A Recombinant Human Glucagon-Like Peptide (GLP)-1–Albumin Protein (Albugon) Mimics Peptidergic Activation of GLP-1 Receptor–Dependent Pathways Coupled With Satiety, Gastrointestinal Motility, and Glucose Homeostasis

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Peptide hormones exert unique actions via specific G protein–coupled receptors; however, the therapeutic potential of regulatory peptides is frequently compromised by rapid enzymatic inactivation and clearance from the circulation. In contrast, recombinant or covalent coupling of smaller peptides to serum albumin represents an emerging strategy for extending the circulating $t_{1/2}$ of the target peptide. However, whether larger peptide-albumin derivatives will exhibit the full spectrum of biological activities encompassed by the native peptide remains to be demonstrated. We report that Albugon, a human glucagon-like peptide (GLP)-1–albumin recombinant protein, activates GLP-1 receptor (GLP-1R)-dependent cAMP formation in BHK-GLP-1R cells, albeit with a reduced half-maximal concentration ($EC_{50}$) (0.2 vs. 20 nmol/l) relative to the GLP-1R agonist exendin-4. Albugon decreased glycemic excursion and stimulated insulin secretion in wild-type but not GLP-1R$^{-/-}$ mice and reduced food intake after both intracebroventricular and intraperitoneal administration. Moreover, intraperitoneal injection of Albugon inhibited gastric emptying and activated c-FOS expression in the area postrema, the nucleus of the solitary tract, the central nucleus of the amygdala, the parabrachial, and the paraventricular nuclei. These findings illustrate that peripheral administration of a larger peptide-albumin recombinant protein mimics GLP-1R–dependent activation of central and peripheral pathways regulating energy intake and glucose homeostasis in vivo. Diabetes 53:2492–2500, 2004

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CCK, cholecystokinin; CNS, central nervous system; DPP-IV, dipeptidyl peptidase-IV; Ex-4, exendin-4; GLP, glucagon-like peptide; GLP-1R, GLP-1 receptor; HSA, human serum albumin; ICV, intracerebroventricular; IP, intraperitoneal; NTS, nucleus of the solitary tract.

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reduced frequency of parenteral administration, relative to native GLP-1. Nevertheless, the molecular interaction with the GLP-1R, volume of distribution, and access to the central nervous system (CNS) would be predicted to be markedly different for a much larger albumin-based molecule (8).

Whether all of the desirable actions of native GLP-1, including the activation of the CNS centers regulating food intake and gastrointestinal motility, would be mimicked by a much larger GLP-1–albumin protein is currently unclear. Accordingly, we have examined the biological actions of Albogen, a recombinant GLP-1–human serum albumin (HSA) fusion protein, using a combination of cell line studies in vitro and both wild-type and GLP-1R–/– mice in vivo.

RESEARCH DESIGN AND METHODS

Reagents. Tissue culture medium, serum, and G418 were purchased from Invitrogen (San Diego, CA). Forskolin and 3-isobutyl-1-methylxanthine were obtained from Sigma (St. Louis, MO). HSA and Albogen were provided by Human Genome Sciences (Rockville, MD). Ex-4 and extendin (9-39) were purchased from California Peptide Research (Napa, CA).

Measurement of CAMP. Baby hamster kidney (BHK) cells stably transfected with rat cAMP reporter gene were grown and propagated in medium containing 0.05 mg/ml G418 as described previously (9). Before analysis, BHK-GLP-1R cells were allowed to attach and form a confluent monolayer. Cells were incubated with 1 μmol/L extendin (9-30) or medium alone for 5 min at 37 °C, followed by an additional 10-min incubation in the presence of increasing concentrations of Ex-4, HSA, or Albogen. All reactions were carried out in triplicate and terminated by the addition of ice-cold absolute ethanol. Cell extracts were prepared by incubation with 10% volume of chilled solution containing 5,000 KIU/ml Trasylol, 32 μmol/l EDTA, and 0.1 nmol/l Diprotin A. Plasma was separated by centrifugation at 4 °C, followed by ice-cold 4% paraformaldehyde solution. Brains were removed immediately after perfusion, kept in ice-cold 4% paraformaldehyde solution for 3 h, and then transferred to a solution containing paraformaldehyde and 10% sucrose for 12 h. Brains were cut into 25-μm sections using a Leica SM2000R sliding microtome (Leica Microsystems, Richmond Hill, Ontario, Canada) and stored at −20 °C in a cold cryoprotecting solution. Sections were processed for immunocytochemical detection of FOS using a conventional avidin-biotin-immunoperoxidase method (Vectorstain ABC Elite Kit; Vector Laboratories, Burlingame, CA) as described (12). The FOS antibody (Sigma-Aldrich, Oakville, Ontario, Canada) was used at a 1:5,000 dilution. Brain sections corresponding to the level of the area postrema, the nucleus of the solitary tract (NTS), the central nucleus of the amygdala, and the parabrachial and paraventricular nuclei were defined according to the mouse brain atlas of Franklin and Paxinos (14) and selected for analyses.

Statistical analysis. Statistical significance was determined by ANOVA and Bonferroni post-test using Prism version 3.03 software (GraphPad Software, San Diego, CA). A P value <0.05 was considered to be statistically significant.

RESULTS

Albogen is a recombinant human protein that contains a dipeptidyl peptidease-IV (DPP-IV)-resistant human GLP-1 analog encoded in the same open reading frame as the HSA amino acid sequence. To evaluate whether the bioactive domain(s) of a much smaller peptide hormone could still recognize and functionally interact with its cognate receptor when constrained within a much larger heterologous protein, we examined whether Albogen activated GLP-1R in vitro. Albogen produced a dose-dependent stimulation of cAMP accumulation in BHK-GLP-1R cells that was significantly diminished by coincubation with the GLP-1R antagonist extendin (9-39) (Fig. 1). Nevertheless, Albogen was not as effective in activating the GLP-1R when compared with the much smaller more potent lizard-derived GLP-1R agonist Ex-4 (Fig. 1; EC50 = 0.2 vs. 20 nmol/l for Ex-4 vs. Albogen, respectively).

These experiments demonstrate that the considerably altered peptide conformation that arises after insertion of GLP-1 sequences into a much larger albumin open reading frame does not eliminate the ability of essential GLP-1 motif(s) to recognize and activate GLP-1Rs. To ascertain whether circulating Albogen is capable of reaching key sites and activating GLP-1R–dependent actions in vivo, we carried out oral glucose tolerance testing in mice. Glycemic excursion was significantly reduced after parenteral Albogen administration to wild-type mice (Fig. 2). Further-
more, the glucose-lowering properties of Albugon depended on a functional GLP-1 receptor because Albugon had no effect on blood glucose in GLP-1R−/− mice (Fig. 2B). Consistent with the known actions of smaller GLP-1R peptide agonists, Albugon, at doses ranging from 0.1 to 10 mg/kg, markedly reduced glycemic excursions after not only oral but also IP glucose loading, in association with a significant increase in the insulin-to-glucose ratios (Fig. 3A and B). Albugon increased the levels of plasma insulin after an IP glucose challenge (Fig. 3C) but did not significantly reduce the levels of plasma glucagon in mice after an overnight fast (Fig. 3D). Taken together, these results demonstrate that Albugon lowers blood glucose and enhances insulin secretion through GLP-1R–dependent mechanisms in vivo.

Smaller 30–to 40–amino acid GLP-1R agonists, including native GLP-1, Ex-4, and liraglutide, have been shown to inhibit food intake, presumably via activation of central hypothalamic neurons regulating satiety (15). To assess whether a much larger recombinant GLP-1–albumin protein would also exert anorectic effects, we studied food intake in normal mice after ICV or IP peptide administration. ICV Albugon significantly lowered food intake in mice (relative to the HSA control) in a dose-dependent manner, and this effect was detectable for up to 7 h (Fig. 4A) but was not sustained over the entire 24-h observation period. In contrast, Ex-4 exerted a more potent and sustained anorectic effect after ICV administration, with a highly significant reduction of food intake observed even at the end of the 24-h study period (Fig. 3).

The blood-brain barrier is relatively impermeable to larger proteins such as albumin under both normal physiological conditions and in the setting of diabetes (16,17). Although GLP-1Rs are expressed on hypothalamic neurons regulating satiety (18,19), whether GLP-1R agonists required direct access to the brain for activation of the central anorectic pathway remains uncertain (20). Accordingly, we examined whether Albugon would also exhibit anorectic effects after peripheral administration. Although lower doses of IP Albugon, 0.16–11 nmol/kg, did not affect food intake, the highest dose tested (110 nmol/kg) reduced food intake at all time points studied, from 2 to 24 h after Albugon administration (Fig. 4C). In contrast, much smaller doses of Ex-4 significantly reduced food ingestion after IP administration in the same experiments (Fig. 4D). Hence, although Albugon is capable of exerting anorectic actions after both central and peripheral administration, it is less potent compared with the smaller GLP-1R agonist, Ex-4. To ascertain whether the anorectic actions of peripherally administered Albugon were due to the nonspecific effects of the larger amount of injected protein, we repeated the experiments using age- and sex-matched wild-type control and GLP-1R−/− mice. Albugon reduced food intake at all time points in wild-type mice but had no effect on food intake in GLP-1R−/− mice. These findings demonstrate that IP Albugon inhibits food intake in a GLP-1R–dependent manner.

GLP-1R agonists markedly inhibit gastric emptying (21), and gastric distension induces c-FOS in GLP-1–expressing neurons in the rat medulla (22). However, whether direct CNS access is required for either GLP-1R–dependent inhibition of food intake or gastric emptying has not been determined. The basal rate of gastric emptying was comparable in wild-type and GLP-1R−/− mice (Fig. 5). Both Ex-4 and CCK-8 significantly inhibited gastric emptying in wild-type mice; however, CCK-8, but not Ex-4, also inhibited gastric emptying in GLP-1R−/− mice. Similarly, Albugon potently inhibited gastric emptying in wild-type but not in GLP-1R−/− mice (Fig. 5).

The anorectic actions of small peptide GLP-1R agonists are associated with c-FOS activation in CNS centers...
coupled with the control of energy intake (18,20,23). To determine whether peripherally administered Albugon was capable of activating neuronal FOS expression, we examined the pattern and extent of CNS FOS expression after IP Albugon administration. Ex-4 markedly increased c-FOS expression in the area postrema, the NTS, the central nucleus of the amygdala, the parabrachial nucleus, and the hypothalamic paraventricular nuclei (Fig. 6A–E).

Similarly, Albugon significantly activated c-FOS expression in the identical brain regions, although much less robustly in the NTS and paraventricular nucleus than in Ex-4 (Fig. 6A–E). Analysis of the murine CNS after peripheral (IP) administration of HSA did not detect immunoreactive HSA in brain parenchyma (data not shown), consistent with the inability of HSA to rapidly cross the normal blood-brain barrier (8).

**DISCUSSION**

GLP-1–based therapies for type 2 diabetes are attracting increasing attention in part because of the preliminary efficacy demonstrated in clinical studies (6) and because...
of unique yet complementary mechanisms of action (1). Unlike some antidiabetic agents that reduce blood glucose while promoting weight retention, GLP-1R activation is coupled with short-term inhibition of food intake in both rodent and human studies (15). Moreover, prolonged GLP-1 administration for 6 weeks to diabetic human sub-
jects was associated with a significant reduction in body weight over the study period (24). The anorectic properties of GLP-1 and its peptide analogs are thought to be due in part to both inhibition of gastric emptying and activation of central satiety centers coupled with reduction of energy intake (20,25). Although development of GLP-1–
based small peptide analogs resistant to enzymatic inacti-
vation is a major focus of current pharmaceutical activity.

FIG. 4. Albugon (ALB) reduces food intake in fasted mice but is less anorectic than Ex-4. After an overnight fast, wild-type mice were given ICV (A and B) or IP (C–E) injections of PBS or increasing doses (nmol/kg) of Ex-4, HSA, or ALB. E: Wild-type and GLP-1R−/− mice were fasted overnight and then given IP injections of 110 nmol/kg of HSA or ALB. Food intake was measured at 2, 4, 7, and 24 h after recovery from injection. Values are expressed as means ± SE; n = 4–5 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. control-treated (PBS or HSA) mice.

FIG. 5. Albugon (ALB) reduces the gastric emptying rate in wild-type but not GLP-1R−/− mice. Gastric emptying rate in wild-type (WT) (A) and GLP-1R−/− (B) mice at 4 h after IP administration of PBS, Ex-4 (0.17 mg/kg), HSA (2.7 mg/kg), ALB (3 mg/kg), or CCK octapeptide (CCK-8; 4 μg/mouse) is shown. Values are expressed as means ± SE; n = 3–4 mice/group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. PBS; ##P < 0.01 vs. HSA.
The need for once- or twice-daily injection of these peptides has stimulated efforts toward development of even longer-acting molecules that retain the ability to activate GLP-1Rs. The relatively prolonged circulating t_{1/2} of endogenous and exogenously administered albumin has fostered attempts directed at coupling peptide moieties to albumin, thereby increasing the circulating t_{1/2} of the albumin-peptide complex (26). Albuferon is an interferon-α–albumin fusion protein that retains the biological activity of interferon yet exhibits a markedly extended pharmacokinetic profile relative to native interferon in Cynomolgus monkeys (27). Similarly, fusion of the amino acid sequence of human growth hormone to albumin prolonged the circulating t_{1/2} of Albutropin relative to the native growth hormone, yet Albutropin retained the ability to stimulate IGF-I and body weight gain in vivo (28). Moreover, peptide binding to albumin extended the pharmacokinetic properties of several smaller proteins including insulin (29), Fab antibody fragments (26), and coagulation factor VIIa inhibitor 1α (30). In contrast to Albugon, however, these albumin-peptide derivatives exert their predominant actions outside the CNS, and activation of the CNS is not critical for their therapeutic actions.

More recent efforts have been directed at extending the t_{1/2} of the much smaller GLP-1 molecule using albumin-based approaches. Liraglutide is a fatty acylated human DPP-IV–resistant GLP-1 analog that binds to albumin and exhibits a t_{1/2} of ~11–15 h after parenteral administration in humans (31). Liraglutide inhibits food ingestion in rats (32) and decreases gastric emptying in human subjects (33); hence, the transient GLP-1–albumin interaction does not preclude communication with CNS centers important for control of satiety and gastrointestinal motility. However, liraglutide binds to albumin in a noncovalent disso- ciable manner, with the actions of liraglutide attributed to a free peptide unconstrained by its intermittent association with albumin in the circulation.

We recently studied the properties of CJC-1131, a DPP-IV–resistant human GLP-1 analog that covalently couples with HSA after parenteral administration (34). CJC-1131 binds to the GLP-1R and activates a broad spectrum of GLP-1R–dependent actions associated with glucose reduction in db/db mice, including stimulation of insulin secretion and insulin gene expression and expansion of islet mass (34). Intriguingly, CJC-1131 also activates FOS expression in hypothalamic neurons and reduces food intake and weight gain in normal and diabetic mice (23,34).

FIG. 6. IP Albugon and Ex-4 increase c-FOS levels in the mouse CNS. Representative photomicrographs are shown of c-FOS–stained coronal brain sections of area postrema (AP) (A), NTS (B), hypothalamic parabrachial nucleus (PB) (C), central nucleus of the amygdala (CeA) (D), and paraventricular nucleus of the hypothalamus (PVH) (E) from wild-type mice at 60 min after IP injection of PBS, Ex-4 (11 nmol/kg), HSA (110 nmol/kg), or Albugon (110 nmol/kg). No hypoglycemia was detected after administration of either Albugon or Ex-4 in these experiments. Original magnification, ×200. CC, central canal; 3V, third ventricle. The number of c-FOS immunopositive (Fos*) cells are depicted below the corresponding CNS section. Data are presented as means ± SE; n = 3 mice/treatment. ***P < 0.001 for Ex-4 vs. PBS-treated mice at 60 min; #P < 0.05, ###P < 0.001 for Albugon- vs. HSA-treated mice at 60 min; **P < 0.01, ^^^P < 0.001 for Ex-4– vs. Albugon-treated mice at 60 min.
Nevertheless, because CJC-1131 is administered parenterally as the free GLP-1 analog, which subsequently forms a covalent linkage with albumin in vivo (34), it remains likely that some or all of the acute effects of CJC-1131 on the brain reflect the rapid initial actions of free CJC-1131 before covalent coupling with albumin.

In contrast, Albugon contains the sequences of human GLP-1 linked in the same open reading frame with recombinant HSA; hence, no "free" GLP-1 is associated with Albugon administration in vivo. Consistent with studies of CJC-1131 covalently conjugated to albumin (34), Albugon activates the cloned GLP-1R, but with a reduced affinity relative to the potent GLP-1R agonist Ex-4. Because the blood-brain barrier is relatively impermeable to albumin, peripheral administration of the much larger Albugon protein provides an opportunity to determine the relative importance of peripheral versus central GLP-1R networks for control of satiety and gut motility. Remarkably, Albugon inhibited food intake after not only ICV but also after IP administration in mice. Similarly, Albugon significantly inhibited gastric emptying after IP administration. Furthermore, the distribution of neuronal c-FOS activation in different CNS nuclei after peripheral Albugon administration was highly similar, albeit less robust, compared with the pattern of FOS activation observed after Ex-4. These findings support a model whereby peripheral activation of GLP-1R–dependent vagal afferents is capable of activating CNS centers, transducing the effects of GLP-1 in the brain (35,36).

Given the clinical importance of the anorectic actions of GLP-1R agonists for prevention of weight gain in the treatment of diabetes, the mechanisms and pathways activated by GLP-1R agonists that converge on inhibition of feeding centers are of considerable interest. Both native GLP-1 and Ex-4 cross the blood-brain barrier through a GLP-1R–independent pathway (37–39); hence, these peptides are capable of directly penetrating and activating CNS centers after exogenous administration. In contrast, peripheral administration of much larger albumin-based GLP-1R agonists, such as CJC-1131 and Albugon, at doses that do not induce hypoglycemia, rapidly activates neuronal c-FOS in distinct brain regions. Furthermore, intravenous but not ICV CJC-1131 induced tyrosine hydroxylase gene transcription in the area postrema in vivo (23). Hence, our data suggest that peripheral activation of central GLP-1R systems coupled with regulation of FOS expression, gastric emptying, and food intake does not require direct exposure to GLP-1R agonists in the CNS, but may be achieved through activation of ascending pathways coupled with central GLP-1R–dependent networks.

The current data demonstrate that as little as 0.1 mg/kg (1.4 nmol/kg) of Albugon was sufficient for lowering of blood glucose; however, much larger doses (110 nmol/kg), were required for inhibition of food intake after IP admin-
istration in mice. These findings imply that acute peripheral Albagon administration is significantly more effective at reduction of glycemic excursion relative to inhibition of appetite. Nevertheless, the experimental design used here did not examine the anorectic effects of more prolonged sustained administration, a paradigm more representative of the potential therapeutic use of Albagon in humans. Furthermore, the \( t_{1/2} \) of circulating HSA, and by inference Albagon, is much longer in humans than in mice (8). Given the intense interest in the development of long-acting GLP-1R agonists for the treatment of type 2 diabetes, the biological properties and mechanisms of action of GLP-1–albumin derivatives merit further investigation.

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