Original Article

Immunoneutralization of Endogenous Glucagon Reduces Hepatic Glucose Output and Improves Long-Term Glycemic Control in Diabetic *ob/ob* Mice

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In type 2 diabetes, glucagon levels are elevated in relation to the prevailing insulin and glucose levels. The relative hyperglucagonemia is linked to increased hepatic glucose output (HGO) and hyperglycemia. Antagonizing the effects of glucagon is therefore considered an attractive target for treatment of type 2 diabetes. In the current study, effects of eliminating glucagon signaling with a glucagon monoclonal antibody (mAb) were investigated in the diabetic *ob/ob* mouse. Acute effects of inhibiting glucagon action were studied by an oral glucose tolerance test (OGTT) and by measurement of HGO. In addition, the effects of subchronic (5 and 14 days) glucagon mAb treatment on plasma glucose, insulin, triglycerides, and HbA_{1c} (A1C) levels were investigated. Glucagon mAb treatment reduced the area under the curve for glucose after an OGTT, reduced HGO, and increased the rate of hepatic glycogen synthesis. Glucagon mAb treatment for 5 days lowered plasma glucose and triglyceride levels, whereas 14 days of glucagon mAb treatment reduced A1C. In conclusion, acute and subchronic neutralization of endogenous glucagon improves glycemic control, thus supporting the contention that glucagon antagonism may represent a beneficial treatment of diabetes. *Diabetes* 55:2843–2848, 2006

In type 2 diabetes, glucose-induced suppression of glucagon secretion is impaired, causing inappropriately elevated glucagon levels relative to the elevated glucose levels (1). Furthermore, endogenous glucose production is inappropriately increased in type 2 diabetes (2–4). Several studies have demonstrated a correlation between elevated glucagon levels and increased hepatic glucose production in both healthy subjects infused with exogenous glucagon (5–7) and type 2 diabetic patients (8–10). Thus, reduction of glucagon levels is considered an interesting target for the treatment of type 2 diabetes, and therefore it is relevant to further characterize the relationship between hyperglucagonemia and the metabolic disturbances associated with type 2 diabetes.

Immunoneutralization of endogenous glucagon with glucagon monoclonal antibodies (mAbs) provides a selective and complete suppression of glucagon action without directly affecting the secretion of other hormones. Studies using these antibodies have demonstrated significant blood glucose–lowering effects in diabetic animal models (11–13).

In this study, monoclonal glucagon antibodies were used to provide isolated elimination of glucagon signaling in the *ob/ob* mouse, a commonly used model of type 2 diabetes displaying hyperglycemia, hyperinsulinemia, and insulin resistance (14–16). With this approach, the aim was to gain further insight into the effects of glucagon on the regulation of hepatic glucose output (HGO) in an animal model of type 2 diabetes, both in the basal state and during the handling of a glucose load. Furthermore, the effects of prolonged elimination of glucagon signaling were studied. The results support the view that glucagon plays a prominent role in the hyperglycemia associated with type 2 diabetes, and they provide new information as to the in vivo effects of selective glucagon antagonism.

RESEARCH DESIGN AND METHODS

For the acute studies (oral glucose tolerance test [OGTT]) and measurement of HGO, we used male *ob/ob* mice (C57BL/6OlaHsd-Lep^{ob}; Harlan, Indianapolis, IN). For the subchronic (5- and 14-day) studies of glycemic control, we used female *ob/ob* mice of the Umeå strain (obtained from Umeå University, Umeå, Sweden). Female *ob/ob* mice of the Umeå strain develop overt diabetes more consistently compared with other strains of *ob/ob* mice; therefore, this particular strain was chosen for the studies of longer duration. All mice were 6–7 weeks old on arrival and 10–11 weeks old at the initiation of experiments. Mice were individually housed under ambient controlled conditions and a 12-h light/dark cycle with free access to food and water. During the acclimatization period, mice were handled daily to familiarize them with the procedures in order to reduce stress during experiments. All animal studies were conducted in accordance with U.S. National Institutes of Health guidelines for the care and use of laboratory animals and approved by the Danish Animal Experiments Inspectorate.

Antibodies. The murine monoclonal anti-glucagon antibody (Glu-001 glucagon mAb; Novo Nordisk, Bagsvaerd, Denmark) and an "inert" monoclonal anti-2,4,6-trinitrophenyl antibody that was used in control experiments (control mAb) were produced and characterized as described previously (11). The dose (4 mg/kg) of glucagon mAbs that was used has been demonstrated to have the ability to immunoneutralize >90% of circulating glucagon for several hours (12). For all experiments, antibody was diluted to a concentration of 1 mg/ml in sterile 0.9% saline and kept at 4°C until use. Antibodies were injected intraperitoneally.

Study protocols

OGTT. In the morning after an overnight fast, mice were given a single dose of either glucagon mAbs or control mAbs ($t = -120$ min). Then, 2 h later ($t = 0$ min), 4 ml/kg of 20% glucose (~40% enriched with [1-^{13}C]glucose) was

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FFA, free fatty acid; HGO, hepatic glucose output; mAb, monoclonal antibody; OGTT, oral glucose tolerance test.

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administered by gavage. At −120, 0, 15, 30, 60, 90, and 120 min after glucose administration, tail blood samples (40 μl) were collected in heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA), transferred to heparin-lithium–coated centrifuge tubes (Beckman Instruments, Palo Alto, CA), and centrifuged (4°C, 6,000*g*, 5 min). Plasma glucose was measured immediately, and remaining plasma was stored at −20°C for later measurement of plasma insulin concentration and ^{13}C enrichment. To avoid collection of large blood samples, plasma samples from half of the animals in each group were used for insulin measurement, and samples from half were used for measurement of ^{13}C enrichment. At 120 min, the mice were anesthetized with isoflurane (Aerrane; Baxter, Deerfield, IL), and livers were quickly removed, freeze-clamped using aluminum tongs precooled with liquid N_2, and stored at −80°C until assayed for glycogen content and ^{13}C enrichment. Corresponding to the −120-min time point in the OGTT, a separate group of overnight-fasted male *ob/ob* mice was anesthetized with isoflurane, and livers were quickly excised, freeze clamped, and subsequently stored at −80°C until assayed for glycogen content.

HGO. At 1 week before the experiments, mice were anesthetized with ketamine (60 mg/kg) and xylazine (3 mg/kg), and an indwelling catheter was inserted in the left internal jugular vein. The catheters were externalized through an incision in the skin behind the head, and the mice were returned to individual cages after the surgery. The mice were fully recuperated from the surgery before the in vivo experiments, as reflected by their reaching their preoperative body weight.

In the morning after an overnight fast, mice were given a single dose of either glucagon mAbs or control mAbs. Then, 1 h later, a primed (10 μCi) continuous (0.5 μCi/min) infusion of D-[3-^{3}H]glucose (Perkin Elmer, Boston, MA) was initiated. From 75 to 150 min, blood samples (30 μl) were collected from the tail every 15 min into heparinized microhematocrit capillary tubes, transferred to heparin-lithium–coated centrifuge tubes, and centrifuged (4°C, 6,000*g*, 5 min). Plasma glucose was measured immediately, and plasma samples were then stored at −20°C for later measurement of plasma [^{3}H] activity. Calculation of HGO was based on the measurements at the last four time points (see CALCULATIONS section below).

Five-day treatment. All mice first received once-daily injections with control mAbs (4 mg/kg) for 1 week (basal period). At the beginning of the 2nd week, mice were allocated to two groups (n = 10 per group) having matching blood glucose profiles during the basal period. The daily treatment continued for 5 days with either control mAbs or glucagon mAbs. Mice had free access to food and water during the entire experiment period. Tail blood samples (5 μl) were collected twice daily, once immediately before the injection and once 5 h after. Whole blood was collected into 5-μl capillary tubes and immediately immersed into 250 μl of analysis buffer (see BIOCHEMICAL ANALYSES section below) for blood glucose measurement. On the 5th day, 5 h after administration of antibody, mice were sedated with CO_2 and killed by decapitation. Trunk blood was sampled into precooled tubes containing Na_2-EDTA (7.5 mg/ml blood) and aprotinin (600 kallikrein inactivation units/ml blood; Novo Nordisk) and immediately centrifuged (4°C, 6,000*g*, 10 min). Plasma was stored at −20°C until assayed for end point levels of lactate, free fatty acids (FFAs), triglycerides, and insulin. A plasma sample was used to determine the remaining glucagon-binding capacity of the glucagon mAbs as an indication of the extent to which glucagon had been neutralized during the study. Livers were quickly excised, freeze clamped with aluminum tongs precooled in liquid N_2, and subsequently stored at −80°C until assayed for glycogen content.

Fourteen-day treatment. In parallel, two groups (n = 10 per group) of Umeå *ob/ob* mice with matching baseline HbA_{1c} (A1C) levels were treated once daily for 14 days with glucagon mAbs or control mAbs. Food and water were weighed for estimation of 24-h intake. After 2 weeks of treatment, blood was collected from the tail vein into 5-μl capillary tubes and immediately immersed into analysis buffer (see BIOCHEMICAL ANALYSES section below) for the measurement of A1C.

Biochemical analyses

OGTT and HGO measurement. Plasma levels of glucose were measured using 10 μl plasma by a glucose oxidase method on a glucose analyzer (Beckman Instruments, Fullerton, CA). Plasma insulin was measured by radioimmunoassay using a kit from Linco Research (St. Charles, MO). For the determination of plasma [3-^{3}H]glucose concentrations, plasma was deproteinized with $ZnSO_4$ and $Ba(OH)_2$ (Sigma, St. Louis, MO), dried to remove ^3H_2O, resuspended in water, and counted in scintillation fluid (Ultima Gold; Packard Instrument, Meriden, CT).

Glycogen was extracted from finely ground liver tissue, using HCl-EtOH, 0.9 mol/l $HClO_4$, and 100% ethanol. Extracted glycogen was resuspended in H_2O and dialyzed overnight in dialysis tubing (Spectrum Laboratories, Los Angeles, CA) against 5 l of deionized water. Glycogen was then digested with amyloglucosidase (Sigma). Concentrations of glycosyl units from digested glycogen were determined on a glucose analyzer, using the glucose oxidase reaction (Glucose Analyzer II; Beckman Instruments, Fullerton, CA).

The ^{13}C enrichments of glycosyl units in hepatic glycogen were determined by gas chromatography–mass spectrometry, using an HP5890-MSD5971 (Hewlett-Packard, Palo Alto, CA) analysis of the penta-acetate derivative of glucose and the glucose moieties of glycogen (m/z [charge/mass ratio] 169–171 and 200–202 in electron impact mode). The difference between these two fragment ions gives the enrichment in C1.

Five- and 14-day treatment. Glucose and lactate concentrations were analyzed by the immobilized glucose/lactate oxidase method, using 5 μl whole blood immersed in 250 μl analysis buffer (EBIO Plus autoanalyzer and buffer solution; Eppendorf, Hamburg, Germany). Plasma insulin concentrations were measured with an enzyme-linked immunosorbent assay method (17), using 5–10 × dilutions of plasma samples. A1C was measured using 5 μl whole blood diluted in analysis buffer (COBAS MIRA Plus autoanalyzer; Roche Diagnostic Systems, Basel, Switzerland). Plasma FFA (Wako Chemicals, Richmond, VA) and triglyceride (Roche, Hvidovre, Denmark) concentrations were measured by the enzymatic calorimetric method, using a total of 30 μl plasma (COBAS MIRA Plus autoanalyzer; Roche Diagnostic Systems). The glucagon mAb binding titer in plasma samples obtained at the end of the 5-day study was measured as previously described (12). For measurement of liver glycogen content, freeze-clamped liver tissue was suspended in H_2O and subsequently homogenized and boiled with HCl. Samples were then centrifuged for 1 min at 2,000*g*, and the supernatant was neutralized with an equal volume of 2 N NaOH. Glucose was determined by measuring NADPH formation, using hexokinase and glucose-6-phosphate dehydrogenase as auxiliary enzymes. Free glucose in the liver tissue was measured in an aliquot of the liver homogenate.

Calculations. Hepatic glycogen content (in micromoles glycosyl units per gram liver tissue) was calculated from an average of the concentration of glycosyl units per milligram in three individual pieces of liver tissue from each animal.

Glycogen synthesis rates were calculated as follows:

$$\text{Glycogen synthesis rate} = \text{Hepatic glycogen concentration} \times \frac{\dfrac{\%1\text{-}^{13}\text{C enrichment in hepatic glycogen}}{\%1\text{-}^{13}\text{C enrichment in plasma}}}{120\text{ min}}$$

The percentage of plasma glucose enrichment was calculated as the average 1-^{13}C enrichment from 15 to 120 min, which was 14.4% for control mAb–treated animals and 15.3% for glucagon mAb–treated animals (nonsignificant P value).

The area under the curve (AUC) for glucose excursions during the OGTT was calculated using the trapezoidal rule. The rate of HGO was calculated as the ratio of [^{3}H]glucose infusion rate (disintegrations per min [dpm]/min) to the specific activity of plasma glucose (dpm · min^{-1} · μmol^{-1}) at the time of the blood sample, according to Steele (18): $R_a = I/\text{SA}$, where R_a is rate of appearance of glucose, I is tracer infusion rate, and SA is the tracer specific activity in plasma.

Data analysis. Statistical analysis was performed using GraphPad Prism version 4.02 (GraphPad, Monrovia, CA). Data are the means ± SE and were compared using Student's unpaired t test, except for the comparisons in the 5-day study where a paired t test was used. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Acute inhibition of glucagon reduces plasma glucose excursion during OGTT. To examine the effect of glucagon on glucose handling in diabetic *ob/ob* mice, an OGTT was performed in overnight-fasted mice pretreated with a single dose of either control mAb or glucagon mAb (Fig. 1). Immediately before glucose administration, at t = 0 min, plasma glucose was lower in the mice that had been treated with glucagon mAb compared with control mAb–treated mice (7.2 ± 0.6 vs. 12.7 ± 1.7 mmol/l, $P < 0.01$). Treatment with glucagon mAbs reduced plasma glucose levels during the OGTT and resulted in a reduction in the baseline-subtracted AUC for the glucose excursion curve. Glucagon mAb treatment did not lead to significant changes in the AUC for insulin during the OGTT compared with control mAb treatment (Fig. 1).

Acute inhibition of glucagon results in increased hepatic glycogen content after OGTT. To evaluate the effects of glucagon on hepatic glucose metabolism in *ob/ob*

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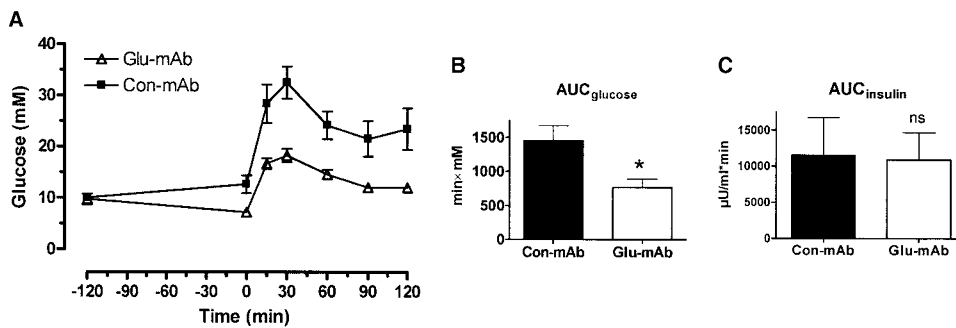

FIG. 1. Effect of glucagon mAb treatment on glucose excursion during an OGTT in *ob/ob* mice. Glucagon mAbs or control mAbs were injected at $t = -120$ min, and glucose was given by gavage at $t = 0$ min (*A*). Baseline-subtracted AUC is shown for the glucose (*B*) and insulin (*C*) excursions observed during the OGTT in *ob/ob* mice pretreated with either control mAbs or glucagon mAbs. Data are the means ± SE, $n = 6$–7 for glucose values and $n = 3$ for insulin values. *$P < 0.05$, Student's unpaired *t* test. Con, control; Glu, glucagon.

mice during an oral glucose challenge, hepatic glycogen concentration was measured in glucagon mAb– and control mAb–treated mice after the OGTT. For reference, glycogen content was also determined in a group of overnight-fasted untreated *ob/ob* mice (basal). At the end of the OGTT, liver glycogen concentration was elevated in the glucagon mAb–treated mice compared with both the control mAb–treated and the basal groups (Fig. 2). In contrast, there was no significant difference between the glycogen levels in the basal group and the control mAb–treated group after the OGTT.

The net rate of glycogen synthesis during the OGTT was estimated using the total hepatic glycogen content and the 1-^{13}C enrichment of glycosyl units in hepatic glycogen (see CALCULATIONS section above). By this calculation, the net rate of hepatic glycogen formation was increased in glucagon mAb–treated mice compared with control mAb–treated mice (0.25 ± 0.04 vs. 0.12 ± 0.02 μmol glycosyl units · g liver^{-1} · min^{-1}, $P < 0.05$). Correspondingly, the amount of 1-^{13}C–labeled glucose that was incorporated into hepatic glycogen was higher in the glucagon mAb–treated mice than in the control mAb–treated mice (4.5 ± 0.7 vs. 2.4 ± 0.4 μmol [1-^{13}C]glycosyl units/g liver, $P < 0.05$).

HGO is reduced in the absence of circulating glucagon. To gain more information on the mechanism behind the glucose-lowering effects of glucagon immunoneutralization and to provide support for the theory that glucagon antagonism reduces HGO, a tracer infusion study was conducted in *ob/ob* mice pretreated with a single dose of either glucagon mAbs or control mAbs to measure basal HGO. During tracer infusion the average plasma glucose levels were lowered in mice treated with glucagon mAb compared with control mAb–treated animals (12.0 ± 0.2 vs. 17.6 ± 0.3 mmol/l, $P < 0.001$). Glucose levels were stable during the last 60 min of the tracer infusion period, indicating that the effect of the antibody treatment had reached a plateau at the time during which HGO was calculated. Immunoneutralization of circulating glucagon caused a significant reduction in basal HGO compared with control mAb–treated animals (159 ± 31 vs. 63 ± 6 μmol glucose · kg^{-1} · min^{-1}, $P < 0.05$) (Fig. 3).

Reduced plasma glucose and triglyceride levels with subchronic glucagon immunoneutralization. In addition to the studies of effects of acute elimination of circulating glucagon, we also investigated the effects of glucagon immunoneutralization in a long-term treatment regimen. After a 5-day basal period with control mAb treatment, *ob/ob* mice were treated with glucagon mAbs or control mAbs for 5 consecutive days to assess the effects of subchronic treatment on the diabetic state of the animals. At the end of the 5-day treatment period, plasma levels of free glucagon were below detection limits in glucagon mAb–treated mice, as documented indirectly by the presence of massive excess glucagon-binding capacity in plasma samples (data not shown) (12). Average glucose levels 5 h postdose were reduced in the glucagon mAb–treated mice during the 5-day treatment compared with glucose levels recorded in the same mice during the basal period and with those recorded in the control mAb–treated control group (Table 1). Plasma triglyceride levels were decreased after 5 days of glucagon mAb treatment, whereas hepatic glycogen content and plasma levels of FFAs and insulin were not altered by the treatment. In addition, there was no indication of lactate acidosis because plasma lactate levels remained unchanged (Table 2). Food intake was also not affected by glucagon mAb treatment (data not shown).

As an additional measure of the long-term treatment effect of selective glucagon inhibition in the diabetic *ob/ob* mouse, a parallel group of mice received glucagon mAb or control mAb treatment for 14 days, and plasma A1C levels were measured at the end of the treatment period. The worsening A1C observed in the control mAb–treated mice

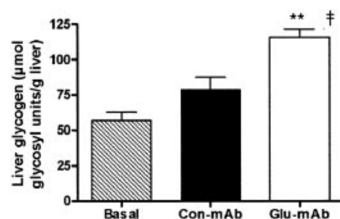

FIG. 2. Effect of glucagon elimination on hepatic glycogen concentration after an oral glucose challenge in *ob/ob* mice. Hepatic glycogen concentration was measured in a group of fasted untreated mice (basal) and in control mAb– or glucagon mAb–treated mice after OGTT. Data are the means ± SE, $n = 6$–8. **$P < 0.01$ for control mAbs vs. glucagon mAbs, P = NS for control mAbs vs. basal; ‡$P < 0.001$ for glucagon mAbs vs. basal in Student's unpaired *t* test. ▨, basal; ■, control mAbs; □, glucagon mAbs.

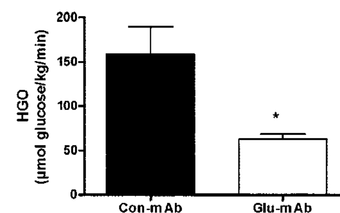

FIG. 3. Effect of glucagon elimination on hepatic glucose production in *ob/ob* mice. Trace amounts of [3-^{3}H]glucose were infused to determine hepatic glucose production in the basal state in *ob/ob* mice treated with either glucagon mAbs or control mAbs. The bar graph shows hepatic glucose production measured during the last 60 min of tracer infusion in *ob/ob* mice treated with glucagon mAbs or control mAbs. Data are the means ± SE, $n = 4$–5. *$P < 0.01$, Student's unpaired *t* test. ■, control mAbs; □, glucagon mAbs.

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TABLE 1
Mean daily 5-h postdose blood glucose levels in mice treated with control or glucagon mAbs for 5 days

	Mean postdose blood glucose (mmol/l)	
	Control mAb treatment	Glucagon mAb treatment
Basal (control mAbs)	13.0 ± 0.4	13.3 ± 0.6
Treatment (control/glucagon mAbs)	12.3 ± 0.4	9.1 ± 0.3*†

Data are means ± SE, $n = 10$ mice/group. *$P < 0.01$ vs. control mAbs 1st week in a paired t test; †$P < 0.001$ vs. control mAbs 2nd week in unpaired t test.

during the course of treatment, taken as an indication of continuous progression of diabetes, was prevented by glucagon mAb treatment (Table 3). Although food intake did not differ between the two treatment groups, weight gain was slightly increased by the glucagon mAb treatment. In addition, as a secondary measure of improved glycemic control, water intake was reduced by the glucagon mAb treatment (Table 3).

DISCUSSION

The acute glucose-lowering effects of glucagon removal with mAbs have previously been demonstrated in different diabetic and nondiabetic animal models (11–13). In the current study, we have further examined the acute and subchronic effects of selective glucagon inhibition on hepatic glucose metabolism in the *ob/ob* mouse, an animal model with phenotypic characteristics resembling type 2 diabetes.

Immunoneutralization of circulating glucagon reduced glucose excursion during an oral glucose challenge, without significant changes in insulin levels compared with control mAb–treated *ob/ob* mice. The marked reduction of AUC for glucose found in the glucagon mAb–treated animals demonstrates that selective glucagon inhibition improves glucose tolerance in the diabetic *ob/ob* mouse. One explanation for the improved glucose tolerance observed in *ob/ob* mice after acute glucagon immunoneutralization might be provided by the increased rates of glycogen formation and increased hepatic glycogen levels found in glucagon mAb–treated mice after glucose challenge. Possibly, hyperglucagonemia in diabetic *ob/ob* mice contributes to a state of elevated glycogenolysis, thus reducing net glycogen formation in response to a glucose load. Selective inhibition of glucagon signaling might reduce simultaneous glycogen breakdown during uptake of the glucose load, thus increasing net formation of hepatic glycogen in the glucagon mAb– compared with control mAb–treated animals.

It is well known that increased HGO contributes to hyperglycemia in type 2 diabetes (2,8). A connection between hyperglucagonemia and increased HGO has previously been established in diabetic patients by correlation (9) and in studies reducing glucagon levels nonselectively with somatostatin (4,10). By selectively inhibiting glucagon without directly affecting other glucoregulatory hormones, this study directly demonstrates the association of increased HGO with hyperglucagonemia in an animal model of type 2 diabetes. Thus, these results provide further support for the concept of glucagon antagonism for the treatment of type 2 diabetes.

The increased glycogen storage found after a glucose challenge in overnight-fasted glucagon mAb–treated *ob/ob* mice might indicate that increased glycogen accumulation would also take place during chronic glucagon immunoneutralization, resulting in a gradual increase in hepatic glycogen content. However, afternoon liver glycogen levels in ad libitum–fed *ob/ob* mice treated with glucagon mAbs for 5 days were not increased compared with control mAb–treated mice (Table 2), indicating that a continuous accumulation of glycogen does not take place when glucagon action is inhibited. Notably, hepatic glycogen content was increased in *ob/ob* mice compared with their lean littermates, irrespective of antibody treatment (Table 2). Furthermore, afternoon hepatic glycogen levels in ad libitum–fed *ob/ob* mice were much higher than the levels found in overnight-fasted *ob/ob* mice (Fig. 2). This suggests that in the ad libitum–fed *ob/ob* mice, hepatic glycogen content is near maximal and is therefore not likely to increase much further. The *ob/ob* mice are known to be hyperphagic, and therefore a situation of prolonged fasting is not likely to occur in these mice during conditions of ad libitum feeding. Our results indicate that increased glycogen accumulation caused by glucagon mAb treatment will occur only when hepatic glycogen stores are reduced as they are after an overnight fast.

Because glucagon is known to stimulate gluconeogenesis, elimination of glucagon signaling may lead to a reduction of glycogen synthesis via the indirect pathway (from glucose synthesized via gluconeogenesis). Glycogen synthesis via the indirect pathway has been shown to contribute ~40% to total glycogen synthesis after meal ingestion (19). Elimination of glucagon signaling could therefore reduce net hepatic glycogen synthesis via suppression of the indirect pathway of glycogen synthesis, thus explaining the lack of glycogen accumulation in *ob/ob* mice undergoing daily glucagon mAb treatment.

The current study demonstrates that the blood glucose–lowering effect of glucagon immunoneutralization in *ob/ob*

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TABLE 2
Effects of 5 days' immunoneutralization of endogenous glucagon in male *ob/ob* mice

	Insulin (pmol/l)	Lactate (mmol/l)	FFAs (mmol/l)	Triglycerides (mmol/l)	Liver glycogen (μmol glucosyl units/g liver)
Control mAbs	8,157 ± 1,290	4.6 ± 0.2	0.54 ± 0.05	2.58 ± 0.28	259 ± 22
Glucagon mAbs	6,375 ± 1,144	4.8 ± 0.2	0.53 ± 0.05	1.36 ± 0.10*	224 ± 21
Lean untreated	39.7 ± 7.4†	3.5 ± 0.2*	0.50 ± 0.03	0.93 ± 0.03†	192 ± 16‡

Data are means ± SE, $n = 10$ mice/group. *$P < 0.01$, †$P < 0.0001$, ‡$P < 0.05$ vs. control mAbs.

TABLE 3
Effects of 14 days' immunoneutralization of endogenous glucagon in male *ob/ob* mice

Group	Water intake ($g \cdot mouse^{-1} \cdot 24\ h^{-1}$)*	Food intake ($g \cdot mouse^{-1} \cdot 24\ h^{-1}$)*	ΔBody weight (g)	Pre-A1C (%)	Post-A1C (%)	ΔA1C (%)
Control mAbs	11.3 ± 1.4	12.3 ± 1.6	−0.6 ± 0.5	6.8 ± 0.3	8.1 ± 0.4	1.3 ± 0.2
Glucagon mAbs	7.5 ± 0.3†	13.8 ± 1.0	2.7 ± 0.2‡	6.8 ± 0.2	7.1 ± 0.2†	0.3 ± 0.1†

Data are means ± SE, $n = 10$ mice/group. *Water and food intake were measured over 24 h after 10 days treatment with the antibodies; †$P < 0.05$, ‡$P < 0.001$ vs. control mAbs (Student's t test).

mice is not a transient phenomenon because subchronic inhibition of circulating glucagon resulted in a maintained reduction of postprandial blood glucose levels. Although 14 days of selective glucagon inhibition did not lead to an absolute lowering of A1C levels, the gradual worsening of the diabetic syndrome that was observed in untreated *ob/ob* mice (as indicated by increasing A1C levels) was ameliorated in glucagon mAb–treated mice. This finding suggests that selective inhibition of glucagon of longer duration may improve glycemic control in type 2 diabetes. However, because A1C levels in mice are believed to represent the average blood glucose levels over at least the past 4 weeks, the treatment period in future studies should be extended to thoroughly evaluate the effects of glucagon immunoneutralization on A1C levels.

Plasma triglyceride concentrations were reduced by ~45% in *ob/ob* mice after 5 days of glucagon mAb treatment, whereas plasma FFA levels were unchanged. These findings are in agreement with recent reports of reduced levels of plasma glucose and triglycerides in *ob/ob* and *db/db* mice treated with glucagon receptor antisense oligonucleotides for 3–4 weeks (20,21). The presence of glucagon receptor mRNA has been documented in mouse adipocytes (22), but expression levels are low, and the importance of glucagon signaling in mouse adipose tissue is not fully understood. Our results suggest that subchronic glucagon immunoneutralization does not affect lipolysis in adipose tissues (indicated by the unaltered FFA levels). The triglyceride-lowering effects observed with subchronic glucagon inhibition in diabetic animals might be an indirect effect of the absence of glucagon signaling because prolonged lowering of plasma glucose could cause a shift in metabolism toward fatty acid oxidation, thus increasing the utilization of triglycerides from plasma.

Inhibition of glucagon signaling may affect insulin secretion as an adaptation to reduced glucose levels, or by inhibition of paracrine stimulation of the β-cell. The finding of similar insulin levels in glucagon mAb– and control mAb–treated mice during a glucose challenge suggests that acute removal of glucagon signaling mainly affects hepatic glucose metabolism, not insulin secretion. In addition, 5 days of selective glucagon inhibition in *ob/ob* mice did not lead to changes in insulin levels. A tendency toward reduced levels of plasma insulin was observed, but it did not reach statistical significance. The finding of unaltered insulin levels is in agreement with results obtained with 3–4 weeks of glucagon receptor antisense oligonucleotides treatment in *ob/ob* and *db/db* mice (21,22). It is possible that the observed tendency toward a reduction in insulin secretion is a consequence of the lowered blood glucose and not of glucagon inhibition per se. Because *ob/ob* mice are hyperinsulinemic, with plasma insulin levels 160- to 200-fold elevated compared with lean littermates (Table 2), a decline in insulin secretion in this setting should not be interpreted as decreased β-cell function, but rather as an amelioration of the diabetic state of the animals.

After 14 days of antibody treatment, increased weight gain was found in glucagon mAb–treated mice compared with control mAb–treated animals (Table 3). Because there was no difference in food intake between the two groups, the weight gain in the glucagon mAb–treated mice may be explained by the improvement of glycemic control, preserving water and energy by reduction of glycosuria.

In summary, this study demonstrates that selective inhibition of circulating glucagon by means of immunoneutralization improves glycemic control in diabetic *ob/ob* mice and reduces hepatic glucose production, plasma triglycerides, and A1C levels. These findings point toward a prominent role for glucagon in the pathophysiology of type 2 diabetes and further confirm the concept of glucagon antagonism as a treatment in type 2 diabetes.

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