Glucagon-Like Peptide 1/Glucagon Receptor Dual Agonism Reverses Obesity in Mice

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OBJECTIVE—Oxyntomodulin (OXM) is a glucagon-like peptide 1 (GLP-1) receptor (GLP1R)/glucagon receptor (GCGR) dual agonist peptide that reduces body weight in obese subjects through increased energy expenditure and decreased energy intake. The metabolic effects of OXM have been attributed primarily to GLP1R agonism. We examined whether a long acting GLP1R/GCGR dual agonist peptide exerts metabolic effects in diet-induced obese mice that are distinct from those obtained with a GLP1R-selective agonist.

RESEARCH DESIGN AND METHODS—We developed a pro tease-resistant dual GLP1R/GCGR agonist, DualAG, and a corre sponding GLP1R-selective agonist, GLPAG, matched for GLP1R agonist potency and pharmacokinetics. The metabolic effects of these two peptides with respect to weight loss, caloric reduction, glucose control, and lipid lowering, were compared upon chronic dosing in diet-induced obese (DIO) mice. Acute studies in DIO mice revealed metabolic pathways that were modulated independently of weight loss. Studies in Glp1r−/− and Gcgr−/− mice enabled delineation of the contribution of GLP1R versus GCGR activation to the pharmacology of DualAG.

RESULTS—Peptide DualAG exhibits superior weight loss, lipid lowering activity, and antihyperglycemic efficacy comparable to GLPAG. Improvements in plasma metabolic parameters including insulin, leptin, and adiponectin were more pronounced upon chronic treatment with DualAG than with GLPAG. Dual receptor agonism also increased fatty acid oxidation and reduced hepatic steatosis in DIO mice. The antiobesity effects of DualAG require activation of both GLP1R and GCGR.

CONCLUSIONS—Sustained GLP1R/GCGR dual agonism reverses obesity in DIO mice and is a novel therapeutic approach to the treatment of obesity. Diabetes 58:2258–2266, 2009
study was to leverage the matched GLP1R agonist potencies and pharmacokinetics of peptides DualAG and GLPAG in comparing the metabolic effects and therapeutic potential of a dual GLP1R/GCGR agonist with a GLP1R-selective agonist in a mouse model of obesity.

**RESEARCH DESIGN AND METHODS**

Experiments were performed in lean and diet-induced obese (DIO) C57BL/6 mice and in weight- and sex-matched Geyer−/− (23), Glp1r−/− (24), or lean C57BL/6 control mice. All mice were obtained from Taconic Farms (Germantown, NY) and were maintained on either standard rat diet (Teklad 7012; Harlan Teklad) or high-fat diet (D12492: 60% kcal from fat; Research Diets) in a 12-h light/12-h dark cycle (light: 3:00 a.m. to 3:00 p.m.). Animal protocols used in these studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee (Rahway, NJ).

**Peptides.** The free thiol-containing peptide precursors of DualAG and GLPAG were synthesized by standard solid-phase peptide synthesis using Fmoc-thu chemistry. Peptides were synthesized by reverse-phase HPLC using water/acetonitrile (0.1% trifluoroacetic acid) gradients. Cholesterol-peptide conjugates were synthesized by reaction of the thiol-containing peptide precursors with cholest-5-en-3α-ol (DSer-2-exo-cholest-5-en-3α-azaoctadecan-18-oate), which was previously assembled by standard solution chemistry. The peptide conjugates were purified by reverse-phase chromatography using water/acetonitrile (0.1% trifluoroacetic acid) gradients. Purified peptide conjugates were characterized by electrospray MS on a Micromass LC/2 platform spectrometer.

**Determination of murine GLP1R and GCGR agonist potency.** In vitro agonist potency of peptides was determined in Chinese hamster ovary (CHO) cells stably expressing murine GLP1R or murine GCGR using the Cisbio CAMP Dynamic 2 assay. Peptides were diluted in assay buffer and incubated with cells in the presence of 20% mouse plasma. The assay was terminated with the addition of the Cisbio detection reagents as per the manufacturer’s instructions. CAMP was detected by a decrease in time-resolved fluorescence energy transfer (TR-FRET) using an EnVision platterader (PerkinElmer).

**Ex vivo liver glycogenolysis assay.** The ability of DualAG and GLPAG to stimulate glycogen breakdown was evaluated in ex vivo perfused livers harvested from C57BL/6 mice using a 13C-nuclear magnetic resonance (NMRR)-based assay as previously described (25).

**Measurement of plasma peptide exposures at the end of the chronic study.** DIO mice, 23 weeks old and maintained for 16 weeks on a high-fat diet, were anesthetized with isofluorane, and blood was collected by cardiocentesis into EDTA-coated microtainer tubes containing DPP-4 inhibitor and aprotinin. The in vitro cell-based CAMP bio-assay for determining GLP1R agonist potency was used with CHO cells stably transfected with human GLP1R to determine peptide concentrations by comparing the degree of CAMP accumulation in plasma samples from treated animals against a CAMP standard curve generated by spiking peptide standards into mouse plasma.

**Single-dose studies in DIO mice.** Weight (∼45 g) and age-matched DIO mice (23 weeks old) were subcutaneously injected at 9:00 a.m. with vehicle, DualAG, or GLPAG at a dose of 191 nmol/kg. Food was removed, and 6 h later (3:00 p.m.; T_max for GLPAG and DualAG) the animals were killed for collection of plasma and tissue samples, which were immediately stored at −80°C.

**RNA preparation and quantitative RT-PCR.** RNA was isolated from tissues using Ultraspec Total RNA Isolation Reagent (Biotecx Laboratories). The resulting total RNA was subjected to DNase treatment using RNAse-freeDNase (Qiagen). After reverse transcription of the RNA to generate cDNA, quantitative RT-PCR was performed with TaqMan PCR Reagent using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Taqman probes were purchased from Applied Biosystems: ChEEBP (Mm00409811_m1), Ctp1a (Mm00554384_m1), Gf21 (Mm00410615_g1), Ldlr (Mm00404169_m1), Pck1 (Mm00402063_m1), Pgc-1α (Mm00471833_m1). Expression was normalized to the copy number for β-actin (Actb).

**Quantitation of malonyl-CoA and acetyl-CoA.** Mouse liver samples (∼100 mg) were homogenized in 1 ml of 10% sulfoacetic acid, 10 mmol/l dithiothreitol. The samples were spun at 15,000 g, and the supernatant was analyzed by LC/MS after 10-fold dilution using an Agilent 1100 series capillary pump interfaced to an LTQ ion trap mass spectrometer (Thermo Scientific). The assay was adapted from Minkler et al. (26).

**Chronic dosing of DualAG and GLPAG in DIO mice.** Male DIO mice (23 weeks old, n = 8/six group, maintained for 16 weeks on a high-fat diet), were acclimated to nonspecific stress for 10 days before the onset of the chronic dosing study. DualAG (1.9 μmol/kg), GLPAG (1.9 μmol/kg), or vehicle (water) was injected subcutaneously every other day for 2 weeks. Body weight and food intake were measured daily. An intraperitoneal glucose tolerance test (IPGTT, 1.5 g/kg dextrose challenge) was performed on day 13 of the chronic study at 10:00 a.m. Whole body composition analysis of conscious mice was conducted before (day 0) and at the conclusion of the study by EchoMRI (Echo Medical Systems). Plasma samples for measurement of terminal plasma concentration of active GLPAG and DualAG were obtained 18 h after the last injection by cardiocentesis.

**Food intake and body weight studies in Glp1r−/− and Geyer−/− mice.** Single-caged weight-matched (∼30 g) wild-type (n = 24), Glp1r−/− (n = 24), and Geyer−/− (n = 21) mice were injected daily (subcutaneously) with vehicle, DualAG (1.9 μmol/kg), or GLPAG (1.9 μmol/kg), 30 min before the onset of the dark cycle for 5 days. Food intake and body weight were recorded daily for the duration of the study.

**Histology.** Liver histology was performed as described elsewhere (27).

**Biochemical analyses.** Insulin and leptin levels in plasma were measured by ELISA (Linco/Millipore). Plasma free fatty acids and ketone bodies were measured using commercially available enzyme-coupled spectrophotometric assays (Wako Chemicals). Plasma triglyceride and total cholesterol were determined using an Olympus AU400e Bioanalyzer. Adiponectin was measured using a mouse adiponectin RIA kit (Linco/Millipore). Blood glucose levels were measured using a OneTouch glucometer (Ultra LifeScan).

**Statistics.** All data are presented as means ± SE. Comparisons among groups were made using ANOVA or unpaired Student’s t test, as appropriate. P < 0.05 was regarded as statistically significant.

**RESULTS**

**Development of long-acting GLP1R/GCGR DualAG and GLP1R-selective agonist (GLPAG) peptides.** As summarized in Fig. 1A, glucagon and OXM, the only members of the glucagon superfamily that activate GCGR, incorporate a neutral polar residue at position three (glutamine, Q). In contrast, GLP-1 and the GLP-1 mimetic exendin-4 exhibit no significant GCGR agonist activity and incorporate an acidic residue at this position (glutamate, E), which decreases binding affinity to GCGR (28,29).

**Peptide DualAG is a long-acting analog of OXM with a Ser2→DSer substitution for resistance to DPP-4 cleavage.** A Gln3→Glu substitution was introduced in DualAG to generate analog GLPAG. As illustrated in Fig. 1A, a cholesterol moiety was conjugated to the thiol side chain of a Cys residue appended to the C-terminus of each OXM analog to improve pharmacokinetics and enhance metabolic stability via plasma lipid and protein binding. The insertion of a short polyethylene glycol spacer between the cholesterol moiety and the peptides ameliorated a decrease in potency of the conjugates in the presence of plasma that occurs because of protein/lipid binding. A more complete description of the discovery and development of these peptides will be provided elsewhere (Bianchi et al., manuscript in preparation).

Consistent with a previous report of DSer2-glucagon being resistant to DPP-4 cleavage (30), peptides DualAG and GLPAG exhibited no loss of in vitro potency after overnight incubation at 37°C with human recombinant soluble DPP-4 (31). Protease resistance of the peptides was further supported by pharmacokinetics data. The T_max (time corresponding to peak plasma concentration post-dose) for DualAG administered subcutaneously in lean C57Bl/6 mice at a dose of 3 mg/kg was 5 h, as determined using a bioassay for GLP1R agonism. The corresponding in vivo half-life (T_1/2) for circulating active peptide was 1.7 h (compared with T_1/2 ~8–12 min for native OXM (32)). Peptide GLPAG exhibited comparable in vivo stability to DualAG as reflected by comparable plasma concentrations of bioactive peptides measured at 24 h postdose in mice (Fig. 2B, inset).

As summarized in Fig. 1B, DualAG is a full agonist of both, mGLP1R (EC_{50, CAMP} = 3.4 nmol/l) and mGCGR (EC_{50, CAMP} = 1.5 nmol/l) and is comparable in potency to OXM in vitro, using a cell-based assay that measures CAMP
accumulation in CHO cells stably transfected with the respective recombinant murine receptor. GLPAG is an equipotent mGLP1R agonist (EC50, cAMP = 1.7 nmol/l), with at least 100-fold reduced mGCGR agonist potency compared with DualAG. The GCGR receptor selectivity of these long-acting peptides was further confirmed using an ex vivo assay that monitors glycogenesis and glycogenolysis in a perfused mouse liver (25). Briefly, a 13C-NMR visible pool of glycogen was created by perfusion of the gluconeogenic substrate [2-13C] pyruvate and liver glycogen was monitored in real time via the C1 resonance of the glucosyl units in the glycogen chain. DualAG or GLPAG was subsequently infused, and loss of label from glycogen (glycogenolysis) was monitored by 13C-NMR to assess GCGR activation (supplementary Fig. 1 A and B, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0278/DC1). Peptide DualAG induced full glycogenolysis at a perfusate concentration of 1 nmol/l (EC50, glyco = 0.5 nmol/l), which compares favorably with its in vitro potency against mGCGR. GLPAG, on the other hand, induced full glycogenolysis at a much higher peptide perfusate concentration (300 nmol/l) (EC50, glyco = 208 nmol/l), consistent with >100-fold reduced agonist potency against mGCGR. Peptides DualAG and GLPAG showed no antagonist activity at GCGR and were inactive at other receptors of the glucagon-secretin family (PAC1, VPAC1, VPAC2, and GIPR, EC50, cAMP >10 μmol/l).

**DualAG reverses obesity and improves glucose metabolism in mice.** DIO mice were injected subcutaneously with DualAG or GLPAG (1.9 μmol/kg) in a 14-day chronic study (Fig. 2A). Although peak plasma peptide concentrations (Cmax) were achieved 5 h after subcutaneous injection and the plasma T1/2 suggests daily dosing as optimal, both peptides were injected every other day to minimize the stress caused by frequent injections. DualAG exhibited superior weight loss efficacy compared with GLPAG at day 14 (25 and 12% decrease in body weight, respectively, relative to vehicle-treated mice, Fig. 2B). As noted earlier, plasma peptide exposures measured at 24 h after the last injection were similar for DualAG and GLPAG (Fig. 2B, inset), confirming the matched pharmacokinetics of the two peptides. As depicted in Fig. 2C, cumulative food intake was reduced to a greater extent with the DualAG than with GLPAG (30 and 12% reduction, respectively, relative to vehicle). Body composition analysis confirmed that the decrease in body weight was primarily accounted for by a proportional decrease in fat mass (Fig. 2D). An IPGTT performed on day 13 (20 h after the last injection) revealed that glucose tolerance was significantly and comparably improved in both treatment groups (Fig. 2E). Furthermore, basal blood glucose levels
were normalized by chronic treatment with either peptide (Fig. 2E, t = 0 measurement predextrose challenge in the IPGTT). Several other metabolic parameters in plasma were also improved by chronic treatment with the peptides (Table 1). Increases in adiponectin and decreases in leptin and insulin levels correlated with the decreased adiposity observed at the end of the study in each treatment group. Reduced cholesterol and triglyceride levels, increased ketone bodies, and decreased hepatic steatosis (Fig. 3) relative to vehicle treatment were also noted, especially for animals treated with DualAG.

To identify metabolic pathways acutely altered by DualAG and GLPAG before any significant weight loss, age- and weight-matched DIO mice were injected with vehicle, DualAG, or GLPAG (191 nmol/kg subcutaneously each peptide) and killed 6 h later to collect plasma for metabolites and liver tissue samples for gene expression analysis. Despite the acute treatment, decreased plasma triglycerides and increased ketone bodies were detected albeit only in animals treated with the dual agonist (supplementary Fig. 2). Increased expression of the gluconeogenic genes Pck1, Pgc1α, and Pdha1 was observed with DualAG treatment, but not with GLPAG or vehicle (Fig. 4A–C). Liver pools of acetyl-CoA, the main product of pyruvate decarboxylation, and malonyl-CoA were decreased by DualAG (Fig. 4D and E). In addition, DualAG caused a significant upregulation of genes that induce fatty acid oxidation (FAO) in the liver, including Fgf21 and Cpt1a (Fig. 4F and G), and the downregulation of lipogenic genes such as ChREBP (Fig. 4H). A robust (~15-fold) upregulation of Ldhr by DualAG (Fig. 4I) was also noted in the context of reduced cholesterol levels previously observed with chronic treatment (Table 1).

**Metabolic effects of DualAG and GLPAG in Glp1r<sup>−/−</sup> and Gegr<sup>−/−</sup> mice.** To provide mechanistic insight into the relative contributions of each target receptor to the metabolic effects of the long-acting peptides, we compared the effect of repeat administrations of DualAG and GLPAG...
TABLE 1
Chronic treatment of DIO mice with DualAG and GLPAG: plasma parameters measured at the end of the 14-day study

|                  | DualAG | GLPAG |
|------------------|--------|-------|
|                  | Vehicle  | 1.9 μmol · kg⁻¹ · day⁻¹ | 1.9 μmol · kg⁻¹ · day⁻¹ |
| N                | 6      | 7     | 7     |
| Insulin (ng/ml)  | 13.2 ± 0.7 | 4.0 ± 0.2*‡ | 7.8 ± 1.1* |
| Leptin (ng/ml)   | 32 ± 4 | 14 ± 1*‡ | 19 ± 1* |
| Adiponectin (μg/ml) | 15 ± 1 | 28 ± 2*‡ | 20 ± 1* |
| Fatty free acids (mM) | 0.2 ± 0.0 | 0.4 ± 0.1 | 0.3 ± 0.0 |
| Cholesterol (mg/dl) | 153 ± 6 | 76 ± 7*‡ | 107 ± 5* |
| Triglycerides    | 68 ± 8 | 44 ± 5* | 47 ± 6* |
| β-hydroxybutyrate (mg/dl) | 4.1 ± 0.3 | 9.3 ± 0.9* | 7.2 ± 0.4* |

Data are means ± SE. *P < 0.05 vs. vehicle; ‡P < 0.05 DualAG vs. GLPAG.

(1.9 μmol/kg subcutaneously) on body weight and food intake in wild-type mice with that in animals lacking either GLP1R (Glp1r⁻⁻⁻) or GCGR (Gcgr⁻⁻⁻). Because both receptor knockout mouse lines are resistant to diet-induced obesity, weight-matched lean mice were used in the study. Both peptides significantly reduced cumulative food intake and body weight in wild-type mice. As observed previously, DualAG treatment effected superior body weight loss in wild-type mice compared with animals treated with GLPAG (Fig. 5). In mice lacking either GLP1R or GCGR, however, the efficacy of DualAG was sustained but partially attenuated compared with the weight loss achieved in wild-type mice. These results implicate both GLP1R and GCGR activation in the mechanism of action of DualAG. The weight loss efficacy of GLPAG in Gcgr⁻⁻⁻ mice was comparable to the attenuated body weight effects of DualAG in these animals and was completely abolished in Glp1r⁻⁻⁻ mice, confirming the GLP1R selectivity of this peptide.

DISCUSSION

Herein, we compare the antiobesity effects of a long-acting dual GLP-1/glucagon agonist (DualAG) with those of a long-acting GLP1R selective agonist (GLPAG) in a mouse model of obesity. To avoid confounding effects in these studies, a specific effort was made to match the pharmacokinetics, GLP1R agonist potencies, and plasma exposures of the two peptides during chronic dosing studies. Long-acting peptides DualAG and GLPAG lowered blood glucose, reduced food intake, and decreased body weight in DIO mice. We report for the first time that chronic treatment with a dual GLP1R/GCGR agonist compared with a GLP1R selective agonist causes superior weight loss and lipid lowering in DIO mice, without causing hyperglycemia. Instead, ambient glucose levels were normalized by both peptides, and glucose tolerance was comparably improved in both groups as measured in an IPGTT conducted at the end of the study. Of note, improvements in metabolic parameters such as plasma insulin, leptin, and adiponectin were typically more pronounced upon chronic treatment with DualAG than with GLPAG, consistent with the increased weight loss efficacy of the dual agonist.

To evaluate the contributions of GLP1R versus GCGR agonism to the observed pharmacology of the long-acting peptides, we evaluated the metabolic effects of repeat dosing with DualAG and GLPAG in Glp1r⁻⁻⁻ and Gcgr⁻⁻⁻ mice. Our studies clearly establish the importance of dual GLP1R/GCGR agonism in the increased weight loss effi-
cacy of DualAG. Specifically, weight loss was observed with DualAG treatment in both Glp1r−/− and Gcgr−/− mice, although efficacy was reduced compared with body weight effects observed in weight-matched wild-type mice. In contrast, the metabolic effects of GLPAG were completely ablated in Glp1r−/− mice, confirming its GLP1R selective effects.

A unified hypothesis for the mechanism of action of peptide DualAG is illustrated in Fig. 5G. Under conditions of fasting metabolism, GCGR signaling pharmacologically accentuates the catabolic aspects of metabolism that favor weight loss. DualAG activates GCGR in the liver, rapidly upregulating key gluconeogenic genes. However, increased GLP1R signaling in the postprandial state is sufficient to improve glucose tolerance with a dual agonist peptide, as has been reported with native OXM in mice (6,14). Our studies also confirm the contribution of GLP1R agonism to the chronic antiobesity effect of DualAG, which is driven primarily by a reduction in adiposity. As illustrated in Fig. 5G, DualAG modulates metabolic pathways that decrease acetyl-CoA and malonyl-CoA pools in the liver, increase ketogenesis, and decrease plasma lipids. Hormone-sensitive lipase mRNA, which is downregulated in animal models of obesity (33) and in obese humans (34,35), is significantly upregulated in adipose tissue obtained from DIO mice treated with DualAG (data not shown). Although the role of glucagon in fat cell metabolisim in humans is unclear, pharmacological activation of GCGR in adipose tissue may activate hormone-sensitive lipase, resulting in an increased free fatty acid pool available for β-oxidation (36). Our data suggest that FAO is acutely upregulated in rodents by GLP1R/GCGR dual agonism before any weight loss. The observed upregulation of liver Fgf21 by DualAG may also contribute to the action of a dual GLP1R/GCGR agonist because pharmacological levels of FGF21 stimulate hepatic FAO and increase energy expenditure (37,38).

The increased weight loss efficacy of DualAG compared with GLPAG is consistent with previous research on the pharmacology of glucagon. The hormone has been reported to decrease total cholesterol in rats and to cause a greater reduction in body weight compared with a pair-fed group of animals because of both reduced food intake and increased energy expenditure (19). Conversely, Langhans et al. (39) showed that intraperitoneal injections of anti-glucagon antibodies in food-deprived rats increased meal size and duration. Furthermore, chronic administration of glucagon in humans has been reported to increase satiety and decrease hunger scores (17,18). Of note, however, GCGR-selective agonism is typically associated with the risk of hyperglycemia because elevation of endogenous glucagon levels and concomitant reduction in insulin levels/action are accepted as key players in the pathogenesis of diabetic hyperglycemia (40). According to this
FIG. 5. DualAG lowers body weight and food intake via activation of GLP1R and GCGR. Effect of repeated injections of DualAG or GLPAG on cumulative food intake and body weight in wild-type (A and B), Glp1r<sup>−/−</sup> (C and D), and Gcgr<sup>−/−</sup> (E and F) mice. The antiobesity effects of DualAG are attenuated but not ablated in either receptor knockout mouse. G: Proposed mechanism of action of DualAG. In addition to the known effects associated with GLP1R activation, hepatic GCGR activation increases liver glucose production and stimulates FAO. The acetyl-CoA generated by β-oxidation challenges the processing capacity of the tricarboxylic acid (TCA) cycle and is used in the biosynthesis of ketone bodies. Consistent with the decrease in plasma cholesterol, animals treated with DualAG showed a robust upregulation of liver LDLr expression. In the adipose tissue, pharmacological activation of GCGR and GLP1R may stimulate hydrolysis of triglycerides (TG). Upregulation of liver Fgf21 in animals treated with DualAG may contribute to stimulation of FAO and ketogenesis. *P < 0.05 DualAG and GLPAG versus vehicle; †P < 0.05 DualAG versus GLPAG. FFA, free fatty acid.
bihormonal hypothesis for diabetes, hyperglucagonemia results in excessive hepatic glucose production, which is not balanced by glucose utilization under conditions of hypoinsulinemia and insulin resistance. Indeed, the development of GCGR antagonists for the treatment of type 2 diabetes is being actively pursued (25,41–44) because these agents act toward restoring normal GCGR tone. Distinct from the demonstrated imbalance in endogenous hormonal action that characterizes the pathology of type 2 diabetes, however, we now report for the first time that concomitant activation of GLP1R in rodents mitigates the metabolic risks associated with GCGR activation while leveraging the beneficial pharmacological effects of activating each receptor, including enhanced weight loss efficacy, antihyperglycemic activity, and lipid-lowering effects. Our hypothesis is that pharmacological GLP1R agonism results in enhanced glucose-dependent insulin secretion, which enhances glucose disposal and provides sufficient anabolic tone to balance the glucoregulatory and catabolic effects of concomitant GCGR agonism. Hence, the GLP1R/GCGR dual agonist peptide DualAG mediates effective weight loss and improves glucose tolerance in rodents without causing hyperglycemia or cachexia. Whether the observed rodent pharmacology is predictive of clinical effects with a long-acting dual agonist peptide remains to be determined, although the weight loss observed with native OXM in overweight subjects is encouraging (9), given the rapid clearance of this peptide. In conclusion, we propose that long-acting GLP1R/GCGR dual agonists such as peptide DualAG represent novel pharmaceutical agents for the treatment of obesity.

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