Title page

Transferrin fusion technology: a novel approach to prolong biological half-life of insulinotropic peptides


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Abbreviations:
GLP-1, glucagon-like peptide 1; GLP-1R, glucagon-like peptide 1 receptor; GLP-1-TF, glucagon-like peptide 1 fused to human transferrin; EX-4, exendin-4; EX-4-TF, exendin-4 fused to human transferrin, DPP-IV, dipeptidyl peptidase IV; NEP, neutral endopeptidase; BrdU, 5-bromo-2-deoxyuridine.
Abstract

Fusion proteins comprising glucagon-like peptide 1 (GLP-1) and exendin-4 (EX-4) fused to a non-glycosylated form of human transferrin (GLP-1-Tf or EX-4-Tf) were produced and characterized. GLP-1-Tf activated the GLP-1 receptor, was resistant to inactivation by peptidases, and had a half-life of approximately 2 days, as compared to 1-2 minutes for native GLP-1. GLP-1-Tf retained the acute, glucose-dependent insulin-secretory properties of native GLP-1 in diabetic animals and had a profound effect on proliferation of pancreatic β-cells. Additionally, Tf and the fusion proteins did not cross the blood-brain-barrier but still reduced food intake after peripheral administration. EX-4-Tf proved to be as effective as EX-4 but had longer-lived effects on blood glucose and food intake. This novel transferrin fusion technology could improve the pharmacology of various peptides.
Introduction

According to recent CDC data, 20.8 million people in the U.S. suffer from diabetes (Centers for Disease Control and Prevention), an increase of 2.6 million from 2002. Despite the availability of several new treatments, however, more than 60% of these patients have poor control of their blood glucose (Koro et al., 2004). Hence, more effective anti-hyperglycemic agents are needed. Disease-modifying treatments that would protect β-cells from progressive failure would also provide an advance in the arena of diabetes treatment.

Glucagon-like peptide-1 (GLP-1) is a 30/31-amino acid peptide derived from post-translational processing of the proglucagon gene in intestinal enteroendocrine L cells (Mojsov et al., 1986). Infusion of synthetic GLP-1 to humans suffering from type 2 diabetes mediates an insulinotropic effect in a glucose-dependent manner and improves total insulin secretion. This action results in a lowering of circulating glucose levels (Vilsboll and Holst, 2004; Zander et al., 2002). In addition to its insulinotropic effects, GLP-1 exerts multiple actions throughout the body, including a neuroprotective action in the brain (Perry and Greig, 2005; Martin et al., 2009), an increase in cardiac output (Nikolaidis et al., 2005), reduction of appetite (Zander et al., 2002), and inhibition of gastric emptying (Flint et al., 2001). Continuous GLP-1 infusions also lead to increases in insulin biosynthesis, β-cell proliferation, and β-cell mass in islets of Langerhans in rodents (Perfetti et al., 2000; Stoffers et al., 2000). One pharmacokinetic impediment, however, to the creation of GLP-1-based agents has been its short circulatory half-life ($t_{1/2}$) of about 1-2 minutes. This minimal $t_{1/2}$ is due to a rapid inactivation by dipeptidyl peptidase IV (DPP-IV). DPP-IV cleaves GLP-1 between alanine and glutamic acid in the
second and third positions, respectively, at the N-terminus of the peptide (Kieffer et al., 1995). The remaining peptide fragment of GLP-1 comprising 28/29 amino acids is no longer active and possesses no significant insulinotropic action (Knudsen and Pridal, 1996) and is further hydrolyzed by circulating neutral endopeptidases (NEPs) (Plamboeck et al., 2005). As a single subcutaneous injection of full-length GLP-1 disappears from the circulation within minutes (Deacon et al., 1995), long-acting degradation-resistant GLP-1 receptor agonists have been or are presently under development for routine use in humans. In this regard, exendin-4 (EX-4/Byetta), a potent GLP-1 receptor agonist that is an endogenous product in the salivary glands of the Gila monster (Heloderma suspectum) (Eng et al., 1992), was approved for treating type 2 diabetes. This peptide possesses the insulinotropic activity of GLP-1, inhibits food intake and gastric emptying, and stimulates islet β-cell proliferation (Aziz and Anderson, 2002; Egan et al., 2002; Egan et al., 2003), yet has a different primary amino acid sequence to GLP-1 (replacement of alanine by glycine at position two of its N-terminus as well as changes in the body and C-terminus of the peptide that serve to prevent its breakdown by DPP-IV and NEPs). These substitutions increase the t1/2 of EX-4 to 2-4 h (Fineman et al., 2003), allowing a reduction of injection frequency [twice daily only (Fineman et al., 2003; Kolterman et al., 2003)] to achieve effective lowering of blood glucose concentrations. In addition to Byetta, another GLP-1 analog has been approved for the treatment of type 2 diabetes, i.e. Liraglutide (Victoza). Liralgutide possesses an extended plasma half-life, similarly to EX-4, via a fatty acid acylation mechanism (Drucker et al., 2010; Chapter et al., 2010). Another mechanism of GLP-1 modification that has demonstrated therapeutic potential is fusion of the GLP-1 dimer with recombinant human
albumin, Albiglutide (Baggio et al., 2004). This fusion extends plasma half-life but attenuates the central nervous system actions of GLP-1. Albiglutide is currently reported to be in Phase III clinical trials (Onge & Miller, 2010).

Unlike circulating small peptidergic hormones, such as GLP-1, there are proteins that use the circulation to transport agents throughout the body that possess tremendously long half-lives. For example, transferrin (Tf), a large circulatory protein that transports iron from the intestine, reticuloendothelial system, and liver parenchymal cells to proliferative tissues, possesses a t1/2 in excess of 14-17 days. Glycosylation of Tf reduces this to 7-10 days (Harford et al., 1994; Li and Qian, 2002). If one could create a hybrid molecule between GLP-1 and Tf (GLP-1-Tf) or any peptide and Tf, then perhaps the shuttling and stabilizing properties of Tf could be imbued to the peptide. Therefore, in this study, we have created fusion products of a DPP-IV-resistant GLP-1 analog to non-glycosylated human Tf and expressed them in yeast before harvesting and purification. Any potentiation of these hybrids’ effective t1/2 would facilitate a reduced frequency of parenteral injection, compared to what is currently required for existing GLP-1 receptor agonists. These hybrid molecules were then tested for effective euglycemic capacity in both non-diabetic and diabetic animals.
Methods

Synthesis of human non-glycosylated transferrin (Tf), and GLP-1/ and EX-4/ human non-glycosylated transferrin (GLP-1-Tf and EX-4-Tf) in yeast. GLP-1-Tf is a fusion protein of glucagon-like peptide 1 (GLP-1) attached to the N-terminus of non-glycosylated human transferrin (Tf), with an intervening linker peptide of twelve amino acids (2 copies of PEAPTD). The GLP-1 moiety comprises the 31 amino acids of mature, human GLP-1 (amino acids 7-37) in which the second amino acid is substituted (A to G) in order to prevent degradation by DPP-IV. EX-4-Tf is comprised of full-length EX-4 fused to the N-terminus of nonglycosylated transferrin using the same linker as in GLP-1-Tf. The transferrin sequence for both fusion proteins and Tf was modified to eliminate the two N-linked glycosylation sites in the C-lobe of Tf through two amino acid changes. Additional information concerning the construction of the GLP-1-Tf entities can be found at multiple publicly-accessible patent databases, i.e. (WO/2006/096515: PCT/US2006/007617) MODIFIED TRANSFERRIN FUSION PROTEINS (http://www.wipo.int/pctdb/en/wo.jsp?KEY=06%2F096515&IA=US2006007617&DISPLAY=STATUS). The encoding sequences for the proteins were inserted into a high copy number 2 µm-based plasmid (Sleep et al., 1991) and expressed at high levels in Saccharomyces cerevisiae. The secreted proteins were purified from high cell density fermentations using several chromatographic steps to a purity of >99 %, as judged by SDS-PAGE, SE-HPLC, and N-terminal sequencing.

Cell culture. Chinese hamster ovary (CHO) cells were stably transfected with the GLP-1 receptor (CHO/GLP-1R cells) and cultured with 5% CO₂ at 37°C in Ham’s F-12 medium (Cellgro: Manassas, Virginia), as previously described (Montrose-Rafizadeh et al., 1997). Rat insulinoma cell line, RIN1046-38 cells, was obtained from Dr. Samuel A. Clark and
cultured with 5% CO₂ at 37°C in M199 medium (Cellgro: Manassas, Virginia), as previously described (Wang et al., 2001).

**Pancreatic islet preparation.** Islets from Sprague-Dawley rats were isolated. Briefly, pancreata were digested by Collagenase Type XI (Sigma: St. Louis, MO) and islets separated through a Ficoll-Paque gradient (Amersham Biosciences, Sweden). The isolated islets were washed several times using Hanks Balanced Salts Solution (Biosource, Belgium) containing 0.2% bovine serum albumin (BSA, Sigma: St. Louis, MO), hand-picked, and cultured overnight with 5% CO₂ at 37°C in M199 medium supplemented with 5 mM glucose. Multiple human islet isolation preparations (5,000 islets per batch) were obtained from the National Islet Cell Resource Center to perform GLP-1-Tf-related experiments.

**In vitro experiments: cAMP generation and insulin secretion.** CHO/GLP-1R cells were rinsed with Krebs-Ringer buffer (KRB, Cellgro: Manassas, VA) and incubated for 1 h at 37°C to lower endogenous intracellular cAMP levels. This was followed by a brief incubation in KRB containing 3-isobutyl-1-methylxanthine (4 mM, Sigma: St. Louis, MO) to inhibit intracellular phosphodiesterases that degrade cAMP. Triplicate wells of cells were treated with serial dilutions of GLP-1-Tf, GLP-1 (7-37), EX-4-Tf, and EX-4 for 30 min at 37°C. Afterwards, the supernatants were removed, and cells were lysed in 0.1 N HCl. Cell lysates were assayed to determine intracellular cAMP concentrations using a competition-based chemiluminescent enzyme immunoassay (Assay Designs Inc.: Ann Arbor, MI). RIN 1046-38 cells grown on 12-well plates that had reached 50–60%
confluence were washed in glucose-free insulin secretion buffer (Biofluids: Rockville, MD), and the cells were incubated with the serial dilutions of GLP-1 and GLP-1-Tf for 1 h in insulin secretion buffer (5 mM glucose) at 37°C. Supernatant was collected, the protein content of the cells was subsequently quantified, and the amount of insulin secreted into the buffer was normalized to protein content. Insulin secretion assays from rat (in the presence of GLP-1 and GLP-1-TF) and human (in the presence of EX-4-Tf and EX-4) islets were performed for 1 h 37°C in insulin-secretion buffer. The supernatant was then collected and saved at -80°C for determination of insulin concentration by ELISA (Crystal Chem: Downers Grove, IL, [rodent insulin for RIN and rat islets] and Alpco: Salem, NH, [human insulin for human islets]).

**Animals.** Male diabetic *db/db* mice (C57BLKS/J-Lepr<sup>db</sup>/Lepr<sup>db</sup>) lacking a functioning leptin receptor and their non-diabetic heterozygous littermates were purchased at 4 weeks of age from Jackson Laboratories (Bar Harbor, ME). Male Sprague Dawley rats were purchased at 6 weeks of age from Harlan Sprague Dawley (Indianapolis, IN). Male cynomolgus monkeys (*Macaca fascicularis*) (N=4) received IV and SC boluses of GLP-1-Tf (2.25 mg/kg), and blood was taken at the times indicated in Figure 1D. The blood was centrifuged, and serum was stored at -80°C for assay of GLP-1-Tf. All animal experiments were carried out on approved protocols in accordance with the Animal Care and Use Committee of the NIA.

**Glucose homeostasis experiments and plasma insulin determinations in non-diabetic and db/db mice.** Intraperitoneal (IP) glucose tolerance testing (IPGTT) (0.5 g/kg body weight) was carried out after an overnight fast in non-diabetic mice. Tf and GLP-1-Tf
were administered IP 30 minutes before the glucose injection. Blood glucose levels (Glucometer Elite, Bayer: Tarrytown, New York) and plasma insulin levels were measured after the IPGTT. Tf and GLP-1-Tf were administered subcutaneously to another set of non-diabetic as well as to diabetic mice, and blood glucose was measured frequently for 48 h for prolonged effects. Plasma insulin levels were measured at 1 and 4 h. An IPGTT (0.5 g/kg body weight) was also performed after an overnight 12 h fast in diabetic mice that had received GLP-1-Tf or Tf subcutaneously at the beginning of the fast.

**Food intake and blood glucose measurements after GLP-1-Tf administration in non-diabetic and db/db mice.** Mice (N=5 per group) were conditioned to eat once daily (9-10 am) for five days. On the morning of the sixth day, they received IP Tf (10 mg/kg), GLP-1-Tf (0.1, 1 and 10 mg/kg), or EX-4 (1 nmol/kg) and then allowed eat *ad libitum*. Animal food intake was measured manually on a daily basis by a consistent animal experimenter and blood glucose levels were measured 2, 4, 7, and 24 h or 48 h after peptide administration.

**Food intake and blood glucose measurements after EX-4-Tf administration in db/db mice.** EX-4-Tf (0.01, 0.1, 0.3, 1 mg/kg respectively) and EX-4 (1 nmol/kg) were injected into 2 cohorts (N=6 per cohort) of *ad libitum*-fed db/db mice. In one cohort, food intake was measured 24, 48, 72, and 96 h after IP peptide administration, and the other cohort was used for frequent blood glucose determinations after SC peptide administration.
Analysis of β cell proliferation and β-cell area. *db/db* mice, 6 per time point, received one IP injection of Tf (10 mg/kg) and the bioactive peptides (10 mg/kg) and were euthanized 1, 2, and 3 days later for measurement of β-cell proliferation and 3, 5, 8, and 10 days later for measurement of islet area using methods similar to those described previously (Perfetti et al., 2000).

Analysis of β cell proliferation and β-cell area. Sixty mg/kg 5-bromo-2-deoxyuridine (BrdU, Sigma: St. Louis, MO) was injected IP 6 h before euthanasia. Pancreata were removed, fixed overnight in 4% buffered formalin, processed for embedding in paraffin, and histological sections (4 µm) mounted on poly-l-lysine-coated glass slides. BrdU was detected with a BrdU antibody (Sigma: St. Louis, MO, 1:500) and BrdU staining kit (Zymed Laboratories Inc.: San Francisco, CA). The number of islets that contained BrdU⁺ cells as well as the percent of BrdU⁺ nuclei per total number of islet cells was quantified. For insulin staining and determination of β-cell area, histological sections were treated with a guinea-pig insulin antibody (Linco: St. Charles, MO). Antibody binding was visualized with 3, 3-diaminobenzidine (DAB) and sections counterstained with hematoxylin (Vector Laboratories: Burlingame, CA). Sectioned tissue images were acquired using a phase contrast light microscope (Zeiss, Germany) and digitized by means of a Sony Power HAD digital camera (20-30 images per section). Total pancreatic area and β-cell positive area for every image were quantified using MetaMorph 4.6.3 software (Universal Imaging Inc.: West Chester, PA). In another set of experiments, insulin and BrdU co-staining was carried out with both insulin antibody (Linco: St. Charles, MO, 1:500) and monoclonal anti-BrdU antibody (Sigma: St. Louis, MO, 1:500).
After the antibodies were applied to the slides and incubated at 4°C overnight, the slides were washed with PBS. Secondary antibodies were then applied (1:200) and incubated for 2 h at 37°C. The slides were washed with PBS and mounted. The immunofluorescence staining was observed using Zeiss 410 confocal microscopy.

**GLP-1-Tf ELISA.** A sandwich ELISA was developed in order to measure the concentration of GLP-1-Tf in body fluids. GLP-1 monoclonal antibody (200 ng, Antibody Shop, Denmark) was immobilized on microtiter plates coated with anti-mouse IgG. After washing, sample or standard was added to the plate that was then incubated at 37°C overnight. The plate was next washed and incubated with biotinylated chicken anti-human transferrin antibody (Fitzgerald Laboratories: Niagara Falls, NY). Washing was repeated, and the plate was incubated with horseradish-peroxidase (HRP)-streptavidin. The bound HRP was measured with Pierce Quanta Blu Fluorogenic Substrate. Measurements were performed on a Spectramax Gemini fluorescence plate reader and Softmax Pro software. Buffer used for all steps was phosphate buffered saline (PBS) containing 1% BSA and 0.05% Tween 20.

**Determination of cerebrospinal fluid (CSF) levels of hTf and GLP-1-Tf.** Sprague-Dawley rats were injected IP with hTf and GLP-1-Tf. Animals were euthanized 2 and 24 h later for analysis of the presence of the proteins in plasma, CSF and brain. CSF was removed by insertion of a 30 gauge needle with syringe attached between the atlas and axis, and the CSF was gently aspirated. CSF used for analysis was without blood contamination. Rats were used in this experiment in order to obtain a sufficient quantity
of CSF. The brains were removed, sonicated in SDS lysis buffer, and the homogenate stored at -80°C for later subjection to Western blot analysis.

**Western blot analysis of plasma, CSF and brain homogenates.** Plasma samples (0.1 µl) or 5 µl of CSF samples and brain homogenates were mixed with 10 µl SDS-PAGE sample buffer and were subjected to 10% SDS-PAGE (Novex: San Diego, CA) according to the supplier’s protocol. The proteins were transferred onto PVDF membrane (BioRad: Hercules, CA) in 25 mM Tris + 192 mM glycine. The filter paper was equilibrated with 25 mM borate buffer (pH 8.0 + 150 mM NaCl + 5% non-fat dry milk). Rabbit anti-Tf (Rockland Immunochemicals: Gilbertsville, PA) was added to the blot and was incubated overnight at 4°C. After extensive washing, HRP-conjugated goat anti-rabbit IgG (Pierce: Rockford, IL) was used as a secondary antibody. The bound antibodies to Tf and GLP-1-Tf bands were detected by SuperSignal West Pico chemiluminescent detection kit (Pierce: Rockford, IL).

**Measurement of c-Fos activation in mouse brain.** Briefly, mice (N=6 per group) received IP injections (100 µl) of either, a) PBS, b) EX-4 (1 nmol/kg), c) GLP-1-Tf (0.1, 1 and 10 mg/kg), or d) Tf (10 mg/kg). Another 3 treatment groups were anesthetized by isoflurane inhalation and placed in a stereotaxic head holder. The skull was exposed along the mid-line and 2 µl of the following treatments were injected unilaterally (ICV) into the lateral ventricle: a) PBS, b) 7.2 mg/ml GLP-1-TF, or c) 7 mg/ml Tf. Mice that received IP injections were anesthetized with isoflurane 2 h later, and the mice that received ICV injections were anesthetized 1.5 or 24 h later. Intracardiac perfusion with
ice-cold 4% paraformaldehyde was immediately carried out. Brains were removed, stored in 4% paraformaldehyde for 72 h, and then transferred to a solution containing 4% paraformaldehyde and 25% sucrose for 12 h. Each brain was cut into 30 μm sections using a sliding microtome with a freezing stage. Brain sections from each animal were placed in a 12-well plate and then processed for immunocytochemistry. The free-floating sections from each animal were first incubated in 0.3% H2O2 for 30 min and then washed in PBS. Thereafter, the sections were incubated in blocking serum for 45 min at room temperature, followed by staining for c-Fos with a rabbit anti-human c-Fos antibody directed against the amino acid residues 4-17 (Calbiochem: La Jolla, CA, 1:30,000) for 48 h at 4°C. The sections were then washed with PBS/0.1% Triton before incubation for 1.5 h at room temperature with biotinylated goat anti-rabbit IgG (1:600) in blocking serum (Vector Laboratories: Burlingame, CA). Subsequently, the sections were visualized with DAB. Sections were mounted on superfrost slides (Fisher Scientific: Pittsburgh, PA), air-dried for 1-2 days, and coverslipped with Permount. Brain sections were analyzed using an Olympus BX51 microscope to identify c-Fos-positive cells. The number of c-Fos immunopositive cells was counted by using a constant area and scale grid position under a 10X objective. The position of the counting grid within each brain region was delineated through adjacent landmarks. Both sides of the brain at one representative level of each region were quantified.

Statistical methods. All results are given as means ± SE. Student's t-test was based on the results of the F-test that assessed the equality of variance of the two means. If the variances were statistically significantly different, then the t-test was based on unequal
variances. An ANOVA test was used to calculate the significance of difference among multiple samples, followed by post hoc testing with Scheffé’s test. P values <0.05 were considered statistically significant.

Results

GLP-1-Tf activated the GLP-1 receptor in vitro and was absorbed when given subcutaneously. The GLP-1 receptor (GLP-1R) is a membrane-associated G-protein-coupled receptor, and upon ligand binding, adenylyl cyclase is activated, resulting in a concentration-dependent elevation in intracellular cAMP levels. We first examined whether GLP-1-Tf activated adenylyl cyclase in GLP-1R-transfected CHO cells (CHO/GLP-1R). It produced a dose-dependent increase in intracellular cAMP levels with a similar (albeit slightly higher) maximal efficacy, but reduced potency compared to native GLP-1 (GLP-1-Tf vs. GLP-1: E_{max}-157 vs. 177 pmol/ml; EC_{50}-2.28 vs. 0.16 nM: Figure 1A). We next determined that GLP-1-Tf stimulated insulin secretion from isolated rat islets and rat insulinoma cells in a concentration-dependent manner with a lower potency than native GLP-1 (Figure 1B,C). After subcutaneous (SC) and intravenous (IV) administration of GLP-1-Tf (2.25 mg/kg) to cynomolgus monkeys, the concentration of GLP-1-TF in serum was measured by a specific sandwich ELISA. The t_{1/2} of GLP-1-Tf, after both routes of administration, was approximately 44 h. Standard pharmacokinetic parameters (C_{max}, T_{max} and ‘area under the curve’ (AUC)) recorded from the primate dosing are indicated in Table 1. Calculation of a classical SC versus IV bioavailability ((AUC_{SC}/AUC_{IV}) x (dose_{SC}/dose_{IV}) x 100) demonstrated a very high subcutaneous functional bioavailability for GLP-1-Tf, i.e. 82.83% (1630070.35/1365895.06) x
(2.25/2.25) x 100) (Figure 1D). By treating CHO/GLP-1R with the serum from the injected monkeys, we determined that the GLP-1-Tf measured by ELISA was biologically active and that the slope of decay of intracellular cAMP accumulation in response to the serum over time was similar to that of GLP-1-Tf in the ELISA (for further information see published patent WO/2006/096515-MODIFIED TRANSFERRIN FUSION PROTEINS). The $t_{1/2}$ in mice was determined to be 14 h. The antisera employed in this ELISA was able to distinguish an amino-terminal epitope of GLP-1 indicative of its active form. The specific bioassay data are represented in the specific patent documentation, WO/2006/096515 ‘MODIFIED TRANSFERRIN FUSION PROTEINS’. Based on these findings, a series of experiments in diabetic and non-diabetic mice was initiated in order to determine the duration of biological action, efficacy for controlling blood glucose in the diabetic state, effects on islet morphology, and CNS-activating properties of GLP-1-Tf.

**GLP-1-Tf lowered blood glucose and increased insulin secretion in non-diabetic mice.**

To determine whether GLP-1-Tf could activate GLP-1 receptors *in vivo*, the effect of the fusion protein on insulin secretion was evaluated. Intraperitoneal (IP) GLP-1-Tf (1 and 10 mg/kg) resulted in enhanced insulin secretion in non-diabetic mice in response to IP injected glucose (IPGTT, 0.5 g/kg glucose). The peak blood glucose was decreased, coinciding with elevated insulin levels at 30 min (**Figure 2A**). In addition, SC GLP-1-Tf dose-dependently reduced blood glucose concentration and increased insulin secretion in *ad libitum*-fed mice (**Figure 2B**). In this experiment, blood glucose levels dropped to 70 mg/dl within 3 h of administration and gradually rose to the pre-administration levels.
within 24 h. The lowered blood glucose persisted for up to 36 hours with the higher dose (10 mg/kg).

**GLP-1-Tf lowered blood glucose and increased insulin secretion in diabetic mice.**

Diabetic db/db (C57BLKS/J-Lepr<sup>db</sup>/Lepr<sup>db</sup>) mice are homozygous for a mutated non-functioning leptin receptor, and consequently, they are hyperphagic, eat throughout the 24 h day (instead of the more usual feeding during the dark hours), become obese, and by 4-6 weeks of age, have elevated blood glucose levels in the 300-400 mg/dl range (normal fasting blood glucose levels are 90-120 mg/dl). Their heterozygous littermates (non-diabetic mice) are not hyperphagic, are lean, and do not develop diabetes. Both 1 and 10 mg/kg SC GLP-1-Tf lowered blood glucose to normal levels (from 358 ± 23 to 115 ± 18 mg/dl) in male ad libitum-fed db/db mice, with the effect beginning as early as 1 h after injection (**Figure 3A**). Increased plasma insulin levels were present for at least 4 h after both 1 and 10 mg/kg GLP-1-Tf (**Figure 3B**), and the highest dose sustained the effect on blood glucose, without causing hypoglycemia, for at least 12 h. A low dose of GLP-1-Tf (0.1 mg/kg) caused an acute, unsustained effect of lowering blood glucose for 4 h after administration, concomitant with increased plasma insulin levels at 1 h (**Figure 3A,B**). db/db mice were next given SC Tf or GLP-1-Tf at 9 pm, fasted overnight, and an IPGTT was performed the next morning. As expected, the fasted Tf-treated animals had lower blood glucose than ad libitum-fed animals (238 ± 39 vs. 358 ± 23 mg/dl), compare zero time of fasted animals [**Figure 3C**], with zero time of fed animals, [**Figure 3A**]) and additionally, the animals given 1 or 10 mg GLP-1-Tf had lower fasting blood glucose (132 ± 27 mg/dl) than those given Tf alone. After the IPGTT, the animals given GLP-1-
Tf had lower blood glucose levels and higher plasma insulin levels, compared to hTf-treated animals (Figure 3C,D).

**GLP-1-Tf had short-lived anorectic properties.** In *ad libitum*-fed *db/db* mice, GLP-1-Tf, at concentrations that lowered blood glucose, did not decrease food intake when measured over 24 hours. To assess anorectic effects of GLP-1-Tf more closely, 6-week old non-diabetic and *db/db* mice were conditioned to feed once daily for 1 h from 9-10 am for 5 days. This prevented hyperphagia-related obesity and maintained normal fasting glucose levels in *db/db* animals (99 ± 9 mg/dl). During the feeding hour, both *db/db* and non-diabetic animals ate approximately 0.5 grams of food. Two animals were euthanized on the morning of the sixth day, and no food was present in their stomachs or small bowels. Also on the sixth day, the remaining animals were administered GLP-1-Tf (0.1, 1 and 10 mg/kg), Tf (10 mg/kg), or EX-4 (1 nmol/kg to the *db/db* mice only) before having free access to food. Food intake was quantified, and blood glucose levels (in order to measure post-prandial glucose elevations) were measured at intervals for the following 48 h. Food intake was decreased by approximately half in non-diabetic animals for up to 7 h after GLP-1-Tf (10 mg/kg) and for 2 h by GLP-1-Tf (1 mg/kg). Post-prandial blood glucose levels were lower, in a concentration-dependent manner, with all three doses, as compared to animals that received Tf only, reflecting both decreased food intake (Figure 4A,B) and increased insulin secretion (see Figure 3B). In the time segment 7-24 h after injection, GLP-1-Tf-treated animals had similar food intake to Tf-treated animals. In *db/db* animals, food intake was also decreased by approximately half for 2 h by GLP-1-Tf (1 and 10 mg/kg) and all three GLP-1-Tf doses, in a concentration-dependent manner,
simultaneously lowered post-prandial blood glucose levels, as compared to Tf treatment (Figure 4C,D). EX-4 prevented all measurable food consumption for 7 h and caused a decrease in food intake for the remaining 17 h (Figure 4D). We have previously shown that EX-4 (1 nmol/kg daily) causes a decrease in food intake for up to 4 days in this mouse model of diabetes (Greig et al., 1999).

**GLP-1-Tf increased β-cell proliferation and increased β-cell area.** *db/db* mice (N=6 per group) were given IP GLP-1-Tf (10 mg/kg), Tf (10 mg/kg), and BrdU (60 mg/kg, 6 h before euthanasia) in order to investigate cell turnover in islets. The GLP-1-Tf-treated animals had an increase in the number of BrdU+ nuclei in islets as early as 24 h after GLP-1-Tf injection, compared to Tf treatment (Figure 5A). After 2 days of GLP-1-Tf treatment there was a statistically significant increase in the number of BrdU+ nuclei (days 2, 3 p<0.05; day 5 p<0.01, Figure 5B). The majority of islets observed in GLP-1-Tf-treated animals had at least 1 or more BrdU+ nuclei (data not shown), while the Tf-only-treated animals failed to demonstrate any distinct increase in BrdU+ nuclei compared to vehicle-treated (PBS) animals (Figure 5A,C,D). As shown in Figure 5A and D it was common to note a single BrdU+ nuclei within an islet in Tf-treated animals which probably represented a background rate of turnover. In contrast, with GLP-1-Tf treatment, a profound increase in the number of BrdU+ nuclei were seen, accounting for as much as 10% of the total islet cell nuclei (Figure 5C). The BrdU+ nuclei were mainly in insulin-containing β-cells (Figure 5C,D). After 3 days of treatment with GLP-1-Tf (10 mg/kg), total islet area was 1.0 ± 0.003%, compared to 0.94 ± 0.02% with Tf treatment, and by 5, 8, and 10 days, total islet area was increased even further (1.18 ± 0.017%, 1.34
± 0.02%, and 1.23 ± 0.02%) respectively, with GLP-1-Tf (Figure 5E,F). The increase in BrdU incorporation was comparable to the increase in BrdU incorporation observed with EX-4 (1 nmol/kg daily, Figure 5B).

**GLP-1-Tf-mediated central c-Fos activation after peripheral administration occurs in conjunction with minimal cerebrospinal fluid detection.** Rats were administered IP GLP-1-Tf and Tf. Plasma and cerebrospinal fluid (CSF) were obtained 2 and 24 h after injection, and brains were removed and homogenized. The presence of functional levels of GLP-1-Tf in the CSF could not be detected by ELISA after IP administration at 2 h or 24 h. Western blotting of plasma with human-specific anti-Tf antibodies demonstrated the presence of the expected 77 kDa size band for Tf and 79 kDa for GLP-1-Tf, representing the full-length size; no other fragments were seen (either for Tf or GLP-1-Tf), and a concentration-dependency was evident (Figure 6A). As our anti-Tf sera was human specific, no human Tf was detected in plasma in the absence of Tf or GLP-1-Tf treatment (data not shown). In agreement with the ELISA, no immunoreactive Tf was seen in CSF by Western blot analysis. Additionally, we incubated CHO/GLP-1R cells with CSF taken after 2 h from the GLP-1-Tf-treated rats, and no increase in intracellular cAMP occurred. Finally, homogenized brain slices from the treated animals did not contain Tf as assessed by Western blot analysis. Taken together, this data suggested that a profound blood-brain barrier penetrance by the GLP-1-Tf did not occur during our experimental paradigms. In order to determine whether peripherally administered GLP-1 analogues were capable of activating the central nervous system, we examined the pattern of c-Fos expression in the brain. We compared the central effects of IP administered
GLP-1-Tf (0.1, 1, 10 mg/kg) to those of EX-4 (1 nmol/kg=4 μg/kg), which is known to activate specific brain areas after IP injection (Baggio et al., 2004). As a control for our hybrid GLP-1 compound, we administered IP Tf (10 mg/kg). In addition, we also administered intracerebroventricular (ICV) Tf and GLP-1-Tf in another series of experiments and measured c-Fos immunoreactivity 1.5 and 24 h later. Peripherally IP administered GLP-1-Tf (10 mg/kg) activated c-Fos expression in neurons of the area postrema (AP) (4/6 animals), the nuclei of the solitary tract (NTS) (6/6 animals) and the paraventricular nuclei of the hypothalamus (PVH) (6/6 animals) 1.5 h after administration (Figure 6B,D). A lower dose of GLP-1-Tf (1 mg/kg, 0.1 mg/kg) activated the NTS (6/6 animals) and PVH (6/6 animals), but not the AP region (0/6 animals) (data not shown). EX-4 activated similar areas to GLP-1-Tf in all injected animals, but the AP response was much more robust compared to GLP-1-Tf. IP injection of Tf (10 mg/kg) had no significant effect on c-Fos immunoreactivity in the AP, NTS, and PVH regions (Figure 6B). 1.5 h after ICV injection of GLP-1-Tf (7.2 mg/kg), c-Fos activation occurred in PVH and NTS regions but not in the AP region (Figure 6C,E). c-Fos activation was not present in any of the three brain regions 24 h after ICV injection (data not shown). ICV injection of Tf (7 mg/kg) had no significant effect on c-Fos immunoreactivity in the AP, NTS, and PVH regions (Figure 6C,E). Therefore, from our current data, it seems likely that despite demonstrating a minimal physical penetrance into the central nervous system GLP-1-Tf could still engender the activation of c-Fos expression in the PVH and NTS. It is almost impossible to completely attribute these central effects of GLP-1-Tf to a peripheral-to-central signaling process, as even negligible levels of neuropeptides in the central nervous system can cause alterations in protein expression. Our data suggests
though that with nearly undetectable levels of GLP-1-Tf blood-brain barrier penetrance, functional central responses to this ligand are effectively created. The relative contribution of peripheral and central actions may take many subsequent years of experimentation to resolve.

**EX-4-Tf was more potent than GLP-1-TF and had long-lasting effects on food intake.** Because EX-4 is already in clinical use and is reported to have significant effects on food intake and weight loss (Szayna et al., 2000), we next compared the activity of EX-4 to EX-4-Tf. In CHO/GLP-1R cells, EX-4-Tf produced a dose-dependent increase in intracellular cAMP levels. Unlike GLP-1-Tf compared to GLP-1, EX-4-Tf only demonstrated a slightly reduced potency compared to its non-Tf conjugated form (EC$_{50}$: 0.06 [EX-4], 0.16 [GLP-1], 0.3 [EX-4-Tf] nM). However, in a similar manner to GLP1-Tf and GLP-1, EX-4-Tf again demonstrated a slight increase in maximal efficacy compared to EX-4 (E$_{max}$ 155 [EX-4], 157 [GLP-1], 173 [EX-4-Tf] pmol/ml) (Figure 7A, Figure 1A). EX-4-Tf also stimulated insulin secretion from isolated human islets in a concentration-dependent manner and with similar potency to EX-4 (EC$_{50}$: 0.03 vs. 0.02 nM, EX-4 vs. EX-4-Tf) (Figure 7B). EX-4-Tf (1mg/kg) normalized blood glucose for at least 24 hours in db/db mice, even in mice whose basal blood glucose was as high as 500 mg/dl. Additionally, the compound was 10-20-fold more potent, and its dose-lowering effect was longer lasting than GLP-1-Tf (Figure 7C, Figure 3A): injection of EX-4 reduced blood glucose for just 8.5 hours. Food intake was also significantly decreased by EX-4-Tf (0.3 and 1 mg/kg) for at least 48 hours in ad libitum-fed db/db mice, again showing its effects to be longer lived than GLP-1-Tf and EX-4 (Figure 7D).
Discussion

Our data indicate that GLP-1-Tf and EX-4-Tf retain the pharmacological effects of native peptides, while extending their length of action. This work also illustrates an example of transferrin fusion technology using peptides with high therapeutic potential but poor practical utility, demonstrating the potential of this technology as a method of producing biopharmaceuticals with patient-friendly pharmacology. EX-4-Tf was a more potent hypoglycemic agent than GLP-1-Tf, and fusion of EX-4 to Tf had minimal deleterious effects on its biological activity.

The fused compounds also retained the insulinotrophic properties of GLP-1 receptor agonists, and the robust and prolonged response to just one injection of GLP-1-Tf (10 mg/kg) exceeded what we and others have experienced with native GLP-1 and EX-4 (Perfetti et al., 2000; Stoffers et al., 2000; Egan et al., 2003; Park et al., 2005). In an occasional islet of GLP-1-Tf-treated animals, up to 10% of its β-cells showed evidence of mitosis 3 days after injection. In type 2 diabetes, β-cell area in post-mortem pancreata is decreased when compared to pancreata from subjects matched for obesity, but without diabetes (Butler et al., 2003). In addition, with increasing duration of diabetes, β-cell function continues to deteriorate (Butler et al., 2003; Clark et al., 2001), presumably because β-cell numbers decrease. Thus transferrin fusion technology offers a promising therapeutic avenue for the treatment of type 2 diabetes.

GLP-1-Tf and EX-4-Tf could potentially also be used for the treatment of type 1 diabetes. If introduced to newly diagnosed patients who still have sufficient β-cells remaining, preservation and expansion of β-cell area may occur in conjunction with agents now under investigation for preventing the inexorable autoimmune β-cell
destruction (Ogawa et al., 2004). As injection of a fusion protein might be as infrequent as weekly, ease of use and lack of detrimental side effects in this vulnerable population would be added bonuses.

With respect to the therapeutic extension of serum half-life of biologically active peptides such as GLP-1, many diverse chemical strategies have been attempted (Lovshin et al., 2009; Chapter et al., 2010). As a result of these multiple strategies, several modified GLP-1 compounds have recently demonstrated clinical promise. For example, Liraglutide contains a Ser34Arg amino-acid substitution and has a C16 palmitoyl fatty-acid side-chain at Lys26. In a controlled trial, once daily injection seemed to be well-tolerated and effective in treating Type 2 diabetes (Seino et al., 2008). Lixisenatide (Christensen et al., 2009) is a modified exenatide molecule with six additional lysine residues in the carboxy terminal and, again, once-daily injections seemed to be specifically required for adequate anti-diabetic efficacy (http://clinicaltrials.gov/ct2/show/NCT00975286). Additional compounds that have been developed with extended serum stability include: Albiglutide (two tandem copies of a modified GLP-1 in serum albumin); Taspoglutide (a GLP-1 based molecule containing amino-isobutyric acid substitutions at positions 8 and 35); and exenatide-LAR, i.e exenatide encapsulated in biodegradable polymer (D,L lactic-co-glycolic acid) microspheres. Other long-acting GLP-1 receptor agonists include CJC1134, a protein that contains an exenatide moiety covalently linked to human albumin through a chemical linkage (Baggio et al., 2008), and LY2428757, a pegylated GLP-1 molecule: long-term therapeutic efficacy of these promising molecules however is not yet fully known. In addition to their euglycemic activities, it is interesting to note that GLP-1-Tf analogs have
recently also been shown to possess therapeutic benefit in cardiovascular disorders, as GLP-1-Tf can attenuate myocardial reperfusion injury (Matsubara et al., 2009).

In our experimental paradigms GLP-1-Tf treatment was associated with a significant, though short-lived, decrease in food intake in animals that had not eaten for the proceeding 24 h (even in \(db/db\) animals, which lack functioning leptin receptors) and had been conditioned to eat for a specific time interval. EX-4 is a more potent and long-lasting inhibitor of food intake than native GLP-1, and this was again demonstrated in this report. However, EX-4-Tf inhibited food intake over a far longer timeframe than EX-4 even in \(db/db\) animals given free access to food. In free-feeding mice, one EX-4-Tf (0.3 and 1 mg/ml) injection had highly significant effects on food intake for 48 hours. This effect was probably due to stimulation of enteric vagal afferents that terminate in the NTS, and neurons from the NTS that then project to satiety centers in the hypothalamus, in addition to effects on gastric emptying (Hornby, 2001). It is unlikely that the fusion proteins directly activated satiety centers; all evidence suggests that peripherally administered fusion proteins do not cross the blood-brain-barrier (BBB) because they could not be detected in brain tissue or in CSF by several assays, including a biological one. This is unlike EX-4 and native GLP-1, which do cross the BBB (Kastin and Akerstrom, 2003; Kastin et al., 2002; ).

EX-4 has been shown to activate GLP-1 receptors on tyrosine hydroxylase-containing neurons in the AP nucleus after IV but not ICV administration (Yamamoto et al., 2003). Therefore, activation of AP neurons by peripheral EX-4 and GLP-1-Tf administration is due to their presence in circulation, and because EX-4 has a 3-fold higher affinity for the GLP-1 receptor than native GLP-1, it more readily activates the AP. We found that ICV-
administered fusion protein also did not activate the AP, corroborating that AP excitation after peripheral administration is most likely due to the lack of a specific blood-brain barrier at that nucleus. Avoidance of food for 48 hours by the animals after one EX-4-Tf injection could be a significant added bonus in the treatment of type 2 diabetes. The administration of GLP-1 or EX-4 as fusion proteins results in no ‘free’ GLP-1 or EX-4 in the circulation at any time, unlike many other compounds under investigation (Agerso et al., 2002), and this again should lessen the gastrointestinal side effects. A caveat however, with respect to potential side-effect reduction, should be made when considering the dosing regimens of transferrin bio-conjugated peptides such as GLP-1, i.e. with the high molecular mass of the transferrin moiety higher drug doses are required to obtain comparable therapeutic molarities as unconjugated peptides.

Transferrin naturally has a long circulating half-life (7-10 days for glycosylated transferrin and 14-17 days for the non-glycosylated form). Non-glycosylated transferrin is normally present in the body (2-8% of total transferrin) and is amenable to large-scale production in a variety of expression systems, such as yeast and insect cells. The high concentration of endogenous transferrin (3-4 mg/ml serum) precludes any effect on iron homeostasis by exogenously added fusion protein. Transferrin fusion technology was investigated several years ago while attempting to target proteins to brain cells and intracellular targets, but these were not successful, as the Tf receptor is saturated with endogenous Tf concentrations at several orders of magnitude higher than its affinity constant (Li and Qian, 2002).

Our novel technology could be used to deliver other agents known to activate receptors on β-cells. Furthermore, this method could be applicable to deliver an imaging agent
attached to the C-terminus of the Tf moiety into islets. This could allow monitoring of natural history, disease progression, and changes in islet area over time in humans, which is not currently possible. Additionally, it could allow imaging of transplanted islets in the liver and hopefully help ascertain the contributions of functional abnormalities vs. loss of transplanted islet area to the worsening hyperglycemia seen in transplanted patients. We propose that Tf-based fusion technology is a broadly applicable and promising approach for prolonging the half-life of many bioactive proteins that are now used to treat a myriad of human diseases.

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References


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Footnotes

This work was supported by the Intramural Research Program of the National Institute on Aging, National Institutes of Health. BJK and JZ contributed equally to the manuscript.
Legends for figures

Figure 1. GLP-1-Tf exhibited similar efficacy but lower potency than native GLP-1, was insulinotropic, and had a prolonged half-life. (A) Stably transfected CHO/GLP-1R cells were treated with increasing concentrations of native GLP-1 and GLP-1-Tf for 30 min followed by assay of intracellular cAMP (N=2 separate experiments). (B) Isolated rat islets (N=40 per treatment group) were treated with increasing concentrations of native GLP-1 or GLP-1-Tf for 1 h at 37°C, and the insulin secreted into the buffer was assayed (N=4 different islet preparations). (C) Rat insulinoma (RIN1046-38) cells were treated with increasing concentrations of native GLP-1 and GLP-1-Tf for 1 h at 37°C, and the insulin secretion into the buffer was assayed (N=4 separate experiments). (D) Cynomolgus monkeys were injected with GLP-1-Tf (2.25 mg/kg) subcutaneously (SC) and intravenously (IV), and their serum was assayed at the time points shown (N=4 animals).

Figure 2. GLP-1-Tf decreased peak glucose levels and increased insulin secretion after an intraperitoneal glucose tolerance test (IPGTT, 0.5 g glucose/kg) and had a prolonged biological action in non-diabetic mice. (A) Blood glucose and plasma insulin levels following an IPGTT after an overnight fast. GLP-1-Tf and Tf were given IP at the time indicated (30 min). (B) Blood glucose and plasma insulin levels in ad libitum-fed mice. GLP-1-Tf and Tf were given SC at time 0. *p<0.05, compared to hTf-treated animals alone, †p<0.05, compared to GLP-1-Tf (1 mg/kg)-treated animals, ††p<0.05, compared to GLP-1-Tf (0.1 mg/kg)-treated animals, N=4-6 animals per treatment group.

Figure 3. GLP-1-Tf had prolonged biological activity, normalized blood glucose, and increased insulin release in ad libitum-fed db/db mice after intraperitoneal glucose tolerance test (IPGTT, 0.5 g glucose/kg). (A,B) Blood glucose and plasma insulin levels
after SC administration at time 0 of GLP-1-Tf and Tf. (C,D) Blood glucose and plasma insulin levels after IPGTT in overnight fasted mice that had received GLP-1-Tf and hTf 12 h prior to testing. *p<0.05, compared to Tf-treated animals alone, †p<0.05, compared to GLP-1-Tf (1 mg/kg)-treated animals. N=6 animals per treatment group.

Figure 4. Food intake was acutely decreased in non-diabetic and db/db mice that had been previously conditioned for 5 days to eat for 1 h daily, and post-prandial glucose levels were blunted by GLP-1-Tf. (A,B) Blood glucose and food intake in non-diabetic mice measured during the time intervals shown after IP GLP-1-Tf and Tf at time 0. (C,D) Blood glucose and food intake in db/db mice measured during the time intervals shown after IP GLP-1-Tf, Tf, and EX-4 at time 0. Values are expressed as means ± SE, N=5 animals per treatment group, *p<0.05, compared to hTf-treated animals alone.

Figure 5. Morphological and histological analysis of pancreatic islets from db/db mice after one IP of GLP-1-Tf and Tf. (A) Representative sections from pancreata of mice treated with IP GLP-1-Tf, Tf, EX-4, and PBS. Cell mitosis was measured by counting BrdU+ nuclei (brown in A: indicated by black arrows) per islet, BrdU having been injected 6 h prior to sacrifice. (B) Quantification of the number of BrdU+ cells from Panel A, values are expressed as means ± SE. (C,D) Representative co-staining for BrdU+(red) and insulin+(green) after one IP dose of GLP-1-Tf and Tf treated mice. BrdU+ nuclei were almost always in insulin+ cells. (E) Single staining for insulin, and (F) quantification of total beta-cell area as a percentage of the total pancreatic area. N=5-6
animals per treatment group. (F) Total β-cell area values. * $p<0.05$, ** $p<0.01$, magnification bar =100µm.

**Figure 6. Peripherally administered GLP-1-Tf activated c-Fos in certain brain areas but was not present in CSF.** (A) Western blot analysis for Tf protein levels in plasma and CSF 2 h after IP GLP-1-Tf and Tf administration to rats. P, plasma; C, CSF. The number of c-Fos immunopositive (Fos+) cells was counted in each field of view (B,D). IP administration of EX-4 (1 nmol/kg) significantly increased c-Fos immunoreactivity in the AP, NTS, and PVH regions. However, intraperitoneal administration of GLP-1-Tf (10 mg/kg) caused a significant elevation of immunoreactivity in the NTS only. There was no significant increase in c-Fos immunoreactivity in the AP, NTS, and PVH regions after intraperitoneal administration of Tf (10 mg/kg). ICV injection of GLP-1-Tf (7.2 mg/kg) caused a significant increase in c-Fos immunoreactivity in the NTS and PVH regions but not in the AP region (C,E). ICV administration of the Tf compound on its own (7 mg/kg) caused no significant changes in c-Fos immunopositivity in the AP, NTS, and PVH regions. N=6 animals per treatment group, ***$p< 0.001$ and **$p< 0.01$. AP = area postrema, NTS = nuclei of the solitary tract, PVH = paraventricular nuclei of the hypothalamus.

**Figure 7. Biological effects of EX-4-Tf on CHO/GLP-1R cells, human islets, and db/db mice.** (A) CHO/GLP-1R cells were treated with increasing concentrations of native EX-4 and EX-4-Tf for 30 min followed by assay of intracellular cAMP (N=3 separate experiments). (B) Isolated human islets (N=1000 per treatment group, one pancreatic
isolation) were treated in triplicate with increasing concentrations of native EX-4 and EX-4-Tf for 1 h at 37°C, and the insulin secreted into the medium was assayed. (C) Blood glucose levels after SC administration at time 0 of EX-Tf and EX-4 in ad libitum-fed db/db mice. *p<0.05, compared to EX-4 treated animals, †p<0.05, compared to EX-4-Tf (0.01mg/kg)-treated animals. N=4-6 animals per treatment group. (D) Food intake in ad libitum-fed db/db mice was measured during the time intervals shown after IP EX-4-Tf and EX-4 at time 0. N=5 animals per treatment group, *p<0.05, compared to EX-4-Tf (0.01mg/kg) treated animals.
Tables

Table 1. Pharmacokinetic data of GLP-1-Tf dosing of cynomolgus monkeys (*Macaca fascicularis*). The same bolus dose of GLP-1-Tf, 2.25 mg/kg, was administered to the monkeys either via a sub cutaneous (SC) or intravenous (IV) route. Plasma concentrations of GLP-1-Tf were assessed (by ELISA) at the time points after injection as depicted in Figure 1D.

<table>
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Figure 2

A

- □ hTf 10 mg/kg
- ■ GLP-1-Tf 10 mg/kg
- ● GLP-1/hTf 1 mg/kg

Blood glucose (mg/dl)

- -30 -15 0 15 30 45 60

Time (min)

IP IPGTT(0.5 g/kg BW) Peptides

B

- □ hTf 10 mg/kg
- ■ GLP-1-Tf 10 mg/kg
- ▲ GLP-1-Tf 1 mg/kg
- ● GLP-1-Tf 0.1 mg/kg

Insulin (ng/ml)

- 10 1 10

Basal hTf 30 min 30 min

Blood glucose (mg/dl)

- 0 12 24 36 48

Time (hour)

hTf 4 hrs GLP-1-Tf 4 hrs

Inset: Insulin levels at 10, 0.1, 1, and 10 mg/kg.
a) 

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b) Intraperitoneal injection

AP/NTS  PVH

PBS  A  E

GLP-1-Tf 11 nmol/kg

B  C  G

C  D  H

Tf 7.0 mg/kg

C  F

Tf 10 mg/kg

C  D

c) Intracerebroventricular injection

AP/NTS  PVH

PBS  A  E

GLP-1-Tf 11 nmol/kg

B  C  G

C  D  H

Tf 7.0 mg/kg

C  F

Tf 10 mg/kg

C  D

d) Intraperitoneal injection

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e) Intracerebroventricular injection

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** indicates statistical significance at p < 0.01; *** indicates statistical significance at p < 0.001.