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Development and Characterization of a Glucagon-Like Peptide 1-Albumin Conjugate

The Ability to Activate the Glucagon-Like Peptide 1 Receptor In Vivo

Jung-Guk Kim,[1] Laurie L. Baggio,[1] Dominique P. Bridon,[2] Jean-Paul Castaigne,[2] Martin F. Robitaille,[2] Lucie Jetté,[2] Corinne Benquet,[2] and Daniel J. Drucker[1]

The rapid degradation of native glucagon-like peptide 1 (GLP-1) by dipeptidyl peptidase-IV (DPP-IV) has fostered new approaches for generation of degradation-resistant GLP-1 analogues. We examined the biological activity of CJC-1131, a DPP-IV–resistant drug affinity complex (DAC) GLP-1 compound that conjugates to albumin in vivo. The CJC-1131 albumin conjugate bound to the GLP-1 receptor (GLP-1R) and activated cAMP formation in heterologous fibroblasts expressing a GLP-1R. CJC-1131 lowered glucose in wild-type mice, but not in GLP-1R−/− mice. Basal glucose and glycemic excursion following glucose challenge remained significantly reduced 10–12 h following a single injection of CJC-1131. Twice daily administration of CJC-1131 to *db/db* mice significantly reduced glycemic excursion following oral and IP glucose challenge ($P < 0.01$ to 0.05) but did not significantly lower body weight during the 4-week study period. Levels of random fed glucose were significantly lower in CJC-1131–treated +/+ and *db/db* mice and remained significantly lower even 1 week following discontinuation of CJC-1131 administration. CJC-1131 increased levels of pancreatic proinsulin mRNA transcripts, percent islet area, and the number of bromodeoxyuridine-positive islet cells. These findings demonstrate that an albumin-conjugated DAC:GLP-1 mimics the action of native GLP-1 and represents a new approach for prolonged activation of GLP-1R signaling. *Diabetes* 52:751–759, 2003

Glucagon-like peptide 1 (GLP-1) is a proglucagon-derived peptide secreted from intestinal L-cells in response to nutrient ingestion (1,2). GLP-1 acts as an incretin to lower postprandial glycemic excursion via stimulation of insulin secretion and inhibition of glucagon secretion. GLP-1 also exerts actions independent of islet hormone secretion, including inhibition of both gastric emptying and food intake (3,4), and stimulation of β-cell proliferation (5,6).

Although the structurally related gut hormone glucose-dependent insulinotropic peptide (GIP) also potentiates glucose-dependent insulin secretion (7), unlike GLP-1, the insulinotropic actions of GIP are diminished in diabetic rodents or in human subjects with type 2 diabetes (8,9). In contrast, GLP-1 administration rapidly lowers glucose in both normal and diabetic subjects (9–11), and 6 weeks of continuous subcutaneous infusion of native GLP-1 significantly decreased blood glucose and HbA_{1c} in human patients with type 2 diabetes (12). Hence there is considerable enthusiasm for the development of GLP-1–based pharmaceutical agents for the treatment of type 2 diabetes (2,13).

Although native GLP-1 effectively lowers blood glucose following acute peptide administration (14,15), both endogenous and exogenously administered GLP-1 exhibit a short $t_{1/2}$ in vivo due primarily to NH_2-terminal cleavage and inactivation by the enzyme dipeptidyl peptidase (DPP-IV) (16,17). The physiological importance of DPP-IV for GLP-1 degradation and glucose homeostasis is exemplified by studies of mice or rats with inactivating mutations in the DPP-IV gene. These rodents exhibit enhanced glucose clearance following glucose challenge and increased circulating levels of intact GLP-1 in vivo (18,19). Similarly, administration of DPP-IV enzyme inhibitors is associated with reduced glycemic excursion, enhanced insulin secretion, and reduced degradation of GLP-1 in normal and diabetic rodents (20–22) and in human subjects (23).

Accordingly, there is considerable interest in complementary strategies for circumventing the rapid cleavage of GLP-1, including the development of GLP-1–based analogs with enhanced resistance to degradation and increased biological potency in vivo (13,24). The majority of these analogs exhibit one or more amino acid substitutions that reduce the affinity of the peptide for DPP-IV and subsequent cleavage both in vitro and in vivo. Similarly, the naturally occurring lizard peptide exendin-4 is a potent GLP-1R agonist that exhibits reduced DPP-IV mediated cleavage and a longer duration of action in both rodents and human subjects (25,26). We have recently initiated studies of GLP-1 derivatives that are resistant to DPP-IV and are synthesized with a short covalent reactive chem-

From the [1]Banting and Best Diabetes Centre, Department of Medicine, University of Toronto, Toronto General Hospital, Toronto, Ontario; and [2]Conjuchem, Montreal, Quebec.

Address correspondence and reprint requests to Dr. Daniel J Drucker, 200 Elizabeth St., MBRW-4R-402, Toronto, Ontario, Canada M5G 2C4. E-mail: d.drucker@utoronto.ca.

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AUC, area under the curve; CHO, Chinese hamster ovary; DAC, drug affinity complex; DPP-IV, dipeptidyl peptidase-IV; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide 1; GLP-1R, GLP-1 receptor; HSA, human serum albumin.

ical linker that interacts with a specific cysteine residue in the albumin molecule following parenteral administration of the modified GLP-1 peptide. The resultant GLP-1–albumin drug affinity complex (DAC) is predicted to retain the actions of GLP-1, yet exhibit a more prolonged duration of action due to a combination of DPP-IV resistance and the longer $t_{1/2}$ conferred by serum albumin in vivo. To test the hypothesis that albumin-bound DAC–GLP-1 derivatives retain the biological properties of native GLP-1, we studied the activity of a DAC–GLP-1 compound, CJC-1131, using cells and in normal GLP-1R−/− and diabetic rodents.

RESEARCH DESIGN AND METHOD

Animals. All animal experiments were carried out in accordance with protocols approved by the Toronto General Hospital Animal Care Committee or the Comité Institutionnel de Protection des Animaux de l'UQAM. C57BL/6 *db/db* mice (The Jackson Laboratory, Bar Harbor, ME) and age- and sex-matched C57BL/6 wild-type mice from the same genetic background were used for chronic administration studies following a minimum 1-week acclimatization period in the animal facility. Wild-type CD-1 mice and GLP-1R−/− mice (27) in the CD-1 background were used for acute peptide administration experiments. Mice were allowed ad libitum access to food and water, except where noted. Animals were on a 12-h light, 12-h dark cycle (lights on 0700 h). For dose-response experiments shown in Fig. 2*B*, female CD-1 mice (Charles River Canada, St-Constant, QC), 7- to 10-weeks old, were studied, whereas for glycemic measurements shown in Figs. 2*C* and *D*, experiments were carried out with female *db/db* mice aged 7–10 weeks. CJC-1131 is a synthetic modification of GLP-1 (Fig. 1*A*) consisting of a single amino acid substitution of L-Ala^8 to D-Ala^8 at position 2, enabling some additional protection from DPP-IV and the addition of a lysine (Lys^{37}) to the COOH-terminal of the peptide. The other modification involves the selective attachment of a [2-[2-[2-maleimidopropionamido(ethoxy)ethoxy]acetamide to the epsilon amino group of Lys^{37}.

In vitro experiments: binding affinity and cyclic AMP generation. Binding affinity was determined by incubating either native GLP-1 or CJC-1131 and 0.03 nmol/l ^{125}I-labeled GLP-1(7–36) amide with Chinese hamster ovary (CHO) cells stably transfected with the human GLP-1R cDNA using an incubation buffer of 20 mmol/l Tris-HCl, pH 7.4, 5 mmol/l $MgCl_2$, 20 mmol/l NaCl, 2% BSA for 90 min at 37°C. For analysis of cAMP generation, CHO–GLP-1R cells were incubated for 20 min with various peptide ligands in the presence of 100 mmol/l IBMX followed by analysis of cAMP as described (28).

Experimental protocol for *db/db* mouse studies. Baseline plasma glucose, oral and intraperitoneal glucose tolerance testing, and food intake were assessed before beginning CJC-1131 treatment. During the 4-week treatment period, wild-type and *db/db* mice were given an intraperitoneal injection of either 25 μg of CJC-1131 or an equal volume of saline twice daily at 800 and 1600 h. Following the 4-week treatment period, a subset of mice from each group was sacrificed for analyses, whereas an additional set of mice continued to be monitored for another 3 weeks.

Glucose tolerance tests and measurement of plasma insulin. Oral and intraperitoneal glucose tolerance tests were carried out following an overnight fast (16–18 h) as described (27,29). Glucose (1.5 mg/g body wt) was administered orally through a gavage tube or via injection into the peritoneal cavity, and blood was drawn from a tail vein at 0, 10, 20, 30, 60, 90, and 120 min after glucose administration. Blood glucose levels were measured by the glucose oxidase method using a glucose meter (Glucometer Elite; Bayer, Toronto, Canada). For plasma insulin determinations, a blood sample was removed from the tail vein during the 10- to 20-min period following glucose administration, and plasma insulin was determined using a rat insulin enzyme-linked immunosorbent assay kit (Crystal Chem, Chicago, IL) with mouse insulin as a standard.

RNA isolation and Northern blot analysis. Mice were anesthetized with CO_2, and pancreata were removed immediately for RNA extraction by the acid-guanidinium isothiocyanate method. Total RNA (10 μg) was electrophoresed in a 1% (wt/vol) formaldehyde-agarose gel and transferred to a nylon membrane (Nytran Plus; Schleicher & Schuell, Keene, NH). For Northern blot analysis, the blot was hybridized to ^{32}P-labeled random-primed complementary DNA probes corresponding to rat proglucagon, rat insulin, or 18S RNA.

Analysis of islet size and β-cell proliferation. Mice were injected with 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO) intraperitoneally 6 h before being sacrificed. The pancreas was removed, fixed overnight in either 10% buffered formalin or 4% paraformaldehyde, and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin using standard protocols. Histological sections were immunostained for insulin using guinea-pig anti-insulin (Dako Diagnostics Canada, Mississauga, ON, Canada) as primary antibody (1:100 dilution) and rabbit anti–guinea-pig immunoglobulin (Dako Diagnostics Canada) as secondary antibody (1:50 dilution). Antibody binding was visualized by 3,3-diaminobenzidine, and sections were counterstained by Meyers hematoxylin. Islet histomorphometry was carried out according to the principles of Delesse (30). The sections were examined using a Leica (Leitz Labor Lux S; Leica Microsystem, Heerbrugg, Switzerland) microscope equipped with a video camera and connected to a computer with imaging software (Q500MC; Leica Microsystem). Estimates of islet area, islet number, number of BrdU immunopositive cells, and total pancreatic area were determined in a blinded manner as described (29,31).

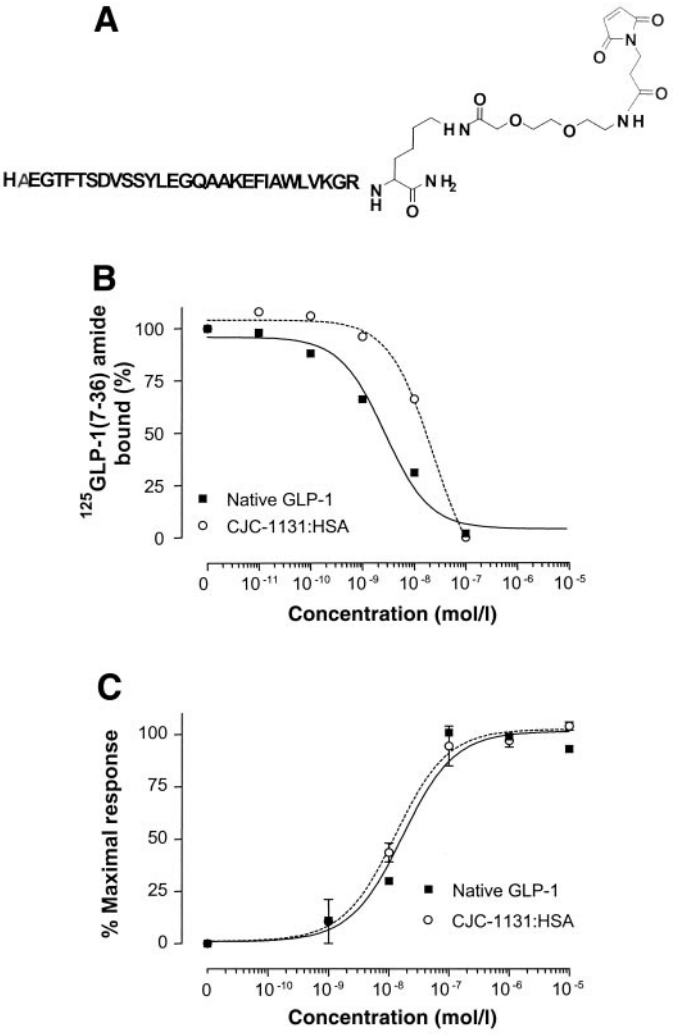

FIG. 1. *A*: Structural representation of CJC-1131 consisting of a single amino acid substitution of L-Ala^8 to D-Ala^8 at position 2 and a Lys^{37} addition to the COOH-terminus with selective attachment of a [2-[2-[2-maleimidopropionamido(ethoxy)ethoxy]acetamide to the epsilon amino group of Lys^{37}. *B*: Binding affinity of native GLP-1 (■) or CJC-1131:HSA (○) in CHO cells transfected with the human recombinant GLP-1R. *C*: cAMP response of native GLP-1 (■) or CJC-1131:HSA (○) in CHO cells transfected with the human recombinant GLP-1R.

Statistics. Results are expressed as mean ± SD or mean ± SE. Statistical significance was calculated by Student's *t* test using SPSS windows version 10 (SPSS, Chicago, IL). $P < 0.05$ was considered to be statistically significant.

RESULTS

To circumvent the short biological $t_{1/2}$ of native GLP-1 due principally to DPP-IV–mediated degradation, we designed a new DPP-IV–resistant GLP-1 derivative that would form

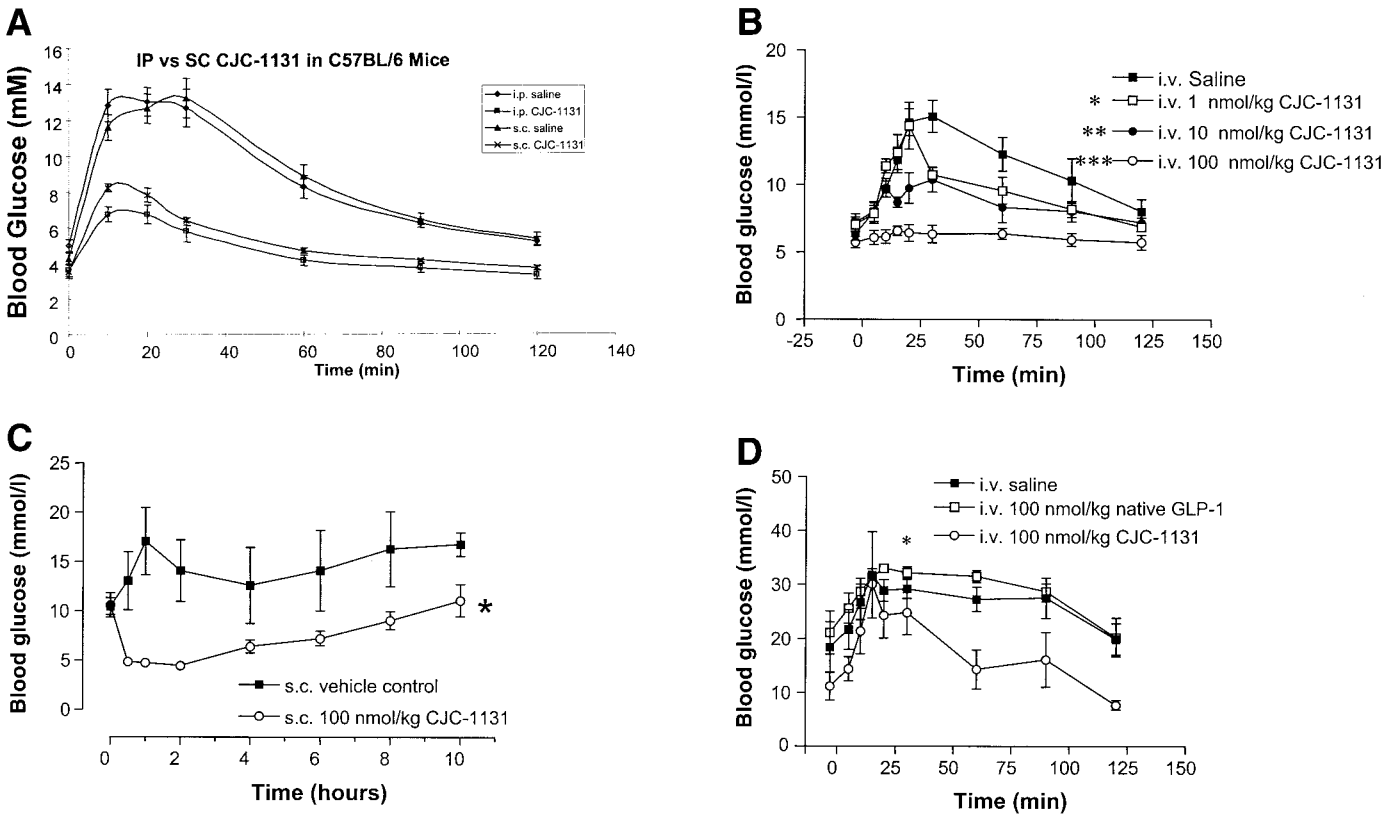

FIG. 2. ***A*****: Blood glucose levels in 9-week-old wild-type C57+/+ male mice during an oral glucose tolerance test performed 30 min after either intraperitoneal or subcutaneous administration of saline or CJC-1131 (25 μg);** ***n*** **= 5 mice/group. Values are expressed as means ± SE.** ***B*****: Blood glucose levels in 7- to 10-week-old female CD-1 mice during an oral glucose tolerance test 5 min after an intravenous administration of saline (■) or 1 (□), 10 (●), or 100 (○) nmol/kg of CJC-1131;** ***n*** **= 10 mice/group. Values are expressed as mean ± SE; *** ***P*** **< 0.05, **** ***P*** **< 0.02, ***** ***P*** **< 0.001 for differences in area under the curve (AUC) glucose for mice treated with saline vs. 1, 10, and 100 nmol/kg CJC-1131, respectively.** ***C*****: Basal glycemia in 6- to 7-week-old female diabetic *db/db* mice after a single subcutaneous injection of either vehicle (■) or 100 nmol/kg CJC-1131 (○);** ***n*** **= 5 mice/group. Values are expressed as means ± SE; *** ***P*** **< 0.02 for AUC glucose in saline versus CJC-1131–treated mice.** ***D*****: Blood glucose levels in 7- to 10-week-old female *db/db* mice during an oral glucose tolerance test performed 12 h after intravenous administration of saline (■), 100 nmol/kg of native GLP-1 (□), or 100 nmol/kg of CJC-1131 (○);** ***n*** **= 5 mice/group. Values are expressed as mean ± SE; *** ***P*** **< 0.05 for AUC glucose in saline versus CJC-1131–treated mice.** ***E*****: Blood glucose levels in wild-type CD-1+/+ (○) and GLP-1R −/− (●) mice during an oral glucose tolerance test performed 60 min after intraperitoneal administration of CJC-1131 (25 μg);** ***n*** **= 9–10 mice/group. Values are expressed as mean ± SE; *** ***P*** **< 0.05; **** ***P*** **< 0.01. #** ***P*** **< 0.001 for AUC glucose in GLP-1R+/+ vs. GLP-1R−/− mice.**

a covalent bond with albumin in vivo, thereby conferring to covalently linked GLP-1 the longer circulating $t_{1/2}$ of albumin (32). A D-alanine residue at position 2 was substituted for the native alanine, and the COOH-terminal end of the GLP-1 molecule was coupled to a reactive chemical linker capable of forming a 1:1 covalent bond to the Cys residue in serum albumin (Fig. 1*A*). The resultant GLP-1 derivative, designated CJC-1131, should retain the biological properties of native GLP-1 yet exhibit DPP-IV resistance and a prolonged $t_{1/2}$ consistent with the clearance of native serum albumin in vivo (33).

To assess the properties of a CJC-1131–human serum albumin (HSA) conjugate, we incubated HSA with CJC-1131 in vitro and utilized the purified CJC-1131:HSA conjugate for studies of GLP-1R binding using CHO cells transfected with human recombinant GLP-1R (Fig. 1*B*). The displacement of ^{125}I-labeled GLP-1 by native GLP-1 versus the CJC1131:HSA complex was highly similar over a range of CJC-1131 concentrations (K_i = 5.16 nmol/l for native GLP-1 vs. 12 nmol/l for CJC-1131:HSA). To assess the bioactivity of CJC-1131, we measured cAMP accumulation using CHO cells transfected with human recombinant GLP-1R. These experiments demonstrated virtually identical dose-response relations for cAMP accumulation, with native GLP-1 exhibiting an EC_{50} of 13 nmol/l, whereas the EC_{50} for CJC-1131–HSA was 11–13 nmol/l (Fig. 1*C*).

We next assessed whether the route of CJC-1131 administration was an important determinant of bioactivity in

C57BL/6 mice following glucose loading. CJC-1131 markedly reduced glycemic excursion following intraperitoneal or subcutaneous administration (Fig. 2*A*). Similarly, intravenous CJC-1131 administration produced a dose-dependent reduction in glycemic excursion following glucose loading (Fig. 2*B*). To ascertain whether a single injection of CJC-1131 would lower blood glucose in diabetic mice, we administered CJC-1131 by subcutaneous injection to *db/db* mice. Remarkably, basal random glycemia decreased rapidly and remained lower in *db/db* mice for up to 10 h following a single subcutaneous injection of CJC-1131 (Fig. 2*C*). Furthermore, the glucose-lowering effect of CJC-1131 was still evident during an oral glucose tolerance test performed 12 h after a single intravenous CJC-1131 injection (Fig. 2*D*). Although CJC-1131 consistently lowered glucose in wild-type mice, no effect was observed in GLP-1R−/− mice (Fig. 2*E*), demonstrating the critical importance of an intact GLP-1R for the biological activity of CJC-1131 in vivo.

These findings demonstrated that single injections of CJC-1131 exert glucose-lowering effects in normal and diabetic mice. We next assessed whether more prolonged repeated administration of CJC-1131 would lower glucose in mice with severe diabetes. Wild-type control C57BL/6 (C57+/+) or *db/db* mice were treated with saline or CJC-1131 twice daily for 4 weeks. Before initiation of CJC-1131, mean fasting glucose was 4.3 ± 0.9 mmol/l in C57+/+ vs. 17.9 ± 6.7 mmol/l in *db/db* mice (Fig. 3*A*). After 2 weeks of twice daily saline or CJC-1131 administration, fasting blood glucose was significantly lower in both control C57+/+ and *db/db* mice treated with CJC-1131 (4.5 ± 0.7 vs. 3.3 ± 0.5 mmol/l and 19.2 ± 5.8 vs. 12.5 ± 3.7 mmol/l, saline vs. CJC-1131 in C57+/+ vs. *db/db* mice, respectively; $P < 0.01$, Fig. 3*A*). Fasting glucose remained significantly lower in C57+/+ mice but not in *db/db* mice treated with CJC-1131 at the end of the 4-week treatment period (Fig. 3*A*). In contrast, fed blood glucose was significantly lower in CJC-1131–treated C57+/+ and *db/db* mice throughout the 4-week experiment (Fig. 3*B*; $P < 0.01$, saline versus CJC-1131). Furthermore, fed blood glucose remained significantly lower in *db/db* mice 1 week following the last injection of CJC-1131 (Fig. 3*B*, $P < 0.05$). Although CJC-1131 produced a small but significant decrease in body weight in C57+/+ mice, no differences in body weight were detected in *db/db* mice treated with saline versus CJC-1131 over the 4-week experiment (Fig. 3*C*).

To determine whether repeated administration of CJC-1131 was associated with improvement in glucose tolerance, we performed oral and intraperitoneal glucose tolerance testing in C57+/+ and *db/db* mice treated with saline or CJC-1131 for 2 weeks. Blood glucose excursion was significantly lower in CJC-1131–treated control C57+/+ mice following either oral or intraperitoneal glucose loading (Fig. 4*A* and *B*). Similarly, both fasting glucose and the glycemic excursion following glucose loading were modestly but significantly reduced in CJC-1131–treated *db/db* mice at multiple time points (Fig. 4*A* and *B*). Although plasma insulin levels were significantly greater in *db/db* mice compared with C57+/+ mice both in the fasting state and after glucose loading, plasma insulin

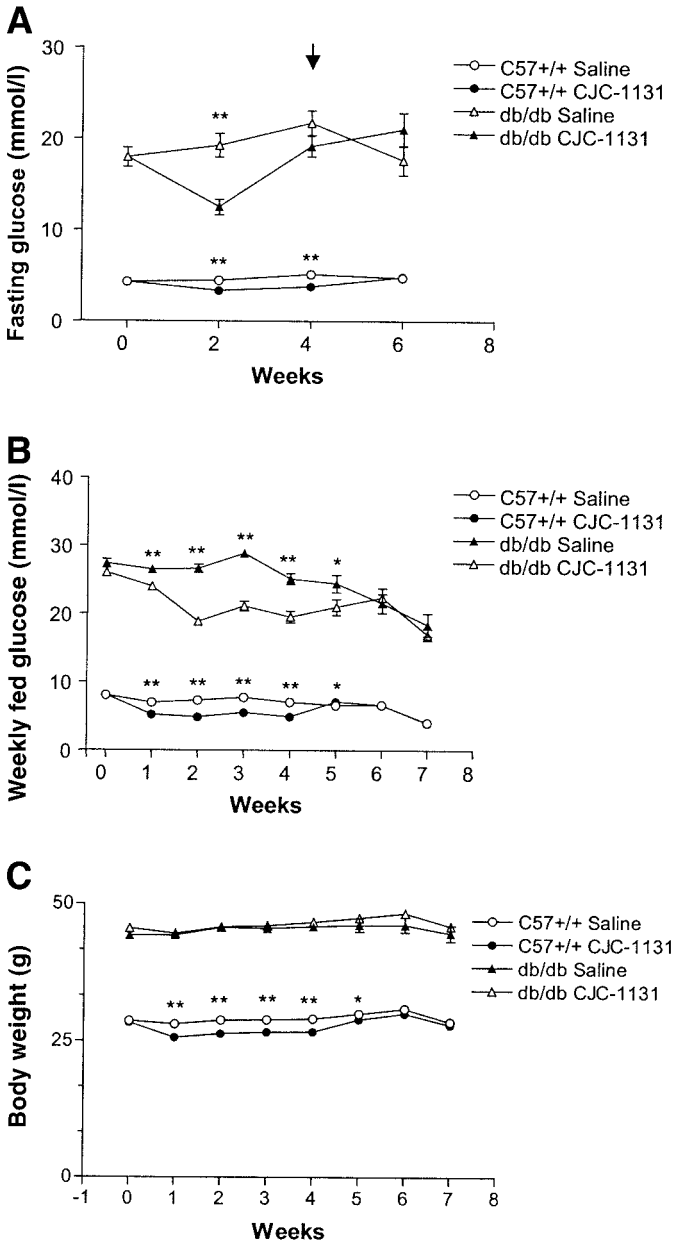

FIG. 3. ***A*: Weekly fasting blood glucose in C57+/+ (○, ●) and *db/db* (△, ▲) mice before and during i.p treatment with saline (○, △) or 25 μg CJC-1131 (●, ▲) twice daily for 4 weeks and for several weeks following discontinuation of active treatment. The arrow denotes the end of the 4-week treatment period. *B*: Weekly fed blood glucose in C57+/+ (○, ●) and *db/db* (△, ▲) mice treated with saline (○, △) or 25 μg CJC-1131 (●, ▲) twice daily observation for 4 weeks and observation for 3 weeks. *C*: Weekly body weight in C57+/+ (○, ●) and *db/db* (△, ▲) mice treated with saline or 25 μg CJC-1131 twice daily by intraperitoneal injection for 4 weeks. Values are expressed as means ± SE; *$P < 0.05$, **$P < 0.01$; n = 10–20 mice per group for experiments depicted in *A–C*.**

levels were not further increased in *db/db* mice treated with CJC-1131 (data not shown).

GLP-1R agonists have been shown to increase proinsulin gene expression and promote islet neogenesis and β-cell proliferation (5,6,34). To determine whether a larger CJC-1131:albumin conjugate exhibits comparable actions on the diabetic pancreas in vivo, we carried out Northern blot analysis using pancreatic RNA from C57+/+ and *db/db* mice. Levels of pancreatic insulin mRNA transcripts were comparable in normoglycemic wild-type C57+/+

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