An Albumin-Exendin-4 Conjugate Engages Central and Peripheral Circuits Regulating Murine Energy and Glucose Homeostasis

LAURIE L. BAGGIO,*,‡,§ QINGLING HUANG,*,‡,§ XIEMIN CAO,*,‡,§ and DANIEL J. DRUCKER*,‡,§

*Department of Medicine, Mt. Sinai Hospital, ‡Samuel Lunenfeld Research Institute, and §Banting and Best Diabetes Centre, University of Toronto, Toronto, Ontario, Canada

Background & Aims: Glucagon-like peptide-1 (GLP-1) regulates glucose homeostasis through multiple mechanisms including direct actions on the endocrine pancreas and indirect activation of central nervous system circuits regulating gastric emptying, satiety, and body weight. Because native GLP-1 is rapidly degraded, there is considerable interest in development of more potent GLP-1 receptor (GLP-1R) agonists with sustained activity; however, the extent to which much larger GLP-1R agonists will mimic some or all of the actions of smaller peptides remains uncertain. **_Methods:_** We studied the actions of CJC-1134-PC, a recombinant human serum albumin-exendin-4 conjugated protein, at the GLP-1R using heterologous cells expressing the GLP-1R in vitro and both wild-type and Glp1r−/− mice in vivo. **_Results:_** CJC-1134-PC activated GLP-1R-dependent signaling in baby hamster kidney-GLP-1R cells and acutely lowered blood glucose in wild-type but not in Glp1r−/− mice. Moreover, acute administration of CJC-1134-PC rapidly activated c-Fos expression in multiple regions of the central nervous system, acutely inhibited gastric emptying, and produced sustained inhibition of food intake in a GLP-1R-dependent manner. Furthermore, chronic daily treatment of high-fat diet-fed wild-type mice with CJC-1134-PC for 4 weeks led to improved glucose tolerance, increased levels of glucose-stimulated insulin, decreased HbA1c, and weight loss associated with decreased hepatic triglyceride content. **_Conclusions:_** These findings illustrate that a high-molecular-weight exendin-4-albumin conjugate retains the ability to mimic a full spectrum of GLP-1R-dependent actions, including activation of central nervous system circuits regulating gastric emptying, food intake, and body weight.

Glucagon-like peptide-1 (GLP-1) is a naturally occurring peptide hormone that is released from intestinal L cells in response to nutrient ingestion. GLP-1 lowers blood glucose levels in both preclinical studies and in human subjects with type 2 diabetes mellitus (T2DM) through multiple distinct actions including stimulation of glucose-dependent insulin secretion, suppression of glucagon secretion, and inhibition of gastric emptying.[1,2] GLP-1 also promotes satiety, suppresses energy intake, and causes weight loss in healthy, obese, and diabetic humans. In preclinical studies, GLP-1 increases β-cell mass via induction of β-cell proliferation and neogenesis and inhibition of β-cell apoptosis.[3–5] The combined properties of GLP-1 to reduce glycemia and promote weight loss, in association with its potential to restore or sustain β-cell mass and function, have sparked considerable interest in the use of GLP-1 as a therapeutic agent for the treatment of T2DM.[6]

A major limitation to the therapeutic use of the native GLP-1 molecule is its very short half-life in the circulation, which has been attributed to both rapid cleavage by the ubiquitous proteolytic enzyme dipeptidyl peptidase-4 (DPP-4) and renal clearance. Consequently, GLP-1 must be injected repeatedly or infused continuously to sustain clinical efficacy in vivo. Thus, pharmaceutic strategies based on sustained GLP-1 receptor (GLP-1R) activation have been focused on the development of long-acting DPP-4-resistant GLP-1R agonists such as exenatide and liraglutide. Exenatide is a synthetic version of the lizard salivary gland-derived peptide exendin-4 (Ex-4) that is resistant to DPP-4.[7] Preclinical and clinical studies demonstrate that exenatide is a long-acting GLP-1R agonist,[8,9] and it has been approved for clinical use in the United States and Europe. Liraglutide is a DPP-4-resistant fatty-acylated GLP-1 peptide analog that binds noncovalently to serum albumin following subcutaneous administration, thereby reducing its renal clearance and extending its pharmacokinetic profile.[10] Liraglutide exhibits more potent and persistent glucose-lowering effects in diabetic patients compared with native GLP-1 and is currently undergoing evaluation in phase III clinical trials in patients with T2DM.

Although exenatide and liraglutide effectively lower blood glucose levels in T2DM patients, the requirement for twice or once daily administration of these agents,

respectively, has fostered ongoing efforts to generate GLP-1R agonists with more sustained effectiveness. One such putative agent is CJC-1134-PC, a conjugate consisting of Ex-4 that has been covalently bonded, ex vivo, to recombinant human serum albumin (HSA) via a chemical linker (see Supplementary Figure 1 online at www.gastrojournal.org). Preliminary results from clinical trials in diabetic humans indicate that CJC-1134-PC has a half-life of approximately 8 days, making it suitable for once weekly dosing.[11] Nevertheless, in short-term studies over 4 weeks, CJC-1134-PC produced only modest effects on body weight; hence, it remains uncertain whether this high-molecular-weight GLP-1R agonist is capable of engaging central nervous system (CNS) circuits regulating appetite and body weight. To determine whether the considerably larger Ex-4-albumin hybrid CJC-1134-PC retains the identical spectrum of biologic actions exhibited by the much smaller GLP-1R peptide agonist Ex-4, we examined the effects of CJC-1134-PC on GLP-1R-dependent actions in vitro as well as in short- and long-term studies in mice.

Materials and Methods

Reagents

Cell culture medium was purchased from Hy-Clone (Logan, UT). Serum, G418, and antibiotics were from Invitrogen-Gibco (Burlington, ON). Forskolin and 3-isobutyl-1-methylxantine (IBMX) were obtained from Sigma Chemical Co (St Louis, MO). HSA and CJC-1134-PC were provided by Conjuchem (Montreal, QC). Ex-4, glucose-dependent insulinotropic peptide, exendin (9–39), and cholecystokinin-8 were purchased from California Peptide Research Inc (Napa, CA).

Animals

Wild-type C57BL/6 mice (4 or 7 weeks old) were from Charles River Laboratories (Montreal, QC). Glp1r$^{-/-}$ mice on the CD-1 genetic background have been described.[12] Glp1r$^{-/-}$ mice backcrossed onto the C57BL/6 genetic background for 6 generations[13] were used in the current studies. All mice were maintained under a 12-hour light/dark cycle (lights on at 7 AM and off at 7 PM) with free access to food and water, except where noted. All experiments were conducted in accordance with protocols and guidelines approved by the University Health Network Animal Care Committee. For acute studies, 8- to 10-week-old wild-type or Glp1r$^{-/-}$ male mice were used. For chronic studies, 4-week-old wild-type male mice were fed either standard rodent chow or a high-fat diet (HFD; 45% kcal from fat; Research Diets Inc, New Brunswick, NJ) for 4 weeks then maintained on their respective diets for an additional 4 weeks. Mice on the standard rodent chow diet were given twice daily intraperitoneal (IP) injections of phosphate-buffered saline (PBS); HFD mice were randomized to receive (1) twice daily IP injections of

PBS, (2) twice daily IP injections of Ex-4 (24 nmol/kg), (3) once daily IP injections of HSA (100 nmol/kg), or (4) once daily IP injections of CJC-1134-PC (100 nmol/kg). Twice daily injections were at ~8 AM and 6 PM. For mice receiving once daily injections, drugs were administered in the evening, but mice were also given IP injections of PBS in the morning to ensure that all mice were exposed to identical experimental conditions.

Biochemical Assays

Blood glucose levels in whole blood were measured using a Glucometer Elite blood glucose meter (Bayer, Toronto, ON). Plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (LINCO Research, St Charles, MO), and pancreatic insulin levels were determined using a Rat insulin RIA kit (LINCO Research). Hb$_{A1c}$ levels were measured using a DCA 2000+ Analyzer (Bayer). Plasma glucagon and leptin were measured using a Mouse Endocrine LINCOplex kit (LINCO Research). Serum monocyte chemoattractant protein-1 (MCP-1), total plasminogen activator inhibitor-1 (PAI-1), and resistin levels were measured using a Mouse Adipokine LINCOplex kit (LINCO Research). Hepatic and/or serum levels of total cholesterol and triglycerides were obtained using specific colorimetric assays (Wako Cholesterol E and Wako L-Type TG-H; Wako Chemicals, Richmond, VA).

In Vitro cAMP Production

Baby hamster kidney fibroblast cells stably transfected with the rat GLP-1R[5] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Cells were incubated with 1 μmol/L exendin (9–39) or medium alone for 5 minutes at 37°C, followed by an additional 10-minute incubation in the presence of increasing concentrations of Ex-4 or CJC-1134-PC. All reactions were carried out in triplicate and terminated by the addition of ice-cold absolute ethanol. cAMP concentration was measured in ethanol extracts using a cAMP RIA kit (Biomedical Technologies, Stoughton, MA).

Glucose Tolerance Tests

Oral glucose tolerance tests (OGTT) and intraperitoneal (IP) glucose tolerance tests (IPGTT) were performed after an overnight fast of 16–18 hours after administration of glucose (1.5 mg/g body weight). For acute studies, CJC-1134-PC or HSA was given via IP injection 1 hour prior to glucose administration, whereas glucose-dependent insulinotropic peptide was injected 10 minutes before glucose administration. For chronic studies, no drugs were administered prior to the glucose tolerance tests.

Feeding Studies

Mice were fasted overnight for 16–18 hours, weighed, and then given IP injections of PBS or 10
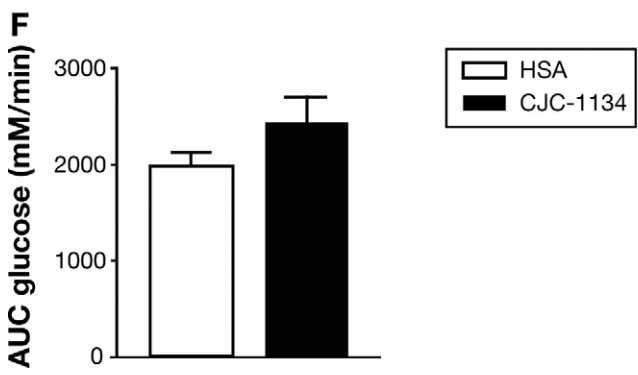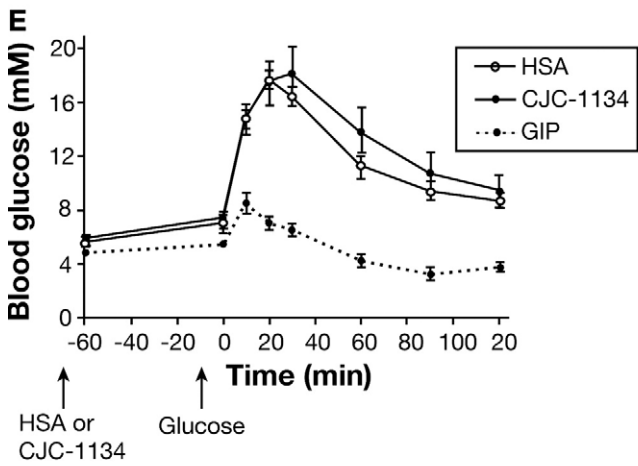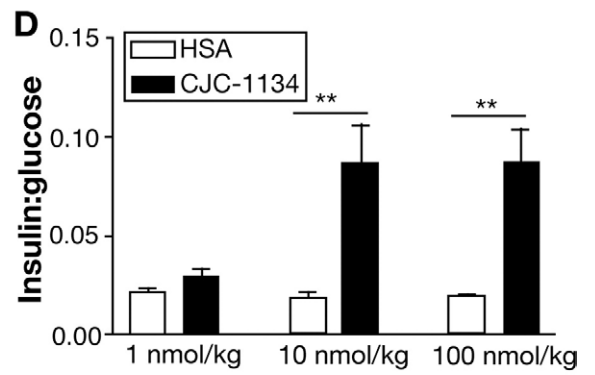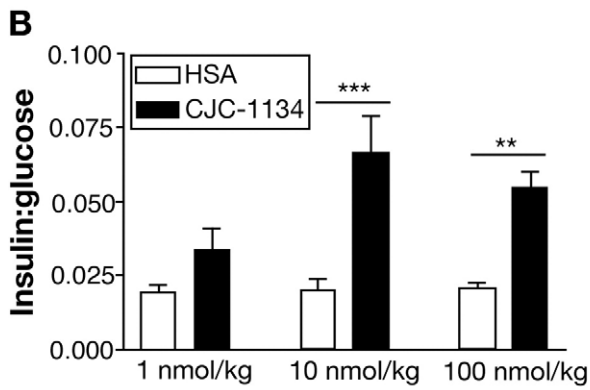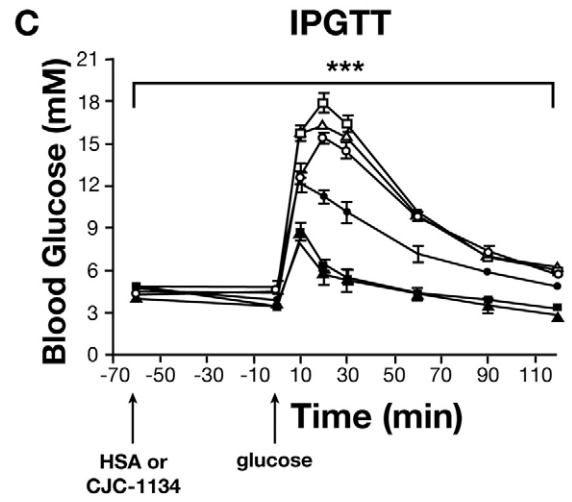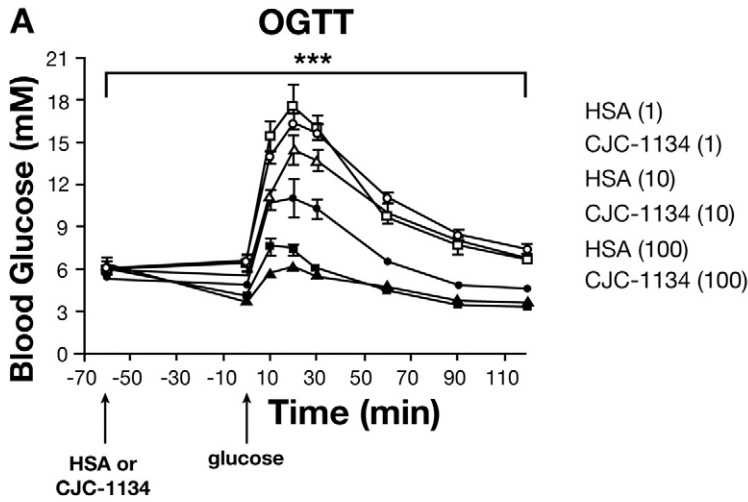
Figure 1. CJC-1134-PC reduces glucose excursions in wild-type but not Glp1r$^{-/-}$ mice. (*A*) Oral (*OGTT*) and (*C*) intraperitoneal (*IPGTT*) glucose tolerance in wild-type mice following IP administration of different doses (nmol/kg) of CJC-1134-PC or human serum albumin (HSA) 60 minutes prior to glucose loading. ***$P < .001$ for AUC data (not shown) for CJC-1134-PC- vs HSA-treated mice at each dose. (*B* and *D*) Plasma insulin-to-glucose ratios at the 10- to 20-minute time point after glucose administration in wild-type mice treated with the indicated doses of CJC-1134-PC or HSA. (*E*) Oral glucose tolerance and (*F*) AUC glucose in Glp1r$^{-/-}$ mice following IP administration of 100 nmol/kg CJC-1134-PC and HSA or 3 μg glucose-dependent insulinotropic peptide (GIP) at 60 minutes (CJC-1134-PC and HSA) or 10 minutes (GIP) prior to oral glucose challenge. Values are expressed as means $\pm$ SE; n = 4–11 mice/group. **$P < .01$, ***$P < .001$ for CJC-1134-PC- vs HSA-treated mice.

BASIC-
ALIMENTARY TRACT

nmol/kg of Ex-4, HSA, or CJC-1134-PC. The mice were then placed into individual cages containing preweighed rodent chow with free access to water. Food was removed from the cages at 2, 4, 8, and 24 hours following treatment, and food intake (g/g body weight) was calculated.

Measurement of Gastric Emptying Rate

The rate of gastric emptying was determined as described[14] 4 hours after peptide injections. The gastric emptying rate was calculated using the following equation: gastric emptying rate (%) = [1 − (stomach content wet weight/food intake)] × 100.

Evaluation of c-Fos Immunoreactivity in the Murine CNS

The number of c-Fos immunopositive neurons was quantified in regions of the mouse CNS as described.[15,16] Sections were processed for immunocytochemical detection of Fos using an avidin-biotin-immunoperoxidase method (Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA) as described.[17] The Fos antibody (Sigma-Aldrich, Oakville, ON) was used at a dilution of 1:50,000. Sections corresponding to CNS regions at the level of the area postrema, nucleus of the solitary tract, and paraventricular nucleus of the hypothalamus were defined according to Franklin and Paxinos.[18]

Body Composition

Total body fat and lean mass were measured using a mouse whole-body magnetic resonance analyzer (Echo Medical Systems, Houston, TX).

β-Cell Mass Determination

Pancreatic sections were immunostained for insulin as described.[13] Morphometric measurements of insulin-positive area and total pancreatic area were determined using ImageScope software (Aperio Technologies Inc, Vista, CA), and β-cell mass was calculated using the following equation: (insulin positive area/total pancreatic area) × pancreas weight. For each pancreas sample, the β-cell mass was averaged from 2 different slides that were obtained from the same pancreas sectioned at 2 different levels.

Hepatic Lipids

For histologic examination of hepatic lipids, the liver was removed, embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrence, CA), frozen rapidly in a dry ice/ethanol bath, and stored at −80°C. Frozen tissue was cut into 8-μm sections and stained with Oil-red O using standard protocols. For hepatic lipid determinations, hepatic lipids were extracted as described.[19]

RNA Isolation and Real-time Polymerase Chain Reaction

RNA was extracted from pancreas samples using TRI reagent (Sigma Chemical Co) and first-strand complementary DNA (cDNA) generated using standard protocols. Primers and polymerase chain reaction (PCR) conditions for the selected genes were those recommended by Applied Biosystems (Foster City, CA). Relative messenger RNA (mRNA) levels were quantified using the $2^{-\Delta\Delta Ct}$ method.[20]

Statistical Analysis

All data are presented as means ± SE. Statistical significance was determined by 1- or 2-way ANOVA and Bonferroni post hoc test using Prism version 4.02 software (GraphPad Software, San Diego, CA). A P value < .05 was considered to be statistically significant.

Results

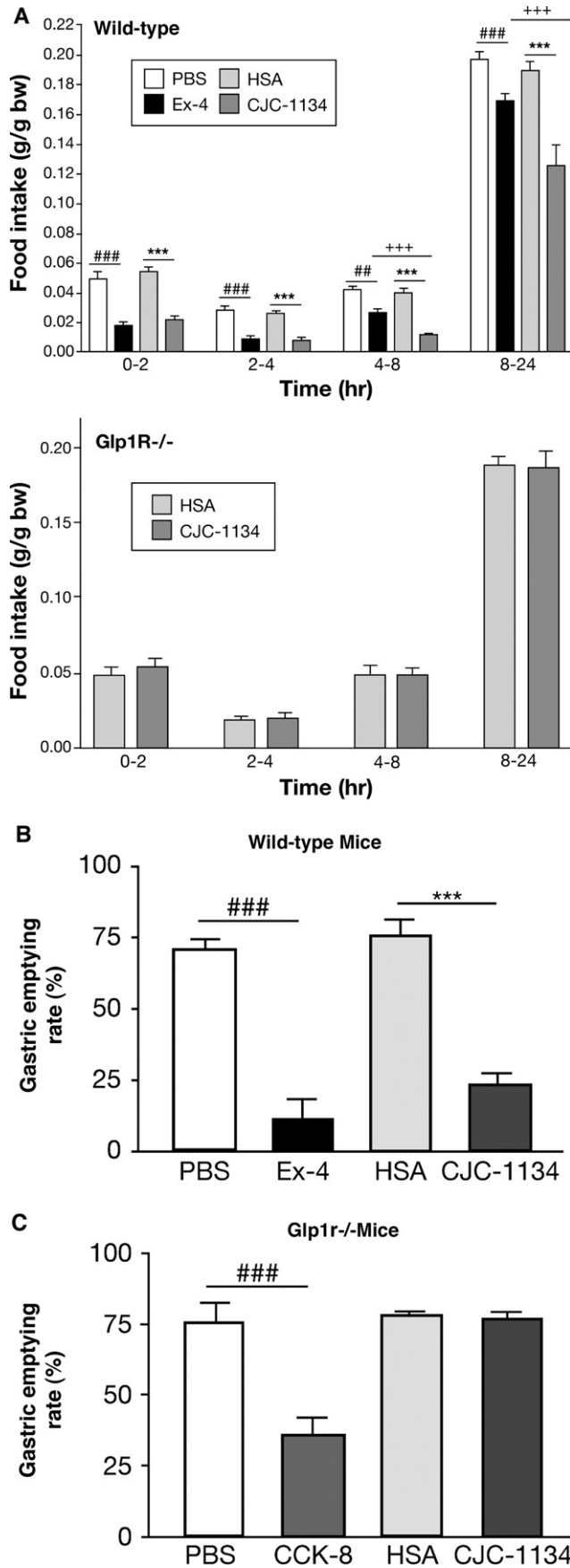### CJC-1134-PC Increases cAMP Production In Vitro

To determine whether covalent attachment of a large molecule like HSA impaired the ability of the Ex-4 moiety within CJC-1134-PC to activate the GLP-1R, we compared the potency of CJC-1134-PC vs Ex-4 in vitro. Both Ex-4 and CJC-1134-PC increased cAMP levels in baby hamster kidney-GLP-1R cells in a dose- and GLP-1R-dependent manner (see Supplementary Figure 2 online at www.gastrojournal.org) with half maximal effective concentration values for CJC-1134-PC and Ex-4 (3.47 nmol/L for CJC-1134-PC and 2.62 nmol/L for Ex-4).

CJC-1134-PC Mimics the Acute Actions of Ex-4 on Glucose Regulation, Feeding, and Gastric Emptying in Mice

We next examined whether CJC-1134-PC can reproduce the repertoire of actions associated with the smaller Ex-4 peptide. CJC-1134-PC significantly reduced glucose excursion after oral and IP glucose challenge in a dose-dependent manner in wild-type mice (Figure 1A and C) but not in Glp1r$^{-/-}$ mice (Figure 1E and F) with significant increases in insulin/glucose ratios at doses of 10 and 100 nmol/kg (Figure 1B and D). In contrast, glucose-dependent insulinotropic peptide potently decreased glucose excursion in Glp1r$^{-/-}$ mice (Figure 1E).

CJC-1134-PC significantly reduced food intake in wild-type mice (Figure 2), and the effect of CJC-1134-PC and Ex-4 to decrease feeding lasted for at least 24 hours (Figure 2A). Moreover, at 4–8 hours and 8–24 hours following injection, the reduction in food intake was significantly greater with CJC-1134-PC compared with Ex-4 (Figure 2). The anorectic action of CJC-1134-PC was not simply a nonspecific aversive response because it required a functional GLP-1 receptor (Figure 2A).

The mechanisms through which GLP-1 regulates gastric emptying remain incompletely understood and may require interaction with neural pathways.[21,22] Both Ex-4 and CJC-1134-PC significantly diminished the gastric emptying rate in wild-type mice (Figure 2B) but not in Glp1r$^{-/-}$ mice (Figure 2C). In contrast, cholecystoki-

nin-8, which inhibits gastric emptying through a distinct mechanism, significantly reduced the gastric emptying rate in Glp1r$^{-/-}$ mice (Figure 2C). To determine whether GLP-1R agonists communicate with the CNS to inhibit feeding and gastric emptying, we first compared the effects of peripheral administration of Ex-4 vs CJC-1134-PC on c-Fos positivity, a marker of neuronal activity (Figure 3). Ex-4 and CJC-1134-PC produced a similar pattern of c-Fos expression in the mouse CNS that included the area postrema, nucleus of the solitary tract, and paraventricular nucleus of the hypothalamus (Figure 3A, B, and C, respectively), regions associated with the control of feeding and gut motility. Moreover, the relative level of c-Fos activation following IP CJC-1134-PC was comparable with that of Ex-4 (Figure 3A–C). Conversely, IP injection of Ex-4 or CJC-1134-PC failed to increase c-Fos expression in Glp1r$^{-/-}$ mice (Figure 3A–C, bar graphs). Immunohistochemical examination of the mouse CNS following IP administration of HSA did not identify any HSA-immunopositive staining (data not shown). Because larger molecules like CJC-1134-PC are unlikely to readily cross the normal blood-brain barrier, these data, taken together with the results of previous experiments,[23] suggest that GLP-1R agonists do not require direct access to the CNS to modify feeding behavior and gastric motility.

Chronic Administration of CJC-1134-PC Improves Glucose Tolerance and Reduces Body Weight and Fat Mass in HFD-Fed Mice

Prolonged administration of GLP-1 or Ex-4 reduces blood glucose levels and promotes weight loss in individuals with T2DM.[6,24,25] Accordingly, mice were rendered obese and glucose intolerant and then treated with once or twice daily IP injection of CJC-1134-PC or Ex-4, respectively, for 4 weeks (see Supplementary Figure 3 online at www.gastrojournal.org). Nonfasting blood glucose levels in HFD-fed CJC-1134-PC- and Ex-4-treated mice were lower compared with control mice (Figure 4A). Mice on the HFD gained more weight compared with mice maintained on the normal chow diet during the first 4 weeks (Figure 4B). However, HFD-fed mice treated with CJC-1134-PC or Ex-4

Figure 2. CJC-1134-PC inhibits feeding and gastric emptying rate in wild-type but not Glp1r$^{-/-}$ mice. (A) Following an overnight fast, wild-type or Glp1r$^{-/-}$ mice were give IP injections of PBS or 10 nmol/kg of exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC, and food intake was determined at 2, 4, 8, and 24 hours postinjection. Gastric emptying rate in (B) wild-type and (C) Glp1r$^{-/-}$ mice at 4 hours after IP administration of PBS, Ex-4 (24 nmol/kg), HSA (100 nmol/kg), CJC-1134-PC (100 nmol/kg), or cholecystokinin-8 (CCK-8; 3 μg). Values are expressed as means ± SE; n = 3–6 mice/group. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ for Ex-4- vs PBS-treated mice. $^{***}P < .001$ for CJC-1134-PC- vs HSA-treated mice. $^{+++}P < .001$ for CJC-1134-PC- vs Ex-4-treated mice.
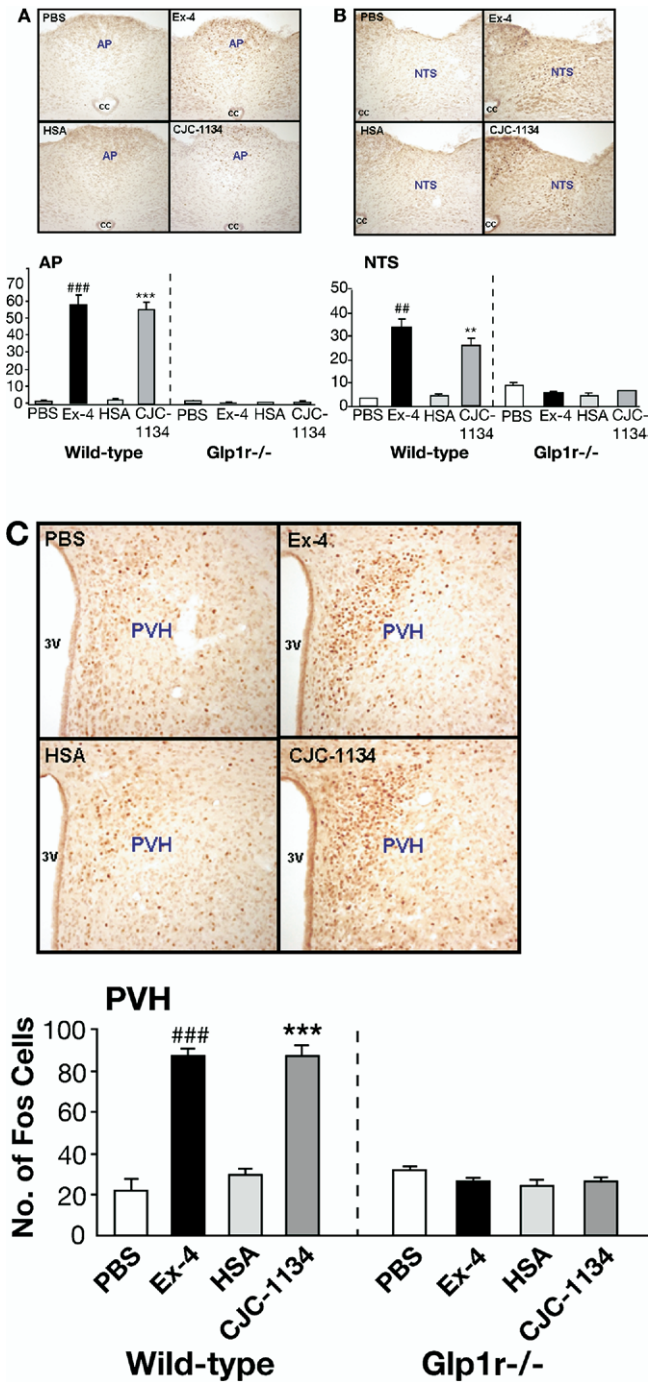
Figure 3. Peripheral administration of CJC-1134-PC increases c-Fos expression. Representative photomicrographs of c-Fos-stained mouse coronal brain sections of (*A*) area postrema (*AP*), (*B*) nucleus of the solitary tract (*NTS*), and (*C*) paraventricular nucleus of the hypothalamus (*PVH*) in wild-type mice at 60 minutes following IP injection of PBS, exendin-4 (Ex-4; 24 nmol/kg), human serum albumin (HSA; 100 nmol/kg), or CJC-1134-PC (100 nmol/kg). Original magnification, ×200. CC, central canal; 3V, third ventricle. The number of c-Fos+ cells in wild-type and Glp1r$^{-/-}$ mice is indicated below the corresponding CNS section. Data are presented as means ± SE; n = 3–5 mice/group. $^{##}P < .01$, $^{###}P < .001$ for Ex-4- vs PBS-treated mice. $^{**}P < .01$, $^{***}P < .001$ for CJC-1134-PC- vs HSA-treated mice.
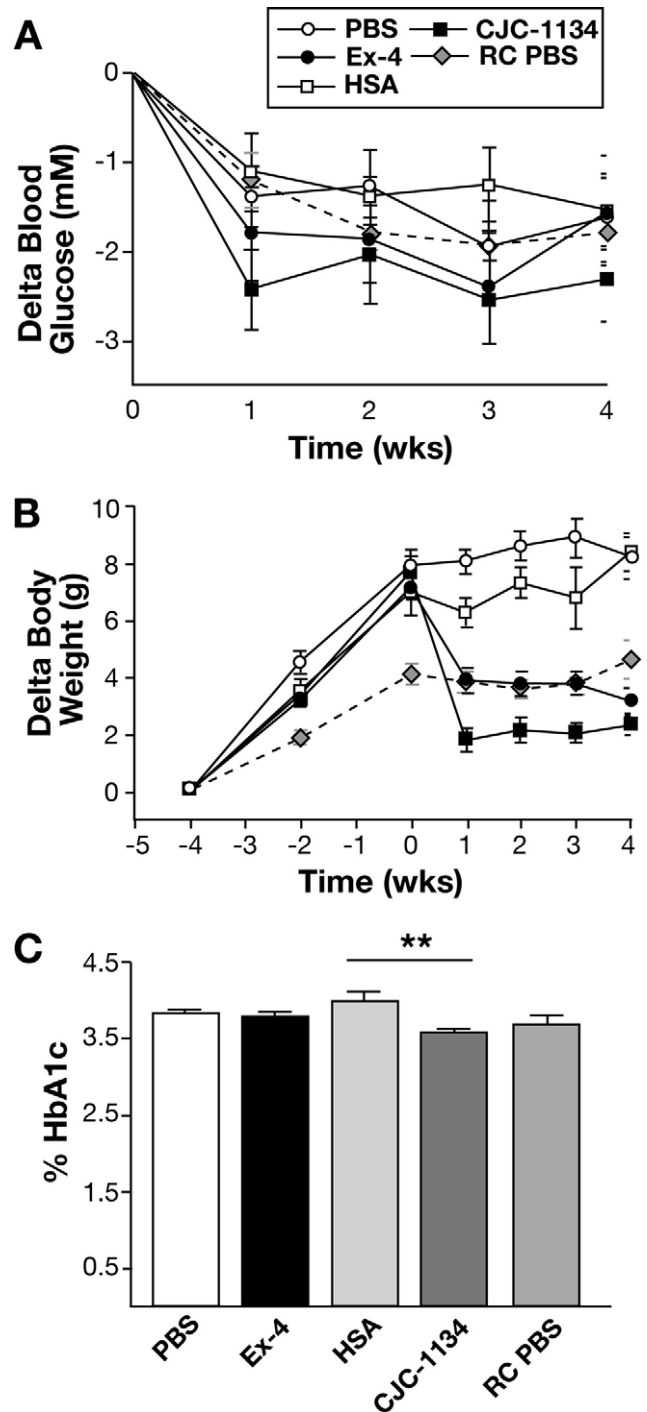

Figure 4. CJC-1134-PC reduces body weight, nonfasting blood glucose, and Hb$_{A1c}$ in high-fat diet (HFD)-fed wild-type mice. Wild-type mice were placed on an HFD for 4 weeks and then treated for 4 weeks with daily IP injections of 100 nmol/kg of human serum albumin (HSA) or CJC-1134-PC or twice-a-day injections of PBS or exendin-4 (Ex-4; 24 nmol/kg). Separate groups of mice maintained on regular chow throughout the entire 8-week study (RC PBS) were given IP injections of PBS twice a day. The change in (*A*) nonfasting blood glucose and (*B*) body weight from baseline was determined weekly. (*C*) Percentage Hb$_{A1c}$ in whole blood after 4 weeks of treatment with PBS, Ex-4, HSA, or CJC-1134-PC in HFD-fed wild-type mice or in regular chow-fed mice treated with PBS (RC PBS). Data represent means ± SE; n = 7–8 mice/group. $^{**}P < .01$ for CJC-1134-PC- vs HSA-treated mice.

Figure 5. CJC-1134-PC improves glucose tolerance in HFD-fed wild-type mice. (*A* and *D*) glucose excursion, (*B* and *E*) AUC glucose, and (*C* and *F*) plasma insulin-to-glucose ratios during an intraperitoneal (*IPGTT*) or oral (*OGTT*) glucose tolerance test in high-fat diet (HFD)-fed wild-type mice treated chronically with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated chronically with PBS (RC PBS). Plasma insulin-to-glucose ratios were determined at the 10- to 20-minute time point following glucose challenge. Values are expressed as means ± SE; n = 7–8 mice/group. ##$P <$ .01, ###$P <$ .001 for Ex-4- vs PBS-treated mice. *$P <$ .05, ***$P <$ .001 for CJC-1134-PC- vs HSA-treated mice.
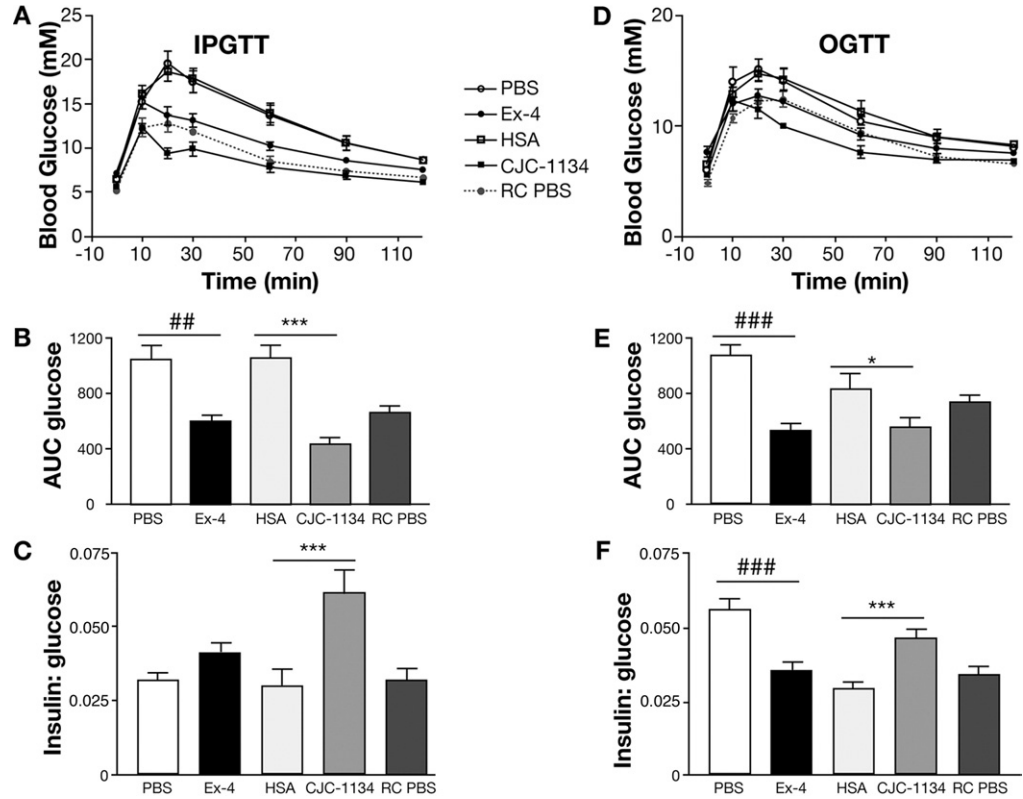
lost weight, and, after 4 weeks of treatment, mice treated with CJC-1134-PC had body weights similar to normal chow-fed PBS-treated mice (Figure 4*B*). Serum Hb$_{A1c}$ levels were reduced in HFD-fed mice treated with CJC-1134-PC but not Ex-4 (Figure 4*C*).

Chronic administration of Ex-4 or CJC-1134-PC in HFD-fed mice produced significant reductions in glucose excursions following glucose challenge (Figure 5*A*, *B*, *D*, and *E*), and CJC-1134-PC but not Ex-4 increased insulin-to-glucose ratios measured following glucose administration (Figure 5*C* and *F*). Chronic treatment with CJC-1134-PC or Ex-4 had no effect on plasma glucagon levels measured in these same samples (data not shown). Magnetic resonance imaging (MRI) data demonstrated that Ex-4 and CJC-1134-PC reduced total and percent body fat mass and increased percent lean mass relative to HFD-fed control treated mice (Figure 6*A* and *B*). Furthermore, body weight and epididymal fat pad weights were significantly decreased in CJC-1134-PC- and Ex-4-treated HFD-fed mice and were comparable with those observed in PBS-treated mice maintained on a normal chow diet (Figure 6*C* and data not shown).

CJC-1134-PC or Ex-4 had no effect on serum MCP-1 or PAI-1 levels in HFD-fed mice (Figure 7*A* and *B*), whereas treatment with Ex-4, but not CJC-1134-PC, led to significant reductions in serum resistin (Figure 7*C*). Serum leptin levels were significantly lower in both CJC-1134-PC- and Ex-4-treated mice (Figure 7*D*).

Serum cholesterol levels were reduced in HFD-fed mice following administration of CJC-1134-PC but not Ex-4 (Figure 8*A*). Surprisingly, chronic treatment with both CJC-1134-PC and Ex-4 resulted in significant elevations in serum triglyceride levels (Figure 8*B*). Although hepatic cholesterol levels were unchanged (data not shown), total hepatic triglyceride content was reduced following treatment with CJC-1134-PC or Ex-4 in HFD-fed mice (Figure 8*C*). Oil-Red-O staining (see Supplementary Figure 4 online at www.gastrojournal.org) further illustrated the reduced hepatic lipid content in CJC-1134-PC- and Ex-4-treated mice. The decrease in hepatic triglyceride, in association with increased plasma triglyceride levels, raised the possibility that mice treated with CJC-1134-PC or Ex-4 fail to accumulate triglyceride in the liver, perhaps leading to increased circulating levels of triglycerides. Hence, we examined the expression of hepatic genes whose products are important for lipid absorption, transport, metabolism, or synthesis. Real-time quantitative PCR demonstrated that treatment with CJC-1134-PC or Ex-4 was associated with significantly reduced levels of mRNA transcripts for fatty acid binding proteins 1 and 2 (Fabp1 and Fabp2) and CD36, respectively (Figure 8*D*–*F*). Conversely, CJC-1134-PC or Ex-4 administration did not alter the mRNA levels of apolipoprotein B, hepatic lipase, peroxisomal proliferator-activated receptor α, or sterol regulatory element binding protein-1 (Srebf1) (data not shown).
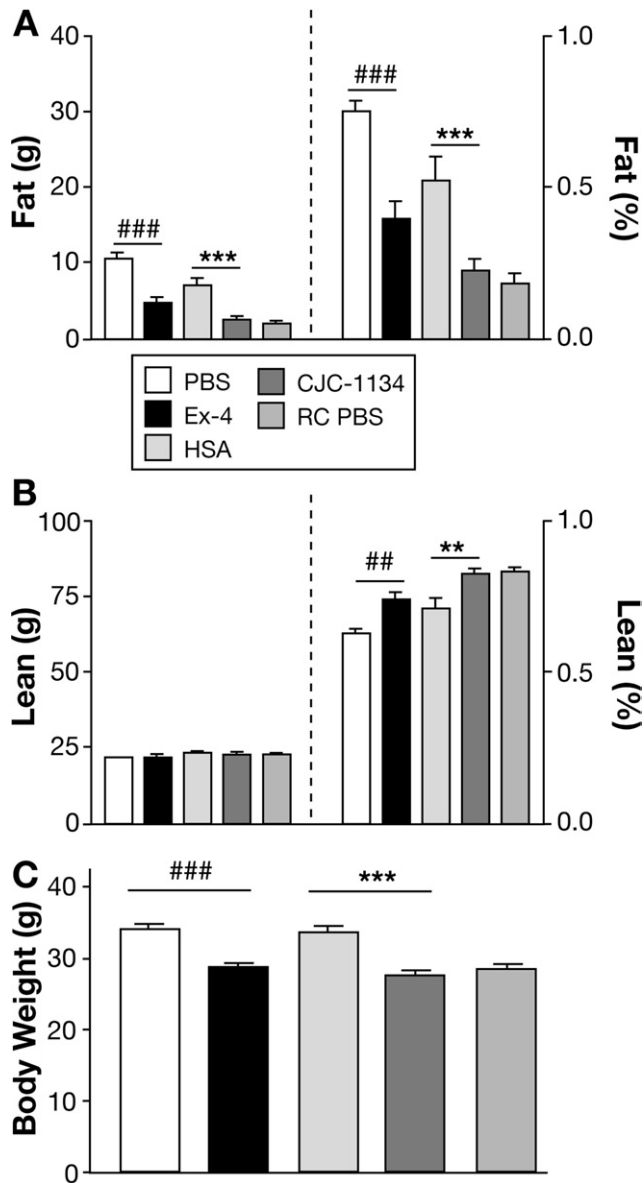
Figure 6. CJC-1134-PC decreases body weight and fat mass in HFD-fed wild-type mice. MRI determinations of (*A*) fat mass and (*B*) lean mass, expressed as total (grams; [*g*]) or normalized to body weight (%), in high-fat diet (HFD)-fed wild-type mice after 2 weeks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated with PBS (RC PBS) for 2 weeks. (*C*) Body weight in HFD-fed mice after 4 weeks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated with PBS (RC PBS). Data are expressed as means ± SE; n = 7–8 mice/group. $^{##}P < .01$, $^{###}P < .001$ for Ex-4- vs PBS-treated mice. $^{**}P < .01$, $^{***}P < .001$ for CJC-1134-PC- vs HSA-treated mice.

Chronic Administration of CJC-1134-PC Up-regulates Expression of β-Cell-Specific Genes and Increases Pancreas Weight but Has No Effect on β-Cell Mass in HFD-Fed Mice

Ex-4, but not CJC-1134-PC, increased insulin mRNA levels compared with saline-treated mice (see Supplementary Figure 5A online at www.gastrojournal.org).

In contrast, pancreas insulin content was significantly reduced in CJC-1134-PC-treated mice relative to levels in mice treated with HSA but was not significantly different in Ex-4- vs control-treated mice (see Supplementary Figure 5B online at www.gastrojournal.org). Although both CJC-1134-PC and Ex-4 increased pancreas weights in HFD-fed mice (see Supplementary Figure 5C online at www.gastrojournal.org), no significant increases in β-cell mass were observed following chronic treatment with either agent (see Supplementary Figure 5D online at www.gastrojournal.org). GLP-1R agonists also increase the expression of genes that contribute to β-cell glucose sensing, proliferation, neogenesis, or cytoprotection.[2,26,27] mRNA levels of *Gck*, *Glp1r*, and *Pdx1* were increased in pancreatic RNA from both CJC-1134-PC- and/or Ex-4-treated mice (see Supplementary Figure 5E–G online at www.gastrojournal.org). Surprisingly however, Ex-4, but not CJC-1134-PC, markedly increased levels of mRNA transcripts for *Irs2* (see Supplementary Figure 5H online at www.gastrojournal.org).

Discussion

Although there is much interest in developing GLP-1-based strategies for the treatment of T2DM,[1,6] its short half-life in the circulation has posed practical limitations on the clinical use of the native peptide. Exenatide is the first DPP-4 resistant GLP-1R agonist approved for the treatment of T2DM. However, the need for twice daily injections of exenatide has spawned efforts to generate GLP-1R agonists with more prolonged pharmacokinetic and pharmacodynamic properties. To this end, albumin binding has been utilized as a strategy to prolong GLP-1R agonist action in vivo, as exemplified by the interaction of acylated liraglutide with albumin, resulting in a circulating half-life of ~11–15 hours in humans.[28] Nevertheless, because liraglutide interacts with albumin in a noncovalent manner and is also cleared by the kidney, it requires once daily administration to achieve continuous GLP-1R activation.

CJC-1134-PC is distinct from liraglutide in that it is a preformed conjugate consisting of Ex-4 that has been covalently bound ex vivo to recombinant HSA via a chemical linker. Our experimental data, encompassing receptor activation studies in vitro and acute and chronic experiments in vivo, demonstrate that the larger albumin-Ex-4 conjugate reproduces the majority of biologic actions associated with the much smaller GLP-1R peptide agonist Ex-4.

Surprisingly, despite its larger size, CJC-1134-PC was equipotent to Ex-4 in its ability to stimulate cAMP production in contrast to Albugon (Albiglutide), a recombinant GLP-1-albumin fusion protein that exhibited reduced affinity and potency for the GLP-1R.[23] Albugon contains the peptide sequences of GLP-1 within the open reading frame of recombinant HSA, whereas CJC-1134-PC contains an Ex-4 molecule linked covalently to a

Figure 7. CJC-1134-PC reduces resistin and leptin levels in HFD-fed wild-type mice. Serum levels of (A) MCP-1, (B) plasminogen activator inhibitor-1 (PAI-1), (C) resistin, and (D) leptin were assayed after 4 weeks of treatment with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC in HFD-fed wild-type mice or in regular chow-fed mice treated with PBS (RC PBS). Data are expressed as means ± SE; n = 4–8 mice/group. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ for Ex-4- vs PBS-treated mice. $^{***}P < .001$ for CJC-1134-PC- vs HSA-treated mice.
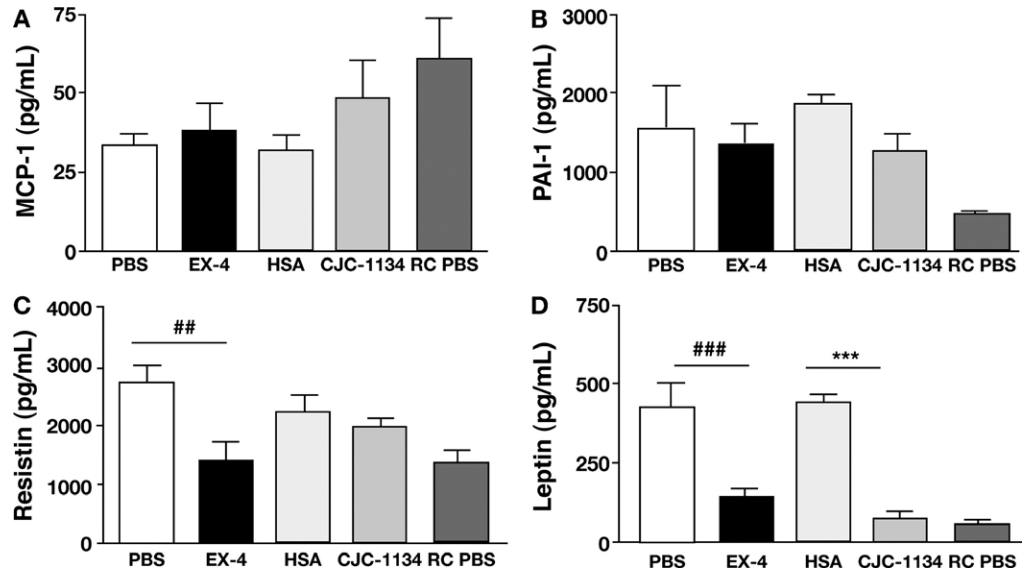
single cysteine residue on HSA. We suspect that the Ex-4 moiety within CJC-1134-PC is less constrained and likely more mobile than the GLP-1 epitope within Albugon, perhaps accounting for the marked difference in potency at the GLP-1R. Hence, one might predict that therapy with CJC-1134-PC may require lower circulating levels relative to treatment with Albugon, a hypothesis that may be tested in future studies. Consistent with the results of receptor studies, CJC-1134-PC and Ex-4 were administered at equimolar doses in the feeding study, and CJC-1134-PC was as effective as Ex-4 at reducing food intake. Hence, the larger molecular weight CJC-
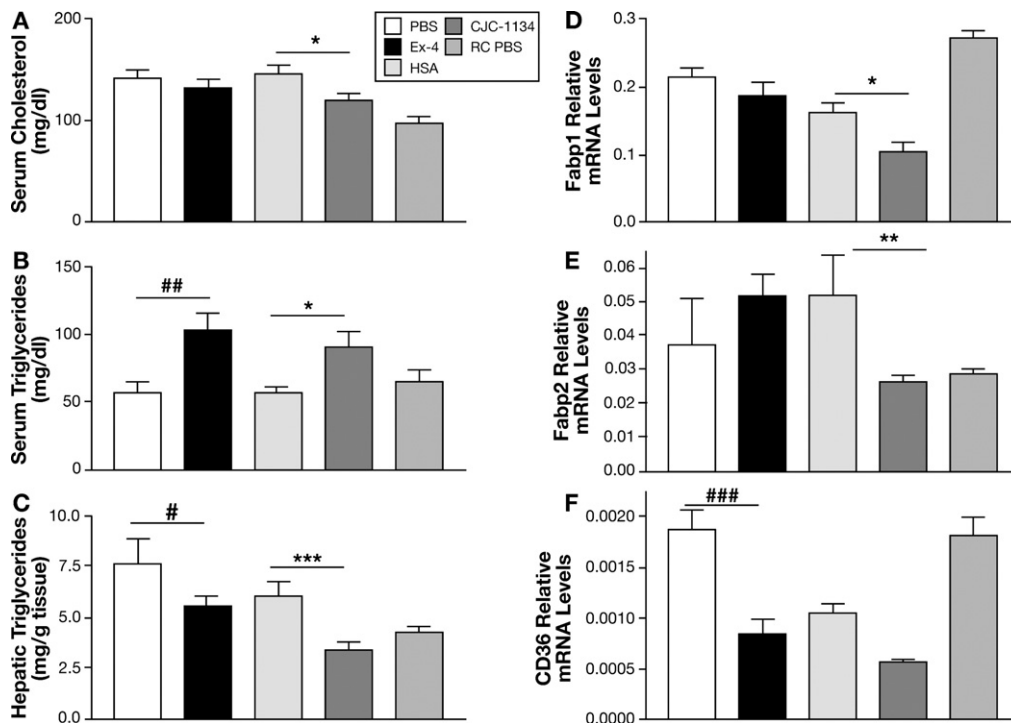
Figure 8. Serum triglyceride levels are increased, but hepatic triglyceride and mRNA levels of fatty acid transport proteins are reduced in mice treated with CJC-1134-PC. Serum cholesterol (A) and triglyceride (B) levels and heptic triglyceride content (C) in HFD-fed wild-type mice treated chronically with PBS, exendin-4 (Ex-4), human serum albumin (HSA), or CJC-1134-PC or in mice maintained on normal chow and treated chronically with PBS (RC PBS). Data are means ± SE; n = 6–7 mice/group. Relative hepatic mRNA levels of (D) Fabp1, (E) Fabp2, and (F) CD36 determined by real-time quantitative PCR in HFD-fed wild-type after chronic treatment with PBS, Ex-4, HSA, or CJC-1134-PC or in RC-fed mice treated with PBS. Data are means ± SE and are normalized to 18S rRNA; n = 3 or 4 mice/group. For A–F, $^{\#}P < .05$, $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ for Ex-4- vs PBS-treated mice and $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ for CJC-1134-PC- vs HSA-treated mice.

1134-PC is as potent an inhibitor of GLP-1R-dependent feeding as the small Ex-4.

Small GLP-1R agonists like GLP-1 and Ex-4 inhibit feeding and gastric emptying either by direct activation of CNS centers or indirectly through activation of vagal afferent pathways.[21,29,30] Because large proteins like albumin cannot readily cross an intact blood-brain barrier,[31] the ability of CJC-1134-PC to inhibit food intake and gastric emptying suggests that GLP-1R agonists do not require direct access to the CNS to mediate these effects, which is in agreement with data demonstrating that GLP-1 and/or Ex-4 modify feeding and gut motility via the vagus.[21,32,33] These results are corroborated by the demonstration that peripherally administered CJC-1134-PC activates c-fos expression in the murine CNS and produces weight loss in mice. Hence, high-molecular-weight GLP-1R agonists such as CJC-1134-PC remain capable of activating circuits regulating gastric emptying and satiety in a GLP-1R-dependent manner.

In humans, GLP-1 or Ex-4 treatment has been associated with significant reductions in postprandial plasma triglyceride levels, whereas chronic treatment with either CJC-1134-PC or Ex-4 led to significantly increased non-fasting plasma triglyceride levels but reduced hepatic lipid content. These results highlight key differences between the control of murine and human hepatic lipid homeostasis. Real-time PCR analysis revealed that CJC-1134-PC- and Ex-4-treatment reduced hepatic levels of genes that encode proteins involved in hepatic lipid uptake and intracellular transport (CD36, Fabp1, Fabp2), suggesting that lipids are being diverted from the liver.

Although demonstrated that GLP-1R agonists increase β-cell mass in diabetic rodents, we detected no increase in β-cell mass in HFD-fed mice that were treated chronically with CJC-1134-PC or Ex-4. Because HFD-feeding can lead to either no changes or increases in β-cell mass to compensate for HFD-induced insulin resistance,[34,35] it is possible that the replicative capacity of the β cell is maximized in our studies and cannot be further increased with CJC-1134-PC or Ex-4. Remarkably, although both Ex-4 and CJC-1134-PC increased levels of mRNA transcripts for the Glp1r, Gck, and Pdx1, only Ex-4 robustly increased levels of Irs2, a GLP-1R-activated signaling protein critically important for β-cell survival.[36] The explanation for this difference in gene expression profiles remains unclear but suggests that structurally different GLP-1R agonists may exhibit unique patterns of GLP-1R activation in different tissues.

Taken together, our findings provide additional evidence supporting the notion that high-molecular-weight GLP-1R agonists can activate a broad spectrum of GLP-1R-dependent actions. Notably, CJC-1134-PC potently activated c-Fos in multiple regions of the CNS, reduced gastric emptying and food intake in acute studies, and promoted weight loss in chronic experiments. These findings extend our understanding of the central and peripheral actions of GLP-1R agonists, with implications for the predicted use and efficacy of these new high-molecular-weight proteins for the treatment of T2DM.

Supplementary Data

References

1. Deacon CF. Therapeutic strategies based on glucagon-like peptide 1. Diabetes 2004;53:2181–2189.
2. Drucker DJ. The biology of incretin hormones. Cell Metab 2006; 3:153–165.
3. Xu G, Stoffers DA, Habener JF, et al. Exendin-4 stimulates both β-cell replication and neogenesis, resulting in increased β-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999;48:2270–2276.
4. Stoffers DA, Kieffer TJ, Hussain MA, et al. Insulinotropic glucagon-like peptide-1 agonists stimulate expression of homeodomain protein IDX-1 and increase β-cell mass in mouse pancreas. Diabetes 2000;49:741–748.
5. Li Y, Hansotia T, Yusta B, et al. Glucagon-like peptide-1 receptor signaling modulates β-cell apoptosis. J Biol Chem 2003;278: 471–478.
6. Drucker DJ, Nauck MA. The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. Lancet 2006;368:1696–1705.
7. Eng J, Kleinman WA, Singh L, et al. Isolation and characterization of exendin 4, an exendin 3 analogue from *Heloderma suspectum* venom. J Biol Chem 1992;267:7402–7405.
8. Young AA, Gedulin BR, Bhavsar S, et al. Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). Diabetes 1999;48:1026–1034.
9. Kolterman OG, Buse JB, Fineman MS, et al. Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. J Clin Endocrinol Metab 2003;88:3082–3089.
10. Agerso H, Jensen LB, Elbrond B, et al. The pharmacokinetics, pharmacodynamics, safety and tolerability of NN2211, a new long-acting GLP-1 derivative, in healthy men. Diabetologia 2002; 45:195–202.
11. Wang M, Kipnes MS, Matheson S, et al. Safety and pharmacodynamics of CJC-1134-PC, a novel GLP-1 receptor agonist, in patients with type 2 diabetes mellitus: a randomized, placebo-controlled, double-blind, dose-escalation study (Poster 498). Propanel Abstracts of the 67th Scientific Sessions of the American Diabetes Association, June 22–26, 2007, Chicago, IL, p A133.
12. Scrocchi LA, Brown TJ, MacLusky N, et al. Glucose intolerance but normal satiety in mice with a null mutation in the glucagon-like peptide receptor gene. Nat Med 1996;2:1254–1258.
13. Hansotia T, Maida A, Flock G, et al. Extrapancreatic incretin receptors modulate glucose homeostasis, body weight, and energy expenditure. J Clin Invest 2007;117:143–152.
14. Asakawa A, Inui A, Yuzuriha H, et al. Characterization of the effects of pancreatic polypeptide in the regulation of energy balance. Gastroenterology 2003;124:1325–1336.
15. Elias CF, Kelly JF, Lee CE, et al. Chemical characterization of leptin-activated neurons in the rat brain. J Comp Neurol 2000; 423:261–281.
16. Yamamoto H, Kishi T, Lee CE, et al. Glucagon-like peptide-1-responsive catecholamine neurons in the area postrema link
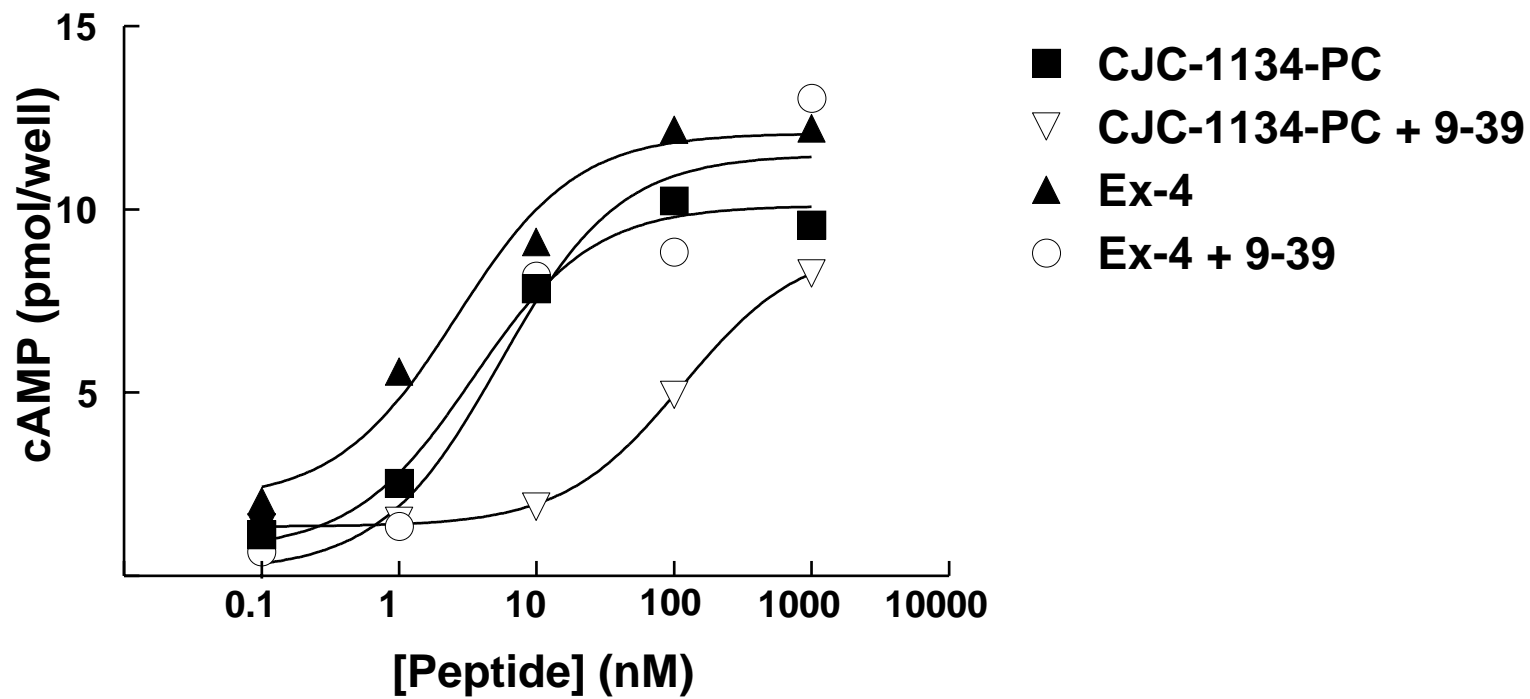
peripheral glucagon-like peptide-1 with central autonomic control sites. J Neurosci 2003;23:2939–2946.

17. Yamamoto H, Lee CE, Marcus JN, et al. Glucagon-like peptide-1 receptor stimulation increases blood pressure and heart rate and activates autonomic regulatory neurons. J Clin Invest 2002;110:43–52.

18. Franklin K, Paxinos G. The mouse brain in sterotaxic coordinates. Burlington, MA: Academic Press, 1997.

19. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497–509.

20. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) method. Methods 2001;25:402–408.

21. Imeryuz N, Yegen BC, Bozkurt A, et al. Glucagon-like peptide-1 inhibits gastric emptying via vagal afferent-mediated central mechanisms. Am J Physiol 1997;273:G920–G927.

22. Nagell CF, Wettergren A, Orskov C, et al. Inhibitory effect of GLP-1 on gastric motility persists after vagal deafferentation in pigs. Scand J Gastroenterol 2006;41:667–672.

23. Baggio LL, Huang Q, Brown TJ, et al. 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. Diabetes 2004;53:2492–2500.

24. Zander M, Madsbad S, Madsen JL, et al. Effect of 6-week course of glucagon-like peptide 1 on glycaemic control, insulin sensitivity, and β-cell function in type 2 diabetes: a parallel-group study. Lancet 2002;359:824–830.

25. DeFronzo RA, Ratner RE, Han J, et al. Effects of exenatide (exendin-4) on glycemic control and weight over 30 weeks in metformin-treated patients with type 2 diabetes. Diabetes Care 2005;28:1092–1100.

26. Wang YH, Egan JM, Raygada M, et al. Glucagon-like peptide-1 affects gene transcription and messenger ribonucleic acid stability of components of the insulin secretory system in RIN 1046-38 cells. Endocrinology 1995;136:4910–4917.

27. Drucker DJ. Glucagon-like peptides: regulators of cell proliferation, differentiation, and apoptosis. Mol Endocrinol 2003;17:161–171.

28. Elbrond B, Jakobsen G, Larsen S, et al. Pharmacokinetics, pharmacodynamics, safety, and tolerability of a single-dose of NN2211, a long-acting glucagon-like peptide 1 derivative, in healthy male subjects. Diabetes Care 2002;25:1398–1404.

29. Kinzig KP, D'Alessio DA, Seeley RJ. The diverse roles of specific GLP-1 receptors in the control of food intake and the response to visceral illness. J Neurosci 2002;22:10470–10476.

30. Hellstrom PM, Naslund E. Interactions between gastric emptying and satiety, with special reference to glucagon-like peptide-1. Physiol Behav 2001;74:735–741.

31. Chuang VT, Kragh-Hansen U, Otagiri M. Pharmaceutical strategies utilizing recombinant human serum albumin. Pharm Res 2002;19:569–577.

32. Abbott CR, Monteiro M, Small CJ, et al. The inhibitory effects of peripheral administration of peptide YY(3–36) and glucagon-like peptide-1 on food intake are attenuated by ablation of the vagal-brainstem-hypothalamic pathway. Brain Res 2005;1044:127–131.

33. Talsania T, Anini Y, Siu S, et al. Peripheral exendin-4 and peptide YY(3–36) synergistically reduce food intake through different mechanisms in mice. Endocrinology 2005;146:3748–3756.

34. Andrikopoulos S, Massa CM, Aston-Mourney K, et al. Differential effect of inbred mouse strain (C57BL/6, DBA/2, 129T2) on insulin secretory function in response to a high-fat diet. J Endocrinol 2005;187:45–53.

35. Hull RL, Andrikopoulos S, Verchere CB, et al. Increased dietary fat promotes islet amyloid formation and β-cell secretory dysfunction in a transgenic mouse model of islet amyloid. Diabetes 2003;52:372–379.

36. Jhala US, Canettieri G, Screaton RA, et al. cAMP promotes pancreatic β-cell survival via CREB-mediated induction of IRS2. Genes Dev 2003;17:1575–1580.

BASIC-ALIMENTARY TRACT

reactive component

linker

Cys 34
of HSA

Gln
Gln
HN
Pro
Phe

S

Pro-Pro-Pro-Ser-Lys

Ala-Gly-Ser-Ser-Pro-Gly-Gly-Asn-Lys-Leu-Trp-Glu

Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile

Ser-Leu-Asp-Ser-Thr-Phe-Thr-Gly-Glu-Gly-His
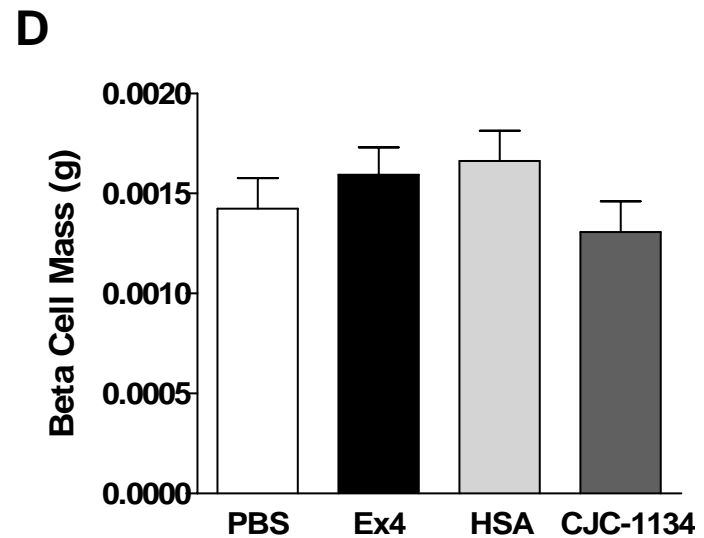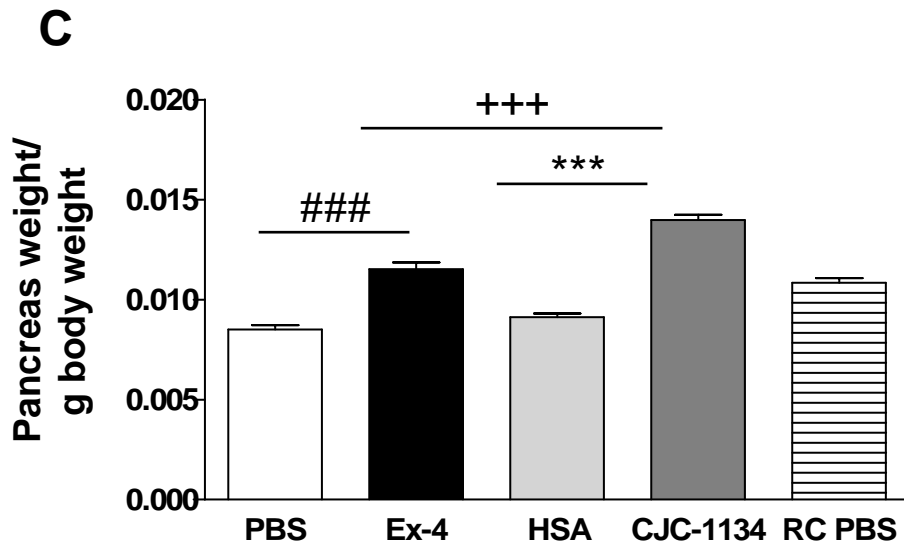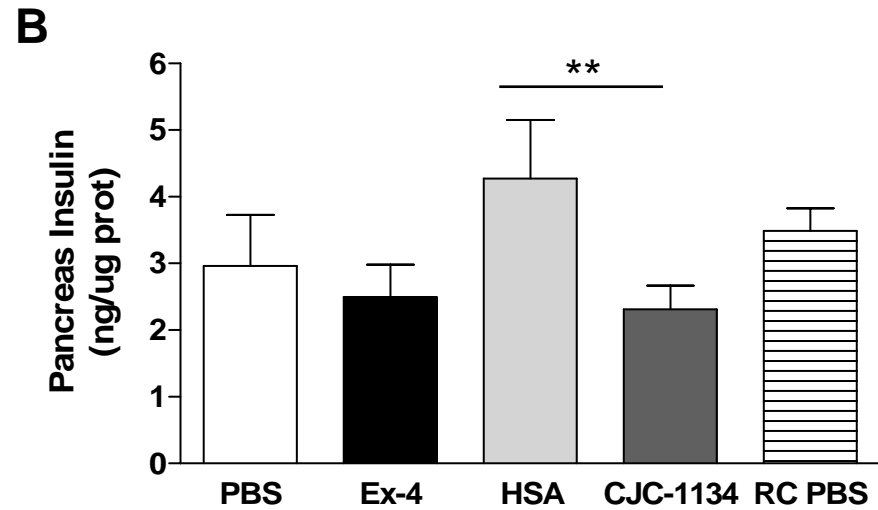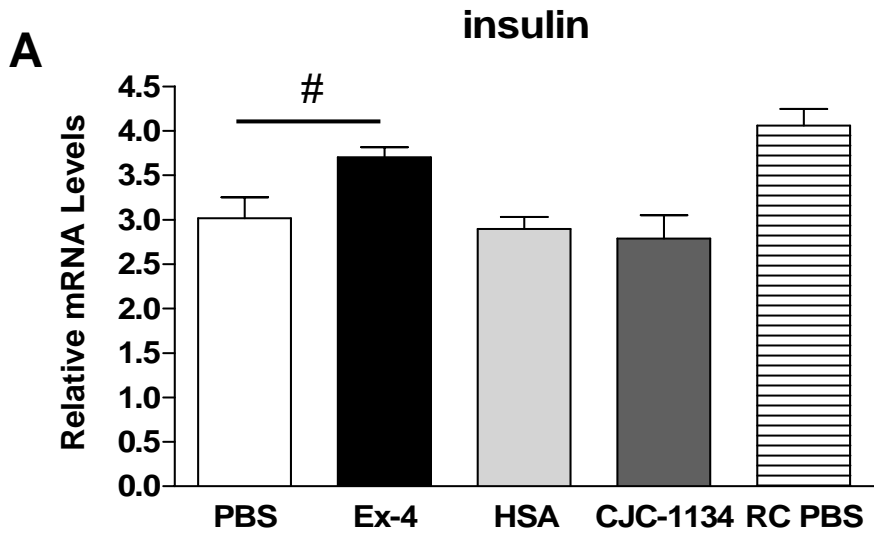
Exendin-4

Supplementary Figure 1

Supplementary Figure 2

Chronic Studies in wild-type C57BL/6 mice

Normal Chow – No Tx	Normal Chow + PBS 2x/day

Time (wks) 0   1   2   3   4   5   6   7   8

High Fat Diet – No Tx	High Fat Diet + Tx

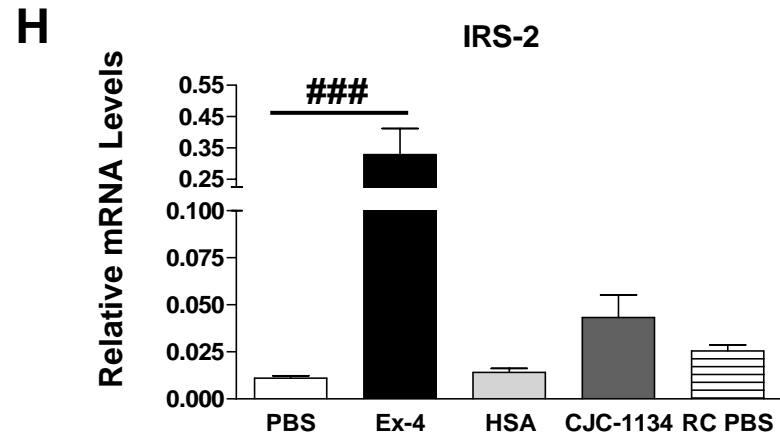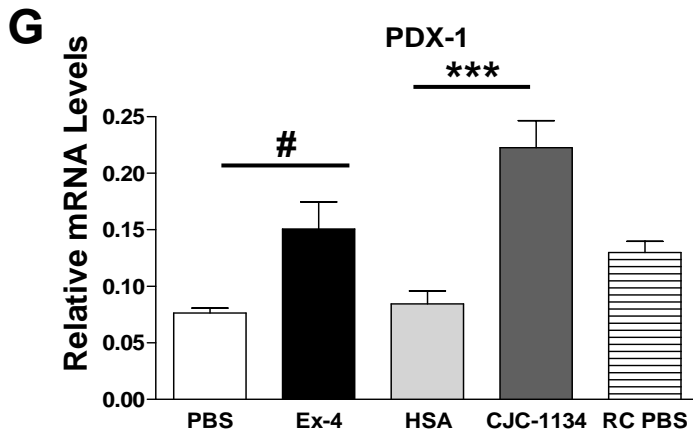Time (wks) 0   1   2   3   4   5   6   7   8

MRI   IPGTT   OGTT & end points
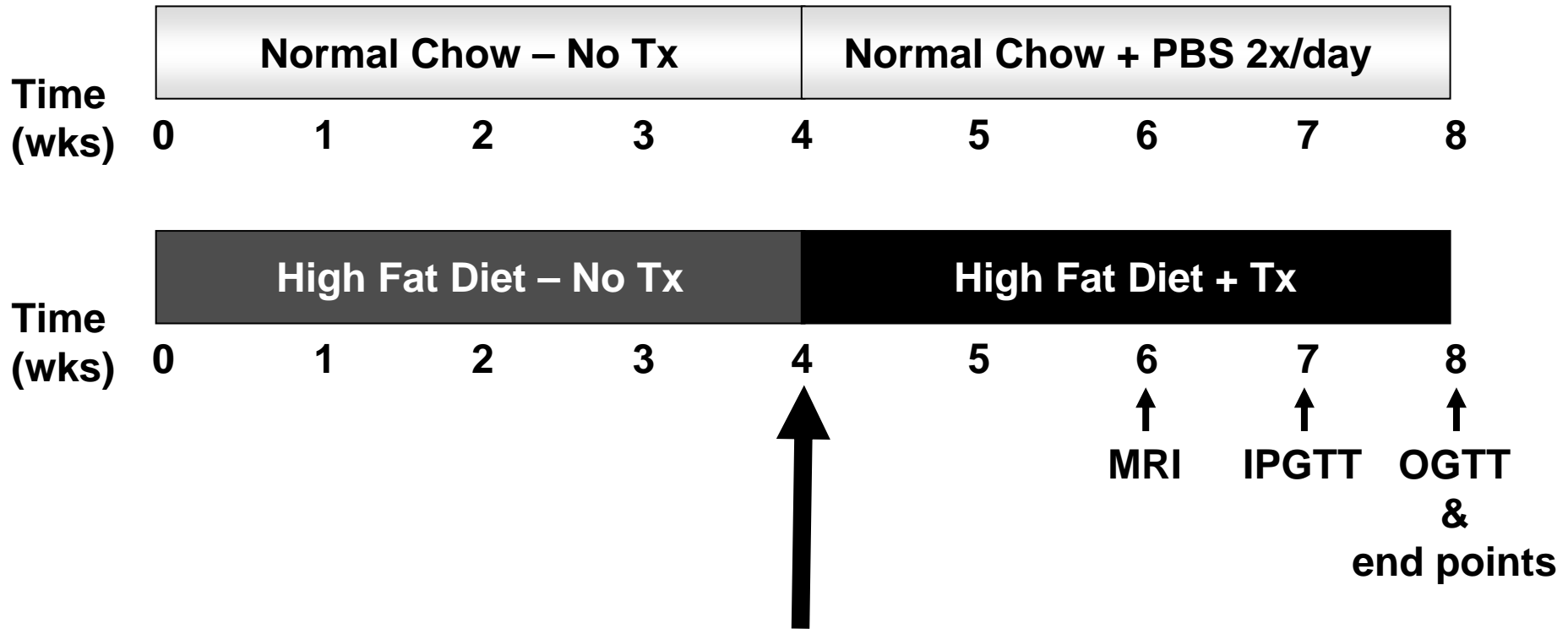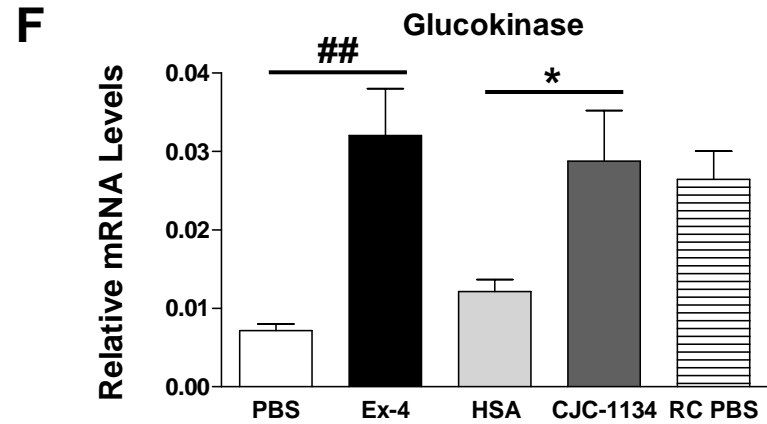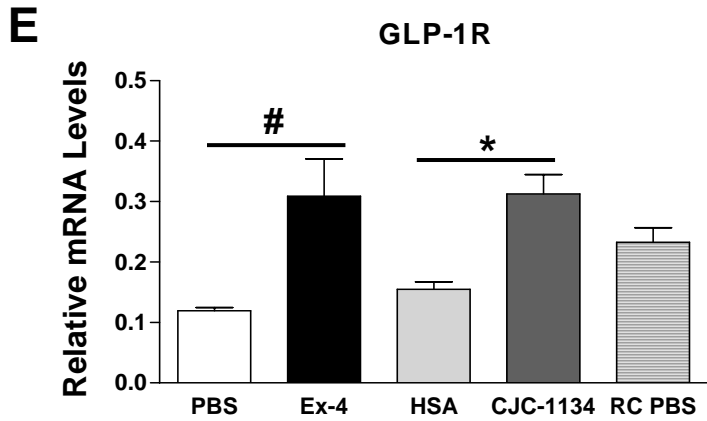
(i) twice daily (am and pm) PBS
(ii) twice daily (am and pm) Ex-4 (24 nmol/kg)
(iii) once daily (pm) HSA (100 nmol/kg)
(iv) once daily (pm) CJC-1134 (100 nmol/kg)

Supplementary Figure 3
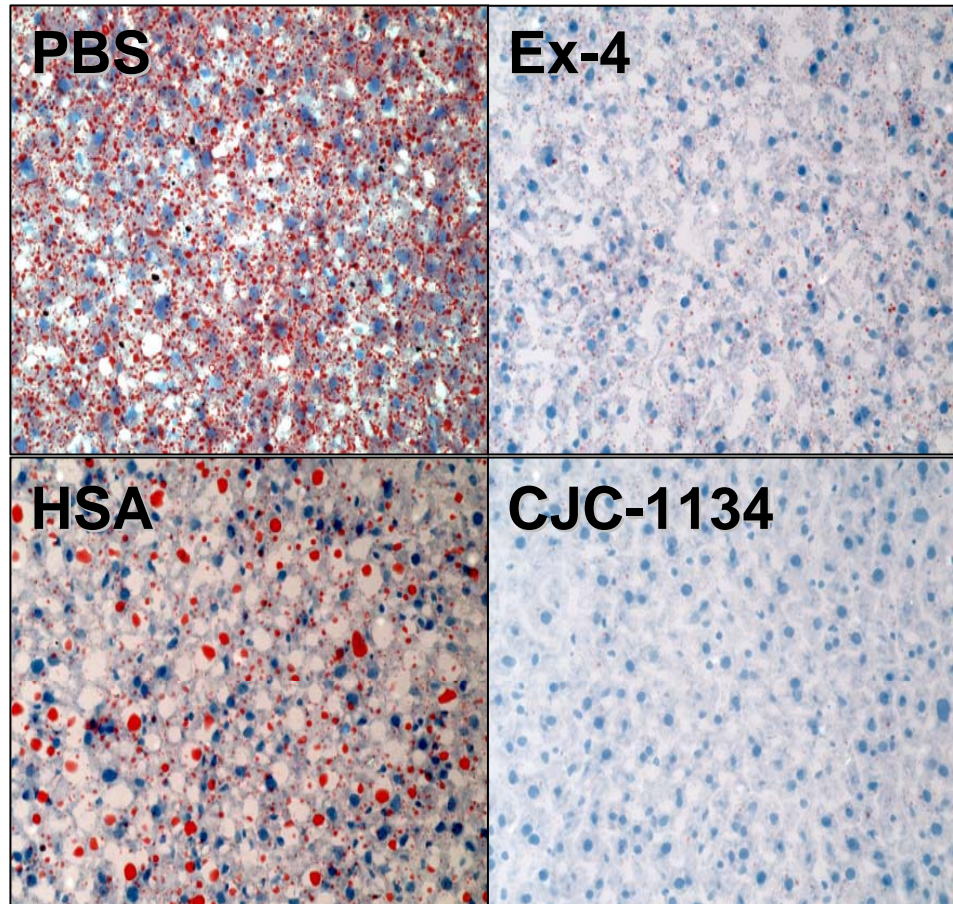
Supplementary Figure 4

Supplementary Figure 5

E GLP-1R

F Glucokinase

G PDX-1

H IRS-2

Supplementary Figure 5