females) were randomized. Mean age of the randomized patients was 55 ± 12 (SD) years, mean duration of diabetes was 11.7 ± 7.6 years, mean duration of insulin therapy was 7.1 ± 5.3 years, mean daily dose of insulin was 39 ± 22 IU, mean daily dose of metformin was 2.1 ± 0.2 g, mean body weight was 99 ± 17 kg, mean BMI was 33 ± 5 kg/m², mean HbA1c was 60.5 ± 3.0 mmol/mol ($7.7 \pm 0.3\%$) and mean fasting glucose was 9.7 ± 0.6 mmol/l. All 18 randomized subjects completed the study. There was no difference in baseline characteristics in patients starting therapy with lixisenatide or placebo (data not shown).

Effects on HbA1c, FBG, body weight and insulin dose (Table 1)

Table 1 shows the baseline levels and effects of treatment in HbA1c and FBG. HbA1c was significantly reduced by lixisenatide but not by placebo treatment. During the six-week treatment with lixisenatide, mean HbA1c was reduced by $-0.5 \pm 0.1\%$ (-5.8 ± 1.2 mmol/mol; $P < 0.001$) whereas there was a non-significant reduction in HbA1c by $-0.1 \pm 0.1\%$ (-1.0 ± 1.3 mmol/mol) by placebo. The between group difference was $-0.4 \pm 0.1\%$ (-4.8 ± 1.0 mmol/mol; $P = 0.042$). Also FBG (between group difference -1.6 ± 0.5 mmol/l; $P = 0.046$), body weight (between group difference -1.1 ± 0.4 kg; $P = 0.043$) and daily insulin dose (between group difference of -2.6 ± 3.9 U; $P = 0.023$) were reduced by lixisenatide compared to placebo. There were similar changes to lixisenatide in the nine patients who started on lixisenatide as in the nine patients who started on placebo (data not shown). In those who started on lixisenatide,

HbA1c remained low for the four week wash-out period to be 57.3 ± 4.3 mmol/l or $7.4\pm 0.4\%$ in HbA1c and 9.0 ± 1.1 mmol/l in FBG at the time of the start of the subsequent placebo period. In those patients, HbA1c (from 57.3 ± 4.3 to 60.2 ± 4.6 mmol/l or 7.4 ± 0.4 to $7.7\pm 0.4\%$; $P=0.015$) but not FBG increased during the placebo period. There was no change in daily dose of metformin during the study.

Hyperinsulinemic hypoglycemic clamp (Fig. 2; Table 2)

Glucose levels. After initiation of the clamp procedure, glucose levels were similarly reduced after lixisenatide and placebo to reach the first steady state level at min 90, which was kept stable at 3.5 ± 0.1 mmol/l after lixisenatide and 3.4 ± 0.1 mmol/l after placebo for 30 min ($P=0.61$). Then, at min 120, glucose infusion rate was reduced, which resulted in a further fall in the glucose level to the 2nd steady state at time 150 min, which was 2.8 ± 0.04 mmol/l after lixisenatide and 2.9 ± 0.07 mmol/l after placebo ($P=0.34$) and kept stable for 30 min. Amount of glucose infused until 180 min was 0.83 ± 0.09 mmol/kg after treatment with lixisenatide and 0.90 ± 0.13 mmol/kg after placebo ($P=0.59$). Total amount of insulin that was infused during the clamp procedure did not differ between lixisenatide (46 ± 9 IU) and placebo (51 ± 11 ; $P=0.65$). At 180 min, insulin infusion was stopped and a standardized 442 kcal mixed meal was given. Glucose levels then increased to 9.3 ± 0.6 mmol/l (lixisenatide) and 9.9 ± 0.5 mmol/l (placebo), respectively, after 60 min ($P=0.363$).

Glucagon levels (Fig. 2, Table 2). Fasting glucagon was not different between the two treatments. During the initial 60 min of the clamp, when glucose levels were gradually reduced from fasting to 3.5 mmol/l, glucagon levels were reduced in both groups, but more after

lixisenatide than after placebo. This resulted in a significantly lower 60 min value for glucagon after lixisenatide compared to placebo ($P=0.005$). Then, when 3.5 mmol/l glucose was reached and clamped, glucagon levels increased after both treatments with no significant difference between the treatments ($P=0.20$). Since glucagon levels were lower after lixisenatide than after placebo at min 60 and the glucagon increase by reducing glucose to 3.5 mmol/l was not significantly different by treatments, glucagon levels were still significantly lower after lixisenatide than after placebo at min 120 ($P=0.045$) as was the mean glucagon levels during the 60-120 min clamp period ($P=0.008$) and AUC for glucagon during min 60-120, i.e., during the 3.5 mmol/l clamp ($P=0.013$). Then, when glucose levels were further reduced to 2.8 mmol/l in the second hypoglycemic step, glucagon levels increased further in both groups. This increase was significantly higher after lixisenatide than after placebo ($P=0.042$). This resulted in glucagon levels at 180 min being not significantly different between the treatments and, also, that the mean glucagon during the 120-180 min study period and the AUC for glucagon during min 120-180 were not significantly different between the treatments. Furthermore, the increase in glucagon levels or the AUC glucagon during the entire clamp was not significantly different between the two treatments (Table 2). At 180 min, a standardized 442 kcal mixed meal was served; thereafter glucagon levels were, again, lower after lixisenatide than after placebo and the 255 min glucagon value, which ended the study, was 13.8 ± 1.9 pmol/l after lixisenatide and 23.8 ± 2.3 pmol/l after placebo ($P<0.001$).

Cortisol, norepinephrine, epinephrine and PP (Fig.2, Table 3). Baseline levels and levels at 120 and 180 min of cortisol, norepinephrine and PP did not differ between the treatments.

Therefore, the 120 and 180 min increases in cortisol, PP or norepinephrine did not differ significantly between lixisenatide and placebo treatment although there was a non-significant trend of a lower norepinephrine levels at both 3.5 and 2.8 mmol/l glucose after lixisenatide compared to placebo. Also baseline epinephrine levels did not differ between the treatments. However, the 120 min epinephrine levels were significantly lower after lixisenatide than after placebo (P=0.021) and, therefore, the epinephrine response to reducing glucose to 3.5 mmol/l was lower during lixisenatide than during placebo as evident both by lower increase in levels (P=0.022) and lower mean epinephrine levels (P=0.023) and AUC for epinephrine (P=0.041) during the 3.5 mmol/l clamp (Table 3). Thereafter, the epinephrine response to reducing glucose to 2.8 mmol/l was not different between the groups, resulting in a non-significant difference in epinephrine levels at 180 min.

Adverse events

In total, 56 AEs were reported during the study, of which 52 occurred during treatment with study medication [29 with lixisenatide (in 13 patients), 23 with placebo (in 12 patients)] and 4 during the washout period between the treatments (3 in the lixisenatide/placebo treatment sequence and 1 in the placebo/lixisenatide treatment sequence). The overall adverse event (AE) profile during treatment with lixisenatide was similar to that during placebo administration, except that nausea was reported in 7 patients during lixisenatide treatment and in 2 patients during placebo treatment. Mild hypoglycemia was reported in one patient during treatment with lixisenatide. No severe hypoglycemia (i.e., requiring third party assistance) was reported.

One SAE was reported in a patient on placebo who had vertigo and was admitted to the hospital overnight because of this; during this night the vertigo subsided.

DISCUSSION

This study evaluated the hormonal counter-regulatory responses to a two-step hypoglycemia clamp procedure in insulin- and metformin-treated patients with type 2 diabetes when lixisenatide or placebo was added. The study had a cross-over design and therefore all patients underwent treatment both with lixisenatide and placebo. The main finding was that 1)the glucagon and epinephrine levels were reduced by lixisenatide at 3.5 mmol/l glucose, whereas 2)their levels at deep hypoglycemia at 2.8 mmol/l glucose were not significantly different between the two treatments,

Glucagon levels were reduced immediately after administration of lixisenatide, which confirms previous studies that lixisenatide inhibits glucagon secretion (25). After this initial 60 min period, and while glucose levels were reduced to 3.5 mmol/l, glucagon levels were still lower during lixisenatide than during placebo, and although the increase in glucagon (glucagon counter-regulation to mild hypoglycemia) was not significantly different between the two treatments, the levels of glucagon remained lower during lixisenatide which was also evident by a lower AUC for glucagon. This shows that at a hypoglycemic level of 3.5 mmol/l glucagon levels are still reduced by lixisenatide. In contrast, during the second hypoglycemic step, when glucose was further reduced to 2.8 mmol/l, the increase in glucagon was more pronounced during lixisenatide than during placebo treatment, resulting in a similar glucagon level at the end of the 2.8 mmol/l hypoglycemic period. Overall, therefore, the study shows that lixisenatide reduces glucagon levels at glucose levels down to \approx 3.5 mmol/l but that the glucagon counter-

regulation to deep hypoglycemia is preserved during treatment with lixisenatide and, therefore, that the inhibition of glucagon secretion stops when glycemic reaches critically low levels.

A preserved glucagon counter-regulation to hypoglycemia has previously been shown for native GLP-1 and the GLP-1 receptor agonist exenatide in nine and twelve healthy volunteers, respectively (11,12) and for the GLP-1 receptor agonist albiglutide in subjects with type 2 diabetes treated with oral agents (13). In these studies the glucagon levels when glucose levels were 3.5 mmol/l were not different from placebo, although glucose was not clamped at this level, which thus may be a difference from the results in the present study. This discrepancy in regard to glucagon levels at 3.5 mmol/l between the different GLP-1 receptor agonists may be explained by different glucose thresholds for glucagon response between the different GLP-1 receptor agonists, by different duration of clamping glucose levels or by different glucagon characteristics in the different study populations. Although important to establish, a most likely explanation is that the present study was performed in patients treated with basal insulin who in general have a longer duration of diabetes and also are given exogenous insulin, which both are conditions that may reduce glucagon secretion. In addition, a so-called hypoglycemia-associated autonomic failure (HAAF), due to recent antecedent hypoglycemia, may have attenuated the counterregulatory response in these patients (26). Since add-on to basal insulin may become an important positioning of GLP-1 receptor agonists, there is, therefore, a need for head-to-head studies between different GLP-1 receptor agonists to examine glucagon counterregulation to mild hypoglycemia in this population.

The reason why the glucagon counter-regulation to deep hypoglycemia is not reduced during treatment with lixisenatide, in spite of the well known effect of lixisenatide to inhibit glucagon secretion at hyperglycemia (26) is probably the glucose-dependent mechanism of action of GLP-1. It has thus been demonstrated both in healthy subjects (27) and in subjects with type 2 diabetes (28) that GLP-1 clearly suppresses glucagon secretion when glucose levels are elevated above fasting levels. However, when reducing glucose levels below fasting, this inhibitory effect on glucagon secretion vanishes, as was demonstrated in the hypoglycemic clamp study using exenatide (12). In that study, exenatide inhibited glucagon secretion at 4.0 mmol/l glucose but not at 3.2 mmol/l glucose, suggesting that the threshold of GLP-1 receptor activation to inhibit glucagon secretion is between these glucose levels. This is confirmed in the present study in insulin-treated patients. In fact, glucagon levels were significantly lower during treatment with lixisenatide compared to placebo when glucose levels were clamped at 3.5 mmol/l, but not at lower levels, which suggests that the inhibitory effect of lixisenatide on glucagon secretion vanishes when glucose levels are reduced to approximately 3.5 mmol/l. Below this level the, hypoglycemia itself could increase glucagon levels as it normally does without inhibition by lixisenatide.

Several mechanisms are involved in the glucagon secretion during hypoglycemia, and these include besides a direct action of low glucose to stimulate glucagon secretion also activation of the autonomic nervous system and the adrenals to increase levels of epinephrine (9,10,29). There is a different glucose threshold for these mediators: whereas autonomic parasympathetic activation is important at a higher glucose level compared to activation through circulating

epinephrine and islet sympathetic activation which become operative at deep hypoglycemia (29). We measured PP which is as a marker of autonomic nerve activation, mainly parasympathetic nerves (30), and we also determined norepinephrine and epinephrine to explore these counter-regulatory factors after treatment with lixisenatide. We showed that all these variables increased by hypoglycemia, although the increase in norepinephrine was not evident until the deep hypoglycemia at 2.8 mmol/l, in line with knowledge that sympathetic nerves are of relevance for hypoglycemia only in deep hypoglycemia (29). The hypoglycemia responses in cortisol, PP and norepinephrine were not significantly different between lixisenatide and placebo although there was a non-significant trend that norepinephrine levels at both 3.5 and 2.8 mmol/l glucose were lower after lixisenatide than after placebo. In contrast, the epinephrine response to reducing glucose to 3.5 mmol/l was significantly reduced by lixisenatide, whereas the epinephrine response to further reduction in glucose to 2.8 mmol/l was not different between the treatments. A normal epinephrine response to deep hypoglycemia by lixisenatide is supported by studies on GLP-1 (11), exenatide (12) and albiglutide (13), whereas the finding that lixisenatide reduces epinephrine levels at glucose levels at 3.5 mmol/l contrasts to the normal response also at this level by exenatide (12) and albiglutide (13) and is in contrast to an increase in epinephrine levels by exenatide during exercise (31). Again, whether this characteristic is due to differences between the GLP-1 receptor agonists, differences in clamp technique or differences between healthy subjects versus subjects with type 2 diabetes treated with oral agents or basal insulin remain to be studied.

A strength of the study is the cross-over design with each subject serving as his/her own matched control, whereas a limitation of the study is that it was only a six week study. Therefore, glucagon counter-regulation to hypoglycemia after a long term treatment with the combination of lixisenatide and basal insulin remains to be established. Another limitation of the study is that the hormonal responses to hypoglycemia were determined after 30 min persistent and stable hypoglycemia, which is different from the clinical situation when there is an actual increase in circulating glucose by the counter-regulation. However, the clamp technique is superior to quantify the counter-regulation since glucose level is stable (32) and therefore suited for the aim of this study to explore the glucagon counterregulation.

In summary, we conclude that in insulin-treated patients with type 2 diabetes at a glucose level of 3.5 mmol/l, lixisenatide reduces glucagon and epinephrine levels compared to placebo, whereas the glucagon and epinephrine counter-regulation to deep hypoglycemia at 2.8 mmol/l is sustained.

CONTRIBUTIONS

The study was designed by J. F. and B. A. The study was conducted and data collected by J. F., M. P. and B. A. Analysis was performed by J. F. and B. A. All authors contributed to writing the manuscript. B.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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CONFLICT OF INTEREST DISCLOSURE

B. A. has consulted for Novartis, Glaxo-SmithKline, Merck, Sanofi, Novo Nordisk, Boehringer Ingelheim and Takeda and has received lecture fees from Novartis A/S, Merck, Novo Nordisk,

Sanofi, Bristol Myers Squibb, AstraZeneca and GlaxoSmithKline. J. F. and M. P. have nothing to declare.

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Table 1. Baseline, six week and change in HbA1c, FBG, body weight and daily insulin dose during the study

	Lixisenatide	Placebo	P-value ^a
Baseline HbA1c (IFCC; mmol/mol)	61.4±3.0	59.9±2.8	0.548
Baseline HbA1c (DCCT; %)	7.7±0.3	7.6±0.3	0.548
6 week HbA1c (IFCC; mmol/mol)	55.6±2.4	58.8±2.9	0.020
6 week HbA1c (DCCT; %)	7.3±0.2	7.5±0.3	0.020
Change in HbA1c (IFCC; mmol/mol)	-5.8±1.2 (P<0.001) ^b	-1.0±1.3 (P=0.432) ^b	0.042
Change in HbA1c (% DCCT)	-0.5±0.1 (P<0.001) ^b	-0.1±0.1 (P=0.432) ^b	0.042
Baseline FBG (mmol/l)	9.7±0.6	9.3±0.7	0.547
6 week FBG (mmol/l)	8.3±0.6	9.3±0.7	0.023
Change in FBG (mmol/l)	-1.6±0.4 (P=0.002) ^b	0.0±0.6 (P=0.986) ^b	0.046
Baseline body weight (kg)	99.5±4.6	99.8±4.5	0.962
6 week body weight (kg)	97.7±4.4	99.1±4.4	0.823
Change in body weight (kg)	-1.7±0.4 (P<0.001) ^b	-0.6±0.4 (P=0.095) ^b	0.043
Baseline daily insulin dose (U) (SD)	39±24	39±25	0.934
6 week daily insulin dose (U) (SD)	37±24	40±24	0.684
Change in daily insulin dose (U) (SD)	-1.8±4.1 (P=0.076) ^b	0.8±2.1 (P=0.135) ^b	0.023

^aP-value for the probability level of random difference between the two treatments

^bP-value for the probability level of random difference before and after treatment within each treatment arm

Table 2. Glucagon levels and counterregulation during the hyperinsulinemic hypoglycemic clamp test in subjects with insulin-treated type 2 diabetes

	Lixisenatide	Placebo	P
Glucagon level at min 0 (pmol/l)	10.7±1.3	9.8±1.2	0.18
Glucagon level at min 60 (pmol/l)	4.5±0.7	6.0±0.8	0.005
Glucagon level at min 120 (pmol/l)	11.2±1.7	16.1±2.6	0.045
Δ glucagon at 3.5 mmol/l (min 60-120) (pmol/l)	6.7±1.6	10.1±2.3	0.20
Mean glucagon at 3.5 mmol/l (min 60-120) (pmol/l)	8.1±1.1	11.4±1.6	0.008
AUC glucagon at 3.5 mmol/l (min 60-120 min) (pmol/l min)	471±63	668±91	0.013
Glucagon level at min 180 (pmol/l)	21.7±3.4	21.1±3.0	0.827
Δ glucagon at 2.8 mmol/l (min 120-180) (pmol/l)	10.5±2.3	4.9±2.3	0.042
Mean glucagon at 2.8 mmol/l (min 120-180) (pmol/l)	19.2±2.9	20.7±2.9	0.63
AUC glucagon at 2.8 mmol/l (min 120-180) (pmol/l min)	988±147	1116±152	0.36
Δ glucagon during whole clamp (min 60-180) (pmol/l)	17.2±3.0	15.0±2.5	0.45
AUC glucagon during whole clamp (min 60-180) (pmol/l min)	1460±208	1782±29	0.12
Glucagon level at min 240	13.8±1.9	23.8±2.3	<0.001

Means±SEM are shown

Δ indicates change in level

Table 3. Cortisol, PP, norepinephrine and epinephrine levels and counterregulation during the hyperinsulinemic hypoglycemic clamp test in subjects with insulin-treated type 2 diabetes

	Cortisol (pmol/l or nmol/l min)		PP (pg/ml or pg/ml min)		Norepinephrine (pmol/l or pmol/l min)		Epinephrine (pmol/l or pmol/l min)	
	Lixisenatide	Placebo	Lixisenatide	Placebo	Lixisenatide	Placebo	Lixisenatide	Placebo
Level min 0	411±29	463±32	171±55	157±55	2.0±0.2	2.1±0.2	0.09±0.02	0.11±0.03
Level min 60	420±28	404±35	157±63	145±53	2.5±0.2	2.7±0.2	0.19±0.06	0.23±0.07
Level min 120	629±78	691±89	321±77	381±96	2.4±0.2	3.0±0.3	1.19±0.34	2.06±0.54 ^a
Δ at 3.5 mmol/l (min 60-120)	219±78	308±85	163±59	235±79	0.0±0.1	0.3±0.2	0.99±0.31	1.82±0.53 ^a
Mean level min 60-120	558±52	574±60	247±66	312±77	2.4±0.58	2.8±0.23	0.78±0.18	1.47±0.39 ^a
AUC at 3.5 mmol/l (min 60-120)	31.2±2.7	32.2±3.1	14.1±4.0	15.8±4.0	144±12	173±15	41.5±11.5	68.7±16.8 ^a
Level min 180	931±46	989±64	660±99	624±96	3.3±0.3	4.1±0.4	2.77±0.51	3.35±0.83
Δ at 2.8 mmol/l (min 120-180)	328±34	287±58	339±88	243±79	1.1±0.23	1.0±0.27	1.54±0.34	1.29±0.49
Mean level min 120-180	716±104	804±106	562±88	482±91	2.4±0.30	3.9±0.53	2.61±0.45	3.15±0.69
AUC at 2.8 mmol/l (min 120-180)	47.6±4.1	50.1±4.3	29.5±4.8	27.2±5.4	166±14	215±19	120±24	162±40
Δ at whole clamp (min 60-180)	511±51	684±66	503±88	479±79	0.77±0.21	1.4±0.31	2.58±0.49	3.11±0.82

^aProbability level of random difference between the two treatments at P<0.05 or less (for detail see result section)

Means±SEM are shown

Δ indicates change in level

Legends to the figures:

Fig. 1 Schematic illustration of the study with the overall cross-over design (upper panel) and the clamp procedure (lower panel)

Fig. 2: Glucose, glucagon, cortisol, pancreatic polypeptide (PP), norepinephrine and epinephrine levels during the hyperinsulinemic, hypoglycemic clamp after six weeks of treatment with lixisenatide (filled symbols) or placebo (open symbols) in 18 patients with insulin treated type 2 diabetes. Dotted lines show the time intervals for the two steps of the hypoglycemia clamp (3.5 mmol/l and 2.8 mmol/l, respectively). Means \pm SEM are shown.



