GLP-1 (9–36) Amide, Cleavage Product of GLP-1 (7–36) Amide, Is a Glucoregulatory Peptide

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Objective: Glucagon-like peptide-1 (GLP-1) (7–36) amide is a glucoregulatory hormone with insulinotropic and insulinomimetic actions. We determined whether the insulinomimetic effects of GLP-1 are mediated through its principal metabolite, GLP-1 (9–36) amide (GLP-1m).
Methods and Procedures: Glucose turnover during two, 2-h, euglycemic clamps was measured in 12 lean and 12 obese (BMI <25 or >30 kg/m^2) male and female subject volunteers with normal oral glucose tolerance test. Saline or GLP-1m were infused from 0 to 60 min in each study. Additionally, seven lean and six obese subjects underwent a third clamp in which the GLP-1 receptor (GLP-1R) antagonist, exendin (9–39) amide was infused from –60 to 60 min with GLP-1m from 0 to 60 min.
Results: No glucose infusion was required in lean subjects to sustain euglycemia (glucose clamp) during saline or GLP-1m infusions. However, in obese subjects glucose infusion was necessary during GLP-1m infusion alone in order to compensate for a marked (>50%) inhibition of hepatic glucose production (HGP). Plasma insulin levels remained constant in lean subjects but rose significantly in obese subjects after termination of the peptide infusions. During GLP-1R blockade, infusion of glucose was immediately required upon starting GLP-1m infusions in all subjects due to a more dramatic reduction in HGP, as well as a delpayed and modest insulinotropic response.
Discussion: We conclude that GLP-1m potently inhibits HGP and is a weak insulinotropic agent. These properties are particularly apparent and pronounced in obese but only become apparent in lean subjects during GLP-1 (7–36) receptor blockade. These previously unrecognized antidiabetogenic actions of GLP-1m may have therapeutic usefulness.

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INTRODUCTION

Glucagon-like peptide-1 (7–36) (GLP-1) amide, an incretin hormone produced in enteroendocrine cells of the gut, has potent insulinotropic activities (1). In addition to modulating insulin secretion (2), GLP-1 increases insulin biosynthesis and gene expression (3,4). It also increases the expression of glucose transporter 2 (GLUT2) and glucokinase (3). In rodents, GLP-1 regulates β-cell mass by decreasing apoptosis (5) and increasing the proliferation rate of β-cells (6,7). Other actions of GLP-1 are the inhibition of glucagon secretion (8), the inhibition of gastrointestinal secretion and motility (9), and reduction in appetite (10) (for recent reviews of the effects of GLP-1, see references (11–14)). A salient feature of GLP-1 is that its insulinotropic action is glucose-dependent, and unlike the incretin hormone gastric inhibitory polypeptide, is preserved in the diabetic state (15,16). Therefore, GLP-1 is an attractive agent for treating type 2 diabetes. Circulating GLP-1 is inactivated within 1–2 min by the enzyme dipeptidyl peptidase-4 (DPP-4) (17,18) that cleaves the first two amino acids at the N-terminus, resulting in the metabolite GLP-1 (9–36) amide (GLP-1m). GLP-1m is known not to be insulinotropic and has thus been considered to be biologically inactive (19).

We demonstrated earlier that total peripheral glucose utilization during the infusion of GLP-1 (7–36) amide could not be fully explained by its insulinotropic actions alone (20). We attributed the increased glucose utilization to one or more insulinomimetic actions of the peptide, distinct from its insulinotropic effects. It has been shown that infusion of GLP-1 (7–36)

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amide results in high levels of GLP-1m due to its cleavage by DPP-4 in plasma (18). Indeed, we showed that when steady-state levels were achieved during infusion of full-length peptide, ≈80% of the circulating plasma levels of peptide were in the form of GLP-1m (20). We hypothesized that the insulinomimetic action of GLP-1 may be due to GLP-1m formation. Therefore, we undertook studies (61 glucose clamp studies) in human subjects with the aim of elucidating the effects of GLP-1m.

METHODS AND PROCEDURES

Selection of subject volunteers

Twelve lean (BMI 20.6–24.9 kg/m^2; %body fat 9.6–30.3, age 19–41 years) and twelve obese (BMI 30.7–47.1 kg/m^2; %body fat 31.8–51.4, age 26–50 years) volunteers were enrolled in the study. There were six men and six women in each group; all were white except for one African-American man in each group. The volunteers' clinical characteristics are presented in **Table 1**. Normal serum electrolytes, liver and renal function, a fasting plasma glucose <6.1 mmol/l (110 mg/dl), oral 75-glucose tolerance test, and hematocrit of ≥38 in men and ≥36 in women were requirements for entry. In addition to BMI in each volunteer, percent body fat was determined using dual-energy X-ray absorptiometry (model Prodigy; General Electric, Madison, WI) on the day of the first study. Volunteers' exclusion criteria included any active hepatic, renal, pulmonary, cardiovascular, gastrointestinal, or endocrine disease, any evidence of autonomic insufficiency or ingestion of any medication that might influence carbohydrate metabolism. All methods and procedures were approved by the Massachusetts General Hospital Institutional Review Board. We obtained an investigator initiated new drug application from the Food and Drug Administration for GLP-1m and the GLP-1 receptor (GLP-1R) antagonist, exendin (9–39) amide. All volunteers provided written informed consent in accordance with the Helsinki II Declaration.

Euglycemic clamps

All volunteers were weight and activity stable and consumed ≥200 g of carbohydrates for at least 3 days before the studies. All volunteers had two euglycemic clamps performed: one during saline only infusion and one during GLP-1m infusion. The two studies were carried out no less than 4 weeks apart. Additionally, seven of the lean and six of the obese volunteers had a third euglycemic clamp performed within 12 months in which both GLP-1m and exendin (9–39) were infused. Thus, a total of 61 clamps were performed.

Two hours before the start of each clamp (−120 min), a priming dose of sterile and pyrogen-free [3-^{3}H] glucose (8.7 kBq/kg, 0.236 nCi/kg) was administered, followed by a continuous infusion of [3-^{3}H] glucose (87 Bq/kg/min, 2.36 μCi/kg/min) for the duration of the experiment. Steady-state glucose specific activity was achieved within −30 min. To confirm this and to assess fasting glucose and hormone/substrate levels, four arterialized blood samples were obtained at 10-min intervals starting at −30 min. With the start of the GLP-1m clamp, at time 0, plasma GLP-1m levels were rapidly raised and then maintained stable with a falling primed (0–10 min, with a change at 2-min intervals) followed by a constant (10–60 min, 1.5 pmol/kg/min, 5 ng/kg/min) infusion rate, as described previously, for the infusion of GLP-1 (7–36) amide (21). With the start of the clamp during saline infusion, at time 0, saline was administered by a falling primed constant infusion as described for the GLP-1m infusion.

After all volunteers had undergone both the saline and the GLP-1m studies, we repeated the GLP-1m study in seven of the lean and six of the obese volunteers during blockade of the GLP-1R with exendin (9–39) within 12 months. These subsets were selected because they had not altered their body weight in the preceding year and were willing to participate. Exendin (9–39) was infused at a constant dose of 300 pmol/kg/min (101 ng/kg/min) from −60 to 60 min. This dose has been demonstrated to suppress the insulinotropic actions of GLP-1 (7–36) amide completely (22). GLP-1m and exendin (9–39) were synthesized by the Protein/Peptide Core Facility at Massachusetts General Hospital. The peptides were >99% pure, displayed a single peak on high performance liquid chromatography (HPLC), and the peptide contents were 82 and 85%, respectively. The peptides were subsequently lyophilized in vials under sterile conditions for single use and certified to be both pyrogen free and sterile.

During the combined GLP-1m and exendin (9–39) study, a blood sample was obtained just before the start of the exendin (9–39) infusion at −60 min and thereafter at 10-min intervals until the start of GLP-1m infusion at time 0. Additionally, two muscle biopsies were obtained from the vastus lateralis from each volunteer ≈10 min before the start of the exendin (9–39) infusion and ≈10 min before the end of the exendin (9–39)/GLP-1m infusions, as described previously (23). The samples were immediately stored in liquid nitrogen for analysis of specific proteins, the samples were placed in buffer containing 20 mmol/l 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid, pH 7.2, 1 mmol/l EDTA, 250 mmol/l sucrose and Complete protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN), homogenized with a Brinkman polytron for 45 s at setting six and membranes prepared for western blot analysis (23).

We employed the euglycemic clamp technique (24) to maintain the prevailing plasma glucose levels within 5 mg/dl of the fasting levels of each subject with an infusion of 20% glucose solution (Travenol, Deerfield, IL) whenever necessary. The glucose solution was spiked with [3-^{3}H] glucose to maintain a stable specific activity (hot Ginf) (25). At the start of GLP-1m or saline infusions, blood samples were obtained every 5 min for 120 min for the determination of plasma glucose and every 10 min for the determination of hormones, nonesterified fatty acids (NEFAs), and plasma glucose specific activity.

Table 1 Subject characteristics

Lean				Obese			
Age (y)	BMI (kg/m^2)	Body fat (%)	HbA_{1c} (%)	Age (y)	BMI (kg/m^2)	Body fat (%)	HbA_{1c} (%)
28[a]	21.39	18.3	5.2	50	34.71	41.3	5.2
19[a]	22.59	13.4	5.3	47[a]	30.73	36.5	5.4
27	24.92	30.3	5.9	43[a]	34.91	31.8	4.9
23	22.24	21.1	5.4	44	34.27	32.1	4.7
24[a]	24.03	9.6	5.4	39	37.22	32.0	5.1
24[a]	21.27	17.0	5.3	37	35.57	34.4	4.4
30	20.85	24.0	5.1	49[a]	47.07	43.6	5.3
33	20.62	29.9	5.1	43	41.60	51.4	5.6
28[a]	21.18	25.9	5.0	38[a]	33.90	47.8	5.3
41[a]	23.34	21.7	5.0	38[a]	45.91	49.4	5.4
27[a]	22.33	27.1	4.9	50	32.00	43.0	5.4
39	23.17	28.4	5.3	26[a]	38.40	49.8	5.9
29[b]	22.33	22.23	5.4	42	37.20	41.1	5.2
1.86[c]	0.39	1.92	0.2	2	1.50	2.2	0.4

The first six volunteers in each group are men.
HbA_{1c}, glycosylated hemoglobin.
[a]Volunteers in exendin (9–39) clamp; [b]Mean; [c]s.e.

Analytical techniques

Blood samples were collected using heparinized syringes. Plasma glucose was immediately analyzed using the glucose oxidize method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA). The remaining blood samples were placed in prechilled test tubes containing peptidase inhibitors as described previously (21). Plasma insulin, C-peptide, glucagon, total GLP-1 (both GLP-1 and GLP-1m), leptin, adiponectin, resistin, NEFA, and specific activity of glucose were

analyzed using methodologies described previously (16). The GLP assay uses an antibody to the C-terminus. Therefore, both endogenous and GLP-1m are equally recognized. Glut-4, total Akt-1, and phospho-AKT-1 protein levels were determined from the muscle membranes using western blot analysis (23). The antibodies used were goat anti-human GLUT4 antibody (1:10,000) from Santa Cruz Biotechnology (Santa Cruz, CA), mouse antihuman AKT-1 antibody (1:2,000) from BD Transduction Laboratories (San Diego, CA) and rabbit antihuman phosphoAKT-1(Ser 473) (1:3,000) from Cell Signaling Technology (Beverly, MA).

The rates of total appearance (R_a) and disappearance (R_d) of glucose were calculated according to the nonsteady-state equations of Steele (26), as modified for the use of hot Ginf. The volume of distribution of glucose was assumed to be 210 ml/kg (27). During the basal period, glucose R_a is equal to hepatic glucose production (HGP) because the liver is the principal source of glucose. When glucose was infused (i.e., during the clamp), endogenous glucose production was estimated as the difference between the calculated total glucose R_a and the exogenous glucose infusion rate for the appropriate time interval. Glucose turnover rates were calculated at 10-min intervals from −60 or from −30 to 120 min. The trapezoidal rule was used to calculate the integrated responses over 30-min intervals. The integrated responses were divided by the time interval, which resulted in mean concentration or rates. All data were analyzed using Statistical Analysis System version 9.1 (Cary, NC). Standard methods were used to compute means, s.e.m., and Pearson correlation coefficients. Mixed-model analysis for repeated-measures design was used to analyze hormone and metabolite responses. When a significant effect was observed, changes between clamps in the same group were evaluated using a paired t-test and differences between groups were evaluated using unpaired t-test, using the Bonferroni adjustment. All statistical tests were two tailed. Except where otherwise stated, data are means ± s.e., and $P < 0.05$ was regarded as statistically significant.

RESULTS

We first analyzed our data to ascertain the requirement of glucose infusion to maintain stable fasting levels during the euglycemic clamps under the various experimental paradigms. In none of the lean subjects either during the saline or the GLP-1m infusions, glucose was required or plasma insulin levels were altered, but there was a gradual decline in R_a. There was a significant drug-time interaction in R_a between the two studies; however, the decline in R_a during saline or GLP-1m infusions was not sufficient to affect plasma glucose levels significantly, and consequently no glucose infusion was required (**Figure 1**). In the obese subjects, during the saline infusions, glucose was required in a single subject and then only for the last 15 min of the study (105–120 min, 4.2 μmol/kg/min) (**Figure 1**). In sharp contrast to the lean group, however, glucose infusion was necessary in each and every obese volunteer between 10 and 50 min after the start of the GLP-1m infusion, and the requirement continued for 40–60 min following termination of the infusion (**Figure 1**). Plasma glucose levels following termination of GLP-1m were slightly higher for the whole of the follow-up period of 60 min compared to the same period following saline infusion and became significantly higher in the obese group, from 90 to 120 min. Plasma insulin levels increased in this group toward the end of the infusion, but had returned to fasting levels by 30 min after discontinuation of the infusion; therefore, the brief insulin elevation cannot be due to the sustained glucose elevation as insulin levels were actually falling as glucose levels were significantly elevated compared

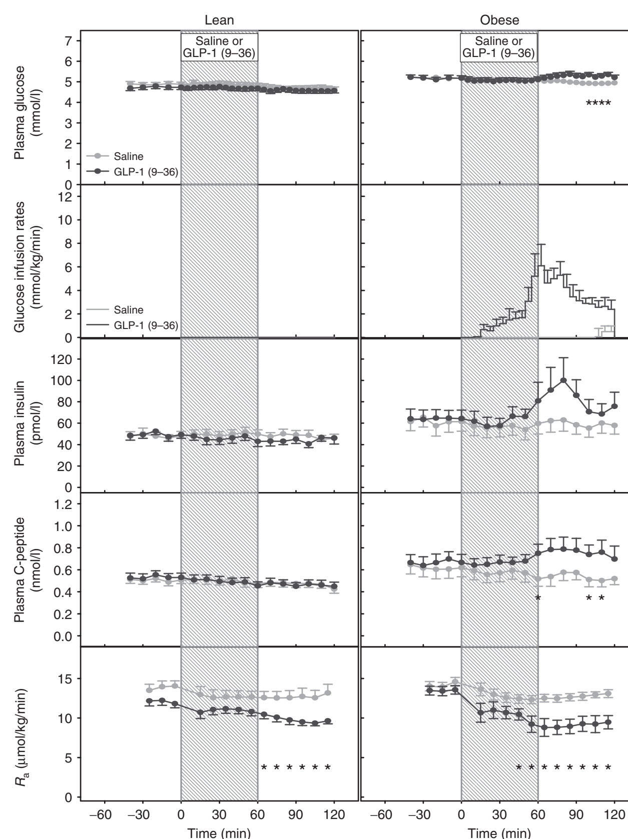

Figure 1 Plasma glucose levels (top panel), glucose infusion rates (second panel), plasma insulin levels (third panel), C-peptide levels (fourth panel), and rates of appearance of glucose (R_a, bottom panel) in 12 lean (left side) and 12 obese (right side) volunteers who received GLP-1 (9–36) amide or saline from 0 to 60 min (mean ± s.e.). *Significant difference between the two studies at indicated times. GLP-1, glucagon-like peptide-1.

to the saline study. C-peptide levels also began to increase after termination of the GLP-1m (**Figure 1**). There was a significant drug-time interaction in C-peptide levels between the saline and the GLP-1m studies in the obese group (**Table 2**, $P < 0.05$). Glucagon, ghrelin, cytokine (leptin, adiponectin, resistin), and NEFA plasma levels were not altered in either group while saline or GLP-1m were being infused (0–60 min) (**Table 2**). GLP-1 (reflecting endogenous production and exogenous GLP-1m administration) plasma levels were ≈145–182 pmol/l during the GLP-1m infusion (**Table 2**, **Figure 3**). These levels are ≈1.5-fold those seen after an oral glucose tolerance test. The areas under the curves for GLP-1m in the lean and the obese groups were not significantly different ($P = 0.408$).

Basal R_a levels were similar in lean and obese subjects (13.8 ± 0.69 vs. 12.8 ± 0.57 (lean, not significantly different) and 14.2 ± 0.54 vs. 13.5 ± 0.56 (obese, not significantly different) μmol/kg/min) before saline and GLP-1m infusions, respectively (**Figure 1**). R_a declined in both lean and obese subjects during both saline and GLP-1m infusions but the decline in R_a during GLP-1m infusion was significantly different from the decline during saline infusion (90–120 min, R_a = 12.8 ± 1.0 vs. 9.5 ± 0.3 (lean) and 12.9 ± 0.4 vs. 9.3 ± 0.9 (obese) μmol/kg/min,

Table 2 Hormone levels in normal (n = 12) and obese (n = 12) groups during the saline or GLP-1m clamps (mean ± s.e.)

	Glucose (mmol/l)	Insulin (pmol/l)	C-pep (nmol/l)	Glucagon (pmol/l)	GLP-1 (pmol/l)	Leptin (ng/ml)	Adiponectin (pg/ml)	Resistin (ng/ml)	Ghrelin (pmol/l)	NEFA (mmol/l)
Lean										
Saline										
Fasting	4.9 ± 0.1	48 ± 4.0	0.5 ± 0.03	19.7 + 1.8	12.2 ± 1.4	5.5 ± 1.1	13.1 ± 2.2	5.4 ± 0.7	0.3 ± 0.04	0.51 ± 0.06
0-60	4.8 ± 0.1	50 ± 3.0	0.5 ± 0.04	19.6 ± 1.7	12.8 ± 1.3	5.3 ± 1.2	13.5 ± 2.5	5.7 ± 0.8	0.3 ± 0.03	0.51 ± 0.05
60–120	4.7 ± 0.1	46 ± 3.2	0.5 ± 0.04	19.6 ± 1.7	13.1 ± 1.3	4.9 ± 1.0	12.1 ± 2.0	5.3 ± 0.7	0.3 ± 0.04	0.51 ± 0.04
GLP-1m										
Fasting	4.7 ± 0.1	49 ± 3.5	0.5 ± 0.04	19.7 ± 1.0	10.1 ± 0.9	5.0 ± 1.0	11.8 ± 1.6	5.0 ± 0.8	0.3 ± 0.04	0.50 ± 0.07
0–60	4.7 ± 0.1	46 ± 4.1	0.5 ± 0.04	19.7 ± 1.0	145.5 ± 13.0**	4.5 ± 0.9	12.8 ± 2.3	4.9 ± 0.6	0.3 ± 0.04	0.46 ± 0.07
60–120	4.6 ± 0.1	44 ± 3.2	0.5 ± 0.04	19.2 ± 1.0	28.6 ± 2.7**	4.4 ± 1.0	13.2 ± 2.5	5.0 ± 0.6	0.3 ± 0.03	0.48 ± 0.06
Obese										
Saline										
Fasting	5.2 ± 0.1	62 ± 9.0	0.6 ± 0.08	20.2 ± 0.1	4.1 ± 0.7*	20.1 ± 3.5*	11.4 ± 1.7	6.2 ± 0.7	0.3 ± 0.04	0.64 ± 0.07
0–60	5.1 ± 0.1	57 ± 9.2	0.6 ± 0.08	19.2 ± 1.2	4.3 ± 0.7*	20.4 ± 4.0*	11.0 ± 1.7	6.3 ± 0.7	0.3 ± 0.03	0.66 ± 0.04*
60–120	5.0 ± 0.1	60 ± 8.7	0.5 ± 0.06	20.0 ± 1.4	4.1 ± 0.7*	20.4 ± 4.1*	10.8 ± 6.0	6.0 ± 0.6	0.3 ± 0.03	0.70 ± 0.05*
GLP-1m										
Fasting	5.2 ± 0.1	64 ± 8.0	0.7 ± 0.07	20.9 ± 1.3	5.0 ± 0.7*	17.7 ± 3.9*	12.2 ± 2.3	6.5 ± 0.9	0.3 ± 0.04	0.70 ± 0.07*
0–60	5.1 ± 0.1	64 ± 7.9**	0.7 ± 0.06*	21.4 ± 1.6	160.7 ± 12.5**	19.1 ± 4.2*	12.8 ± 2.8	6.5 ± 1.0	0.3 ± 0.04	0.68 ± 0.07*
60–120	5.3 ± 0.1	82 ± 14.3*,**	0.8 ± 0.10*,**	20.7 ± 1.7	33.7 ± 2.4**	19.4 ± 4.5*	12.1 ± 2.3	6.7 ± 1.0	0.3 ± 0.03	0.63 ± 0.06

GLP-1, glucagon-like peptide-1; NEFA, nonesterified fatty acid.
*P < 0.05 from corresponding time period of the lean group (unpaired t-test). **P < 0.05 from corresponding time period of the saline study (paired t-test).

saline vs. GLP-1m, respectively) (**Figure 1**). Additionally, the difference in the decline of R_a from basal was also significantly different between the two studies in both lean and obese subjects (**Figure 1**). The greater fall in R_a of the lean group during GLP-1m infusion occurred without any significant changes in plasma insulin levels. It also should be noted that the fall in R_a of the obese subjects precedes the increase in insulin and C-peptide by at least 30 min (**Figure 1**), indicating that the fall is not a consequence of any changes in plasma insulin levels, and the fall in R_a in the lean subjects occurred even though there was no change in insulin and C-peptide levels.

To ascertain whether the observed decline in R_a during GLP-1m infusion was mediated through the known GLP-1R, we repeated the GLP-1m infusion in half of the lean and obese volunteers during GLP-1R blockade, accomplished by the administration of exendin (9–39) (22). For this study, we infused exendin (9–39) for 60 min before beginning, and continued during, GLP-1m infusion for another 60 min (**Figure 2**). In both lean and obese subjects, plasma glucose during the entire clamps were similar, and of the 48 paired t-test comparisons, only one point was statistically different between GLP-1m and GLP-1m/exendin (9–39), in the lean group. In the lean volunteers, there was a nonsignificant decline in insulin levels during the first 60 min of the exendin (9–39) infusion compared to during the GLP-1m infusion but on addition of GLP-1m to exendin (9–39), insulin levels began to increase. The time course of C-peptide levels was very similar to that of insulin (**Figure 2**).

Glucose infusion was immediately necessary during combined exendin (9–39) and GLP-1m infusions in order to maintain euglycemia (**Figure 2**) and the need for the glucose infusion lasted until the end of the study. In the obese volunteers, insulin levels began to increase within 10 min of initiation of GLP-1m superimposed on the on-going exendin (9–39), and by 30-min insulin levels were statistically different from the insulin levels attained during GLP-1m alone (**Figure 2**). C-peptide levels also began to increase ≈30 min after the initiation of the GLP-1m. The pattern of C-peptide response between GLP-1m alone and exendin (9–39)/GLP-1m was similar (**Table 3**). In both studies, C-peptide levels remained elevated for the 60-min recovery period. Glucose infusion was also necessary immediately after beginning GLP-1m in addition to exendin (9–39) (**Figure 2**); lasting until the end of the study. Basal Ras were similar with GLP-1m alone as with GLP-1m/exendin (9–39) in both lean and obese subjects, that is exendin (9–39) alone did not have an effect on R_a. However, R_a began to fall rapidly in both groups with infusion of GLP-1m/exendin (9–39). Consequently, there was a significant drug-time interaction between GLP-1m vs. GLP-1m/exendin (9–39) (P < 0.05) for both groups. In the lean group, the fall in R_a was significantly different during the GLP-1m/exendin(9–39) compared to the GLP-1m studies from 25 to 95 min and in the obese group from the 25- to 55-min period (P < 0.05). The R_a fell by at least 50% in both groups from basal levels (P < 0.05): endogenous GLP-1 did not influence R_a during fasting, when the known GLP-1R was blocked by the exendin(9–39) antagonist.

We also compared the fall of R_a during the three studies to the basal fasting rate. In the lean group, during the saline infusion none of the four consecutive 30-min internal Ras

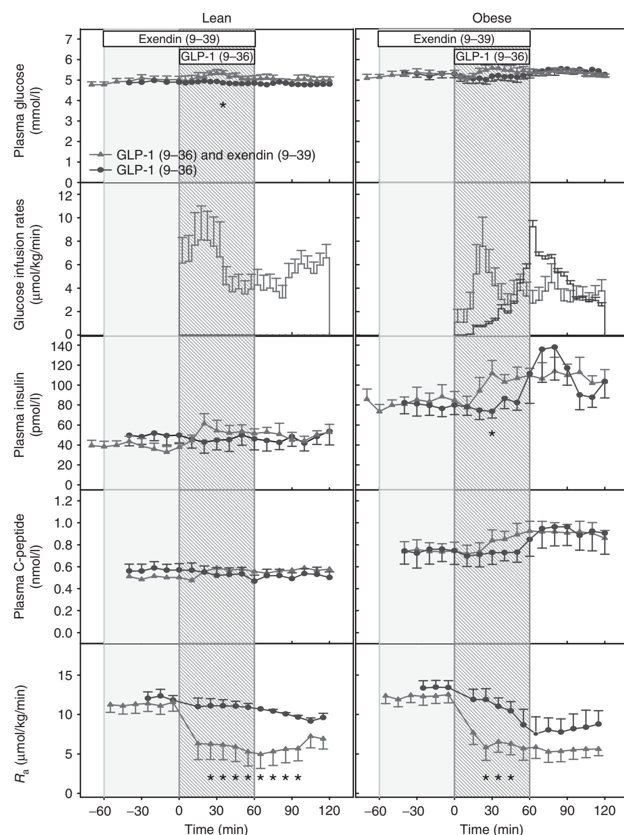

Figure 2 Plasma glucose levels (top panel), glucose infusion rates (second panel), plasma insulin levels (third panel), C-peptide levels (fourth panel), and rates of appearance of glucose (bottom panel) in seven lean (left side) and six obese (right side) volunteers who received GLP-1 (9–36) amide from 0 to 60 min. Exendin (9–39) amide was infused from −60 to 60 min (mean ± s.e.). *Significant difference between the two studies at indicated times. GLP-1, glucagon-like peptide-1.

(e.g., 0–30, 30–60, etc.) were significantly different from the basal R_a. However, during GLP-1m infusion Ras for the 60–90 and 90–120 min intervals were significantly suppressed compared to the basal R_a ($P < 0.05$). In the obese group, during the saline infusion, the Ras for the 30–60 and the 60–90 min were also significantly suppressed compared to the basal R_a ($P < 0.05$). During GLP-1m infusion, the Ras for 30–60, 60–90, and 90–120 min were suppressed compared to basal ($P < 0.05$) and the suppression was greater than the corresponding intervals for the saline infusion ($P < 0.05$). In the lean group, during GLP-1m infusion during GLP-1R blockade, the Ras for the last three consecutive 30-min intervals were significantly suppressed compared to basal ($P < 0.05$) and in the obese group the Ras for all four consecutive 30-min intervals were significantly suppressed compared to basal R_a ($P < 0.05$). Furthermore, when GLP-1m was infused, the suppression during the 30-min intervals was significantly greater when the GLP-1R was blocked ($P < 0.05$). Finally, we compared the changes in plasma insulin levels during the three studies to the basal fasting insulin levels in each group. None of the consecutive 30-min interval insulin levels in any of the studies were significantly different from the basal insulin levels.

Except for the rise in GLP levels from 0 to 60 min, all other hormones and cytokine levels were stable in both lean and obese groups (**Table 3**). However, as expected, lean and obese subjects showed differences in insulin and leptin values. Exendin (9–39) did not change the GLP-1m clearance rate as the plasma GLP-1 levels during GLP-1m infusion alone and GLP-1m/exendin (9–39) infusions were similar in both studies (**Figure 3**). Basal R_d in the lean and obese groups during the saline infusions were 13.7 ± 0.55 and 14.5 ± 0.65 µmol/kg/min, respectively and was not altered significantly during any studies. Akt-1 total protein levels, and phosphoAkt-1 (Ser 473) levels, measured from the muscle biopsies before starting exendin (9–39) and during the last 10 min of GLP-1m/exendin (9–39) did not change in either group (data not shown). GLUT4 protein levels were significantly higher in the obese group compared to the lean group ($P < 0.05$) in the basal state. By the last 10 min of the GLP-1m/exendin (9–39) infusion, GLUT4 levels were lower in both groups compared to basal levels; however, the decrease was significant only in the lean group (pre, 583 ± 106: post, 368 ± 61 optical density units/1.5 µg homogenized protein, $P < 0.05$).

DISCUSSION

In an earlier study, we infused GLP-1 (7–36) amide in fasted obese, insulin-resistant subjects and observed an increase in peripheral glucose uptake that was not attributable solely to increased endogenous insulin secretion (20). Notably, in this study of the effects of the insulinotropic GLP-1(7–36) amide, no inhibition of HGP (R_a) was observed beyond that attributable to the increase in plasma insulin. In other studies, the administration of GLP-1 (7–36) amide to fasted dogs (28) or pigs (29) resulted in increased glucose utilization, independent of changes in insulin (28). Furthermore, the direct infusion of GLP-1 (9–36) amide into insulin-resistant dogs with dilated cardiomyopathy enhances myocardial glucose uptake without changes in plasma insulin levels (30). We hypothesized that these extrapancreatic effects of GLP-1 (7–36) amide shown by us in humans, and by others in dogs and pigs is due to GLP-1's primary metabolite, GLP-1m. This study allowed us to evaluate the effects of GLP-1m alone, and to block any effects mediated through the GLP-1R. Plasma levels of GLP-1m attained in this study were similar to those attained during GLP-1 (7–36) amide infusion in our previous study (20).

Here, we show that GLP-1m has insulinomimetic properties in humans that are particularly evident in the insulin-resistant state of obesity and in lean subjects when GLP-1R is blocked by exendin (9–39), a known GLP-1R antagonist (22). The effects of GLP-1m appear to be primarily due to the direct suppression of endogenous (hepatic) glucose production (R_a), and not as a consequence of increased insulin or decreased glucagon secretion. In the obese group, there was a modest but statistically significant increase in insulin and C-peptide which became evident after 30–40 min of GLP-1m infusion and remained evident for some time after its termination. This insulinotropic response resulted in a further suppression of R_a. To investigate the mechanism underlying suppression of R_a, we neutralized the effects of the known GLP-1R by blocking it with exendin

Table 3 Hormone levels in the normal (n = 7) and obese (n = 6) groups during the GLP-1m and exendin (9–39) clamps (mean ± s.e.)

	Glucose (mmol/l)	Insulin (pmol/l)	C-pep (nmol/l)	Glucagon (pmol/l)	GLP-1 (pmol/l)	Leptin (ng/ml)	Adiponectin (pg/ml)	Resistin (ng/ml)	Ghrelin (pmol/l)	NEFA (mmol/l)
Lean										
GLP-1m										
Fasting	4.89 ± 0.13	50 ± 6.0	0.6 ± 0.06	19.2 ± 1.6	10.4 ± 1.3	4.9 ± 1.7	9.7 ± 2.2	5.0 ± 1.0	0.3 ± 0.05	0.34 ± 0.07
0–60	4.89 ± 0.12	46 ± 7.0	0.5 ± 0.06	18.5 ± 1.4	155.6 ± 21.3	4.6 ± 1.4	9.7 ± 2.1	4.7 ± 0.8	0.3 ± 0.05	0.33 ± 0.07
60–120	4.83 ± 0.11	45 ± 6.2	0.5 ± 0.06	18.6 ± 1.5	31.7 ± 4.3	4.4 ± 1.6	9.9 ± 2.1	4.9 ± 0.7	0.3 ± 0.05	0.36 ± 0.06
GLP-1m and exendin (9–39)										
Fasting	5.00 ± 0.16	40 ± 2.2	0.5 ± 0.05**	21.4 ± 1.6	14.3 ± 1.9**	4.3 ± 1.2	9.8 ± 1.5	9.6 ± 2.0**	0.2 ± 0.02	0.50 ± 0.04
0–60	5.17 ± 0.16	51 ± 3.6	0.6 ± 0.06	20.4 ± 1.2	158.6 ± 10.4	4.5 ± 1.1	9.1 ± 1.4	8.7 ± 1.4	0.2 ± 0.02	0.43 ± 0.05
60–120	5.00 ± 0.17	49 ± 2.7	0.6 ± 0.06	19.5 ± 1.0	35.6 ± 2.2	3.9 ± 1.0	8.9 ± 1.4	9.0 ± 1.8**	0.2 ± 0.02	0.41 ± 0.06
Obese										
GLP-1m										
Fasting	5.30 ± 0.18	80 ± 9.4*	0.7 ± 0.10	21.9 ± 2.5	4.9 ± 1.0*	25.2 ± 5.3*	13.2 ± 3.3	4.9 ± 0.8	0.3 ± 0.06	0.69 ± 0.08*
0–60	5.17 ± 0.12	82 ± 8.2*	0.7 ± 0.10	22.2 ± 3.0	164.3 ± 23.0	27.2 ± 5.7*	14.2 ± 4.4	4.6 ± 0.7	0.3 ± 0.06	0.66 ± 0.08*
60–120	5.46 ± 0.16*	113 ± 19.1*	0.9 ± 0.20*	21.9 ± 3.3	36.3 ± 4.0	22.3 ± 5.9*	13.9 ± 3.8	4.7 ± 0.6	0.3 ± 0.03	0.58 ± 0.04*
GLP-1m and exendin (9–39)										
Fasting	5.31 ± 0.16	84 ± 9.1*	0.7 ± 0.08*	24.7 ± 2.9	3.2 ± 0.5*	23.9 ± 3.7*	12.0 ± 1.7	5.8 ± 1.1	0.2 ± 0.02	0.74 ± 0.07*
0–60	5.38 ± 0.18	98 ± 6.9*,**	0.8 ± 0.08*	23.9 ± 2.5	182.4 ± 7.6	23.8 ± 3.5*	13.0 ± 2.4	5.2 ± 0.8	0.2 ± 0.02	0.72 ± 0.07*
60–120	5.33 ± 0.13	108 ± 9.1*	0.9 ± 0.08*	22.9 ± 2.9	37.7 ± 4.9	24.6 ± 4.7*	10.7 ± 2.1	4.6 ± 0.8	0.2 ± 0.02	0.59 ± 0.08

GLP-1, glucagon-like peptide-1; NEFA, nonesterified fatty acid.
*P < 0.05 from corresponding time period of the lean group (unpaired t-test).
**P < 0.05 from corresponding time period of the GLP-1m study (paired t-test).

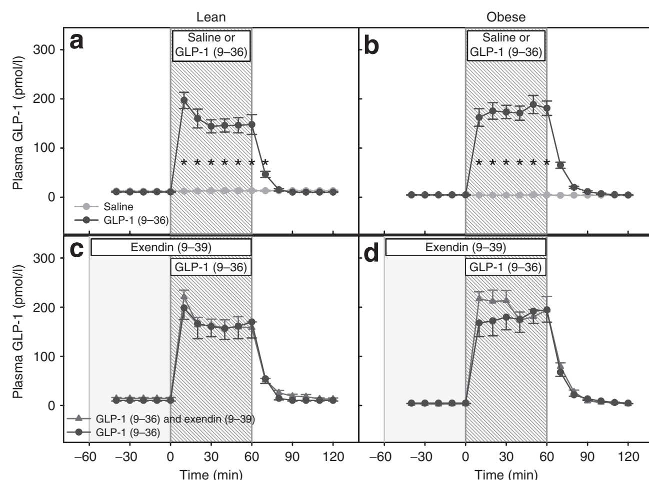

Figure 3 Plasma glucagon-like peptide-1 (GLP-1) levels during the GLP-1 (9–36) amide (GLP-1m) and saline infusions in (**a**) 12 lean and (**b**) 12 obese volunteers, and during exendin (9–39) amide and GLP-1m infusions in (**c**) 7 lean and (**d**) 6 obese volunteers (mean ± s.e.). *Significant difference between the two studies at indicated times.

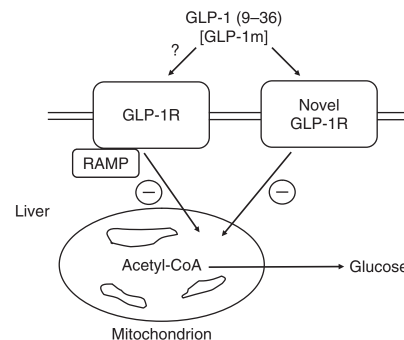

Figure 4 Hypothetical model of putative actions of GLP-1 (9–36) amide (GLP-1m) on the inhibition of glucose formation in the liver of obese human volunteers. Two possible receptor-mediated mechanisms are proposed. The known GLP-1 receptor (GLP-1R) expressed in steatotic livers of obese individuals recruits a specific receptor activity modifying protein (RAMP) that switches ligand specificity of the receptor from that of the insulinotropic hormone GLP-1 (7–36) to the insulinomimetic hormone, GLP-1 (9–36), GLP-1m. Alternatively, there may exist in the liver an as yet unidentified "novel" receptor that recognizes GLP-1m. Both putative receptor signaling pathways would result in the inhibition of glucose formation. GLP-1, glucagon-like peptide-1.

(9–39). We expected that GLP-1R blockade would not affect the response to GLP-1m alone, and would establish the presence of a distinct mechanism, separate from any interaction with the GLP-1R. To our surprise, we found that the effect of GLP-1m on R_a was actually augmented in both lean and obese subjects through GLP-1R blockade. This augmentation might be due, in part, to the slight rise in insulin secretion which occurred after the introduction of the GLP-1m during GLP-1R blockade. It is, therefore, likely that GLP-1m is indeed insulinotropic, but this is only evident when the more potent insulinotropic effect of GLP-1 (7–36) amide is negated by GLP-1R blockade (31). In lean subjects, on introduction of GLP-1m during receptor blockade, insulin levels increase and R_a decreased quickly

and more robustly that when GLP-1m alone was infused. In our study, we found no effect of GLP-1m on rates of glucose disappearance (R_d) in lean or obese subjects, with or without GLP-1R blockade, which suggests that GLP-1m has no effect on peripheral glucose uptake. The levels of adipokines, ghrelin, glucagon, and NEFA do not change and do not explain why the actions of GLP-1m predominate in the obese state.

Our findings are consistent with a prior study in pigs in which GLP-1m was found to reduce blood glucose, although R_a was not studied (29). In addition, a recent article showed that GLP-1m treatment is associated with a reduction in glycemia after a solid meal, although the mechanism was not determined (32). GLP-1m has been shown to antagonize human GLP-1R expressed in baby hamster kidney cells (33) and *in vivo* studies in pigs have demonstrated that GLP-1m antagonizes the inhibitory effects of GLP-1 (7–36) amide on antral motility (29). In mice, GLP-1m has no effect on glucose disposal or insulin secretion. This was demonstrated in transgenic mice that had a complete disruption of GLP-1R as well as in normal mice with suppression of insulin secretion with diazoxide (34). Although species differences probably exist, these data *in toto* suggest that GLP-1m may have a physiological role.

The mechanisms underlying the suppression of R_a by GLP-1m are unknown. The effect of GLP-1m on hepatic glucose metabolism may be due to a novel GLP-1m receptor (GLP-1mR) present on hepatocytes, on the hepatoportal sensor as suggested by Burcelin *et al*. (35), on β-cells, and/or on vagal afferent fibers (36). It is also possible that GLP-1m is interacting with pathways in the central nervous system leading to increased glucose uptake in the liver, as it is now understood that there is a neural component to insulin regulation of HGP, the so-called indirect effect (37,38). Because GLP-1 (7–36) amide has been shown to cross the blood–brain barrier (39) so GLP-1m is similarly likely to do so. Therefore, the effects of GLP-1m on the regulation of HGP may have both an indirect and direct component. That GLP-1m directly affects hepatocytes is the subject of a patent (40). The patent proposes that GLP-1m inhibits the formation of hyperglycemia-induced reactive oxygen species in isolated cultured hepatocytes. The insulinotropic GLP-1 (7–36) amide similarly inhibits hyperglycemia-induced reactive oxygen species formation in hepatocytes but its actions are completely blocked by DPP-4 and neutral endoprotease inhibitors indicating that GLP-1 (9–36) amide, and not GLP-1 (7–36) amide, is the active peptide responsible for the suppression of reactive oxygen species formation in hepatocytes (40). Therefore, there is a direct effect of GLP-1m on hepatocytes, distinct and separate from the hepatoportal sensor, vagus, and brain.

The effects of GLP-1m were previously examined in healthy, normal-weight humans in the presence of GLP-1 (7–36) amide (19). Those investigations found that the insulin secretory response to GLP-1 (7–36) amide was the same whether or not GLP-1m was coinfused. However, HGP was not assessed, and it is likely that the effects of GLP-1m on insulin secretion would not be appreciated due to the more potent insulinotropic properties of GLP-1 (7–36) amide. In contradiction to our results, Zander *et al*. examined the effect of GLP-1m generated from exogenous GLP-1 (7–36) amide by inhibiting its formation using DPP-4 inhibition (41). There was no significant insulin response evident and glucagon levels did not decrease even with a dose of GLP-1 as high as 9.6 pmol/kg/min. Additionally, although GLP-1 and DPP-4 inhibition resulted in lower blood glucose than GLP-1 alone, this does not necessarily mean that the glucose-lowering effects were from insulinotropic augmentation by GLP-1. It is also unclear whether the exogenously administered GLP-1 recapitulated the physiologic formulation of GLP-1 because plasma levels of intact (N-terminal) GLP-1 and its metabolite GLP-1m were not in the usual ratios observed during infusion studies quoted in the literature (20,32).

The cleavage of histadine and alanine from the N-terminus of GLP-1 (7–36) amide by DPP-4, which results in GLP-1m, has generally been regarded as the limiting factor in the therapeutic usefulness of GLP-1 because it necessitates continuous infusion of the peptide in order to have any sustained effect on blood glucose (42,43). To overcome this deficiency, two approaches are currently under investigation. GLP-1 analogs that are resistant to DPP-4 such as exenatide have been developed, and DPP-4 inhibitors such as sitagliptin and vildagliptin, are now either approved for human use or being tested in humans (44,45). DPP-4 inhibitors have been found in obese diabetic subjects, to decrease postprandial glucose levels, and increase postprandial GLP-1 levels, but not change gastric emptying, plasma insulin levels, glucose disposal, or HGP, when compared to placebo (46). This is surprising because one would expect that the increase in plasma GLP-1 levels, accompanied by hyperglycemia, should have resulted in decreased glucose production and increased glucose disposal. We hypothesize that the lack of effects of the DPP-4 inhibitors on HGP are because of prevention of formation of GLP-1m. Moreover, the small but very statistically significant fall in postprandial glucose levels may be due to effects on plasma levels of other gut hormones and neuropeptides similarly degraded by DPP-4.

We speculate that the GLP-1 (7–36) amide has a dual function. First, it binds to the known GLP-1R on the β-cell and stimulates insulin release. Increased insulin levels result in well-known metabolic effects, including reduction of NEFAs, suppression of R_a and decreases in glucagon levels. GLP-1 (7–36) amide is then cleaved by DPP-4, rendering GLP-1 (9–36) amide. This peptide then binds to a second receptor (GLP-1mR) and initiates a series of events that result in further reductions in R_a, independent of plasma glucose levels and the actions of insulin. In the postabsorptive state glucose oxidation is reduced and NEFA oxidation is activated, even in lean subjects. Because the effects of GLP-1m were indeed more pronounced in the obese state we can speculate that GLP-1m is acting in some manner to suppress fatty acid oxidation and thereby inhibiting production of glucose by the liver, through effects on the glucose–fatty acid oxidation cycle (47). It can be further hypothesized that the activity of the putative GLP-1mR can be upregulated in insulin-resistant states. In obesity, the putative receptor is upregulated to compensate for insulin resistance. During blockade of GLP-1-induced insulin release by exendin (9–39), this response is

augmented even further. As it has been previously shown that GLP-1 introduced into the brains of mice results in insulin resistance (48), the effects and location of the putative GLP-1mR may be either central, splanchnic, or both. Therefore, exendin 9–39 is a facilitator or sensitizer of GLP-1m effects, both on insulin secretion from the β-cell and reduction of R_a in the liver. Finally, it is possible that there may be several GLP-1R subtypes and that the binding affinity to a putative GLP-1mR subtype is different in the obese state. An analogy for different actions of GLP-1 and GLP-1m with respect to action and affinity to receptor subtype is the well described physiological functions of peptide YY(1–36) (PYY (1–36)) and the second biologically active form of PYY, PYY(3–36), which is formed by the removal of amino-terminal dipeptide Tyr-Pro by DPP-4 (49). PYY (3–36) has different biological action than intact PPY (1–36), mediated by different receptors (50).

We therefore propose a working hypothesis for the effects of GLP-1m (**Figure 4**). The liver is the major site of glucose formation that is inhibited by GLP-1m because of its role in glucose metabolism. We show here that the actions of GLP-1m are not mediated by the GLP-1R as we know it, which suggests that the actions are mediated by an as yet unidentified "novel" receptor. We propose that the GLP-1mR signals mitochondrial events in hepatocytes by inhibiting fatty acid oxidation. It may also be possible, however, that the GLP-1R is differentially expressed in the liver under special circumstances, such as obesity (51). If the GLP-1R is expressed differentially in the livers of obese individuals we suggest that one mechanism for its unique recognition of GLP-1m is its recruitment of a special receptor activity modifying proteins. Such a mechanism of receptor activity modifying protein recruitment is known to change the hierarchy of ligand recognition of the calcitonin gene–related receptor between the ligands, calcitonin, and adrenomedullin (52). The calcitonin gene–related receptor and the GLP-1R are both members of the structurally highly related Family B of seven-membrane spanning G-protein coupled receptors.

There are some limitations to our study. We did not measure portal venous insulin levels and it is likely that the liver was exposed to higher insulin levels than that measured in the plasma. Therefore, it is possible that part of the observed suppression of HGP is due to a weak and inapparent insulinotropic action of GLP-1m. The GLP-1R antagonist, exendin (9–39), inhibits the insulinotropic action of GLP-1 (7–36) amide by 76% during hyperglycemia (22). Although unlikely, the possibility exists that the insulinotropic effect observed with the infusion of GLP-1m during euglycemia was mediated by the stimulation of the known GLP-1R. We assume that the suppression of endogenous glucose production is due solely to an effect on the liver, but contributions of the kidney, albeit very small, may have also occurred. Regardless of the mechanism(s) responsible, our data demonstrate that GLP-1m is biologically active. This major circulating form of GLP-1 has distinct and potent effects on the inhibition of HGP in the insulin-resistant state of obesity and it is weakly insulinotropic in humans. We therefore conclude that the glucoregulatory effects of GLP-1 are actually mediated, in part, by its metabolite, GLP-1m. These findings corroborate the observations that GLP-1m has cardiac and vascular effects that are equal to the effects of full-length GLP-1 (30,40), and suggest that GLP-1m may have a therapeutic role in the regulation of glucose levels in the diabetic state.

Our findings have implications for the use of DPP-4 inhibitors in humans with type 2 diabetes. By preventing formation of GLP-1m, the effects of GLP-1 on glucose uptake and R_a are abrogated. A very recent study has shown that DPP-4 inhibition resulted in elevated postprandial GLP-1 levels, a very mild decrease in glucose levels and no change in glucose disposal or uptake (46), supporting the observation that the effects of GLP-1m are lost.

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DISCLOSURE

The authors declared no conflict of interest.

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