Supplementary Materials and Methods

Participants

Six men participated in study A and 6 additional men participated in study B.

Study A: Determination of Intestinal and Hepatic TRL Particle Secretion in Response to GLP-2

Six healthy, nonobese, normoglycemic, normolipidemic men, on no medications, were recruited by advertisements in the local press. Their demographic and biochemical parameters are shown in Table 1. They underwent routine biochemical testing to exclude dysglycemia, anemia, coagulopathy, and renal or hepatic dysfunction. Each participant was studied on 2 occasions, 4–6 weeks apart, in a randomized, double-blind, placebo-controlled, cross-over trial. Randomized number generators were used by the hospital pharmacy to allocate patients. Each participant received a single subcutaneous dose of either 1500 μg of GLP-2 or placebo at the start of the lipoprotein kinetic study (at 9 AM or 0 hours, as illustrated in Figure 1*A*). A similar dose has been shown previously to be efficacious in short-bowel syndrome.[1,2]

GLP-2 Preparation

Native GLP-2 was procured in collaboration with Dr David Sigalet (Alberta Children's Hospital, University of Calgary, Calgary, Alberta, Canada). It was synthesized using solid-state peptide synthetic techniques and supplied as bulk, nonsterile powder, by CS Bio Co (Menlo Park, CA), with more than 97% purity, manufactured to good manufacturing practice standards. Identity of the peptide was confirmed by high-performance liquid chromatography analysis, mass spectrometric analysis, amino acid analysis, and elemental analysis.

The bulk compound (1.5 g) was dissolved in saline and pH buffered to 7.4, filter sterilized into sterile capped 10-mL vials (pyrogen-free, latex-free, rubber-capped vials, Afton Scientific, Charlottesville, VA), and stored frozen at -20°C to -80°C until use. The use of this formulation of GLP-2 for investigational purposes in human beings was approved by Health Canada (Ottawa, Ontario, Canada).

Lipoprotein Kinetic Studies

Participants were admitted to the Metabolic Test Centre of the Toronto General Hospital the morning before the lipoprotein kinetic study. Fasting blood samples were taken for measurement of plasma glucose, insulin, and lipids. The same morning, a nasoduodenal tube (Entriflex NG Tube 55" [140 cm] 10fr, item 8884721055; Kendall Products, Tyco Healthcare, Toronto, Ontario, Canada) was inserted into the first part of the duodenum under fluoroscopic guidance as previously described.[3] Volunteers had a standardized mixed meal at 5 PM and were not permitted any food or drinks orally except water until the conclusion of the study. The following day, lipoprotein kinetics was assessed as described previously[3] (Figure 1*A*). Because the apoB-48 concentration in the fasting state is too low to allow accurate assessment of isotopic enrichment, volunteers were studied in the constant-fed state with an intraduodenal infusion of a liquid formula (80 mL/h of Great Shake; Hormel Health Labs, Austin, MN; 13% protein, 38% carbohydrates, and 49% fat by caloric content) from 4 AM until the end of the study. Nasoduodenal feeding was used rather than oral ingestion of the liquid formula to circumvent any potential effect of GLP-2 on gastric emptying.[4] At 7 AM, a pancreatic clamp was started and continued for the remainder of the study (until 7 PM) to neutralize any potential effects of pancreatic hormone fluctuations on lipoprotein production. The clamp comprised the following infusions: somatostatin (Sandostatin; Novartis Pharmaceuticals Canada, Dorval, Quebec, Canada) 30 ug/h to inhibit pancreatic insulin and glucagon secretion with concomitant replacement at basal levels of insulin (Humulin R; Eli Lilly Canada, Toronto, Ontario, Canada) at 0.05 mU/kg/min, human recombinant growth hormone (because somatostatin inhibits growth hormone secretion) at 3 ng/kg/min (Humatrope; Eli Lilly Canada), and glucagon (Eli Lilly Canada) at 0.65 ng/kg/min. All hormones were diluted in 1 L of normal saline and infused with a syringe pump (B. Braun Medical, Inc, Bethlehem, PA). Autologous serum (5 mL), freshly prepared from the subject's blood, was added to the saline as a carrier before hormone dilution.

At 9 AM, a primed, constant infusion (10 μmol/kg bolus followed by 10 μmol/kg/h) of L-[5,5,5-$^2H_3$]-leucine (d3-leucine; Cambridge Isotope Laboratories, Andover, MA) was started and continued for 10 hours for the assessment of lipoprotein kinetics. Blood samples were collected at 1, 2, 3, 4, 5, 7, 8, 9, and 10 hours thereafter for isolation of TRL, stable isotope enrichment, and kinetic analysis. Blood samples for TG, FFA, and hormone analysis were collected at regular intervals. Blood samples for GLP-2 assay were collected into tubes containing DPP-IV inhibitor immediately before, and 2 and 4 hours after, GLP-2 or placebo administration.

Laboratory Methods

Plasma was separated from blood samples, within 2 hours, in a refrigerated centrifuge at 3000 rpm for 15 minutes at 4°C. Tetrahydrolipstatin (a lipase inhibitor, Hoffman-La Roche, Ltd, Basel, Switzerland), sodium azide (Sigma Aldrich, Oakville, Canada), and aprotinin (Sigma Aldrich) were added to the plasma to prevent hydrolysis and protein degradation at the following concentrations: tetrahydrolipstatin (0.55 mg/L blood), sodium azide (70 mg/L blood), and aprotinin (1.94 mg/L blood). TRLs were isolated at each time point with ultracentrifugation at d = 1.006 g/mL for 16 hours, 39,000 rpm at 12°C. The TRL fraction thus corresponded to an Sf of greater than 20, which includes lipoprotein fractions of chylomicrons and very-low-density lipoproteins. Aliquots of TRL fractions (approximately 1 mg protein) were delipidated and separated by preparative 3.3% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Gel bands corresponding to apoB-48 and apoB-100 were excised, hydrolyzed, and amino acids were derivatized to allow

for the determination of leucine isotopic enrichment as described.[5] Plasma free amino acids were extracted, dried, derivatized, and stable isotope enrichments were determined as previously described.[3] Derivatized samples were analyzed with gas chromatography–mass spectrometry (Agilent 5975/6890N; Agilent Technologies Canada, Inc, Mississauga, Ontario, Canada) with electron impact ionization using helium as the carrier gas. Selective ion monitoring at m/z of 200 and 203 was performed and tracer-to-tracee ratios were calculated from isotopic ratios for each sample according to a standard curve of isotopic enrichment.

Commercial kits were used to measure TG (Roche Diagnostics), FFA (Wako Industrials, Osaka, Japan), insulin (Millipore, Billerica, MA), total GLP-2 (Millipore), and glucagon (Millipore). TRL apoB-100 mass was measured with an enzyme-linked immunosorbent assay kit specific for human apoB-100 (Mabtech Inc, Mariemont, OH; intra-assay coeffecient of variability, 2%; interassay CV, 10%). TRL apoB-48 mass was measured by enzyme-linked immunosorbent assay using an apoB-48–specific assay (Shibayagi Co Ltd, Shibukawa, Gunma, Japan; intra-assay CV, 3.5%; interassay CV, 5.6%).

Steady-State Kinetic Analysis

A steady-state lipoprotein kinetic analysis was performed for apoB-100 (placebo and GLP-2 treatment) and apoB-48 (placebo treatment only). For compartmental modeling, isotopic enrichments were expressed as enrichment (E) using the expression E = tracer-to-tracee ratio/(1 + tracer-to-tracee ratio).[6] A multicompartmental model was fitted to the stable isotope enrichment curves for apoB-100 using SAAM II software (version 1.2; University of Washington, Seattle, WA) to derive the FCRs (Figure 1*B*). The model consisted of synthesis of TRL apoB from the precursor pool via a delay element that consisted of 3 subcompartments. Plasma-free leucine enrichment, determined at each visit for each subject, was used as a forcing function and individual enrichment time-course curves were used to derive kinetic rate constants. PRs of each apolipoprotein were calculated as follows: PR = FCR × pool size, where pool size = the average plasma concentration (mg/L) over the 10 hours of the kinetic study × plasma volume (estimated as 0.045 L/kg body weight).

Non–Steady-State Modeling of apoB-48

Because there was a large transient increase in TRL apoB-48 concentrations immediately after GLP-2 treatment, mathematic modeling with 3 different hypothesis-generating nonsteady state solutions were imposed on the steady-state model (Figure 1*B*) for the mean TRL apoB-48 data generated in the GLP-2 arm of study A as described earlier. Predicted TRL apoB-48 concentrations and enrichment based on non–steady-state modeling were compared with actual values to assess the potential validity of each solution. The 3 non–steady-state solutions were as follows. Hypothesis 1: GLP-2 causes a transient Gaussian-shaped increase in de novo–synthesized TRL apoB-48 synthesis (PR) without affecting apoB-48 FCR. Hypothesis 2: GLP-2 causes a transient reduction in TRL apoB-48 FCR, while de novo apoB-48 synthesis (PR) is constant over time. Hypothesis 3: GLP-2 releases preformed, unlabeled TRL apoB-48 into circulation. The de novo synthesis (PR) and FCR of apoB-48 are constant over time.

Study B

Six healthy, nonobese, normoglycemic, normolipidemic men were recruited by advertisements in the local press. Their demographic and biochemical parameters are shown in Table 1. An outline of the study design is shown (Figure 5*A*). They underwent a single-blind, cross-over study with 2 visits (one with GLP-2 and the other with placebo administration) 4 weeks apart. The order of visits was randomized using a randomized number generator. Volunteers were admitted to the Metabolic Test Centre at 6:30 AM on the morning of the study, a single sampling venous catheter was inserted into a superficial forearm vein, and baseline fasting blood samples were taken. At 7 AM (T = -7 hours) (Figure 5*A*) the participants were given a liquid meal comprising 4 small cartons of Hormel Great Shake Plus (Hormel Health Labs; 520 g in total with 52 g of fat, 92 g of carbohydrates, 32 g of protein, and a total caloric content of 960 Kcal), ingested over a 15-minute period. Along with the liquid meal, volunteers chewed and then swallowed 12 capsules (total dose, 120,000 IU) of vitamin A (retinyl palmitate) (Jamieson Natural Sources, Toronto, Ontario, Canada). Blood samples were taken hourly for the next 7 hours. At 2 PM (ie, 7 hours after ingesting the liquid meal, T = 0 hours) (Figure 5*A*) they received either 1500 μg of GLP-2 or placebo subcutaneously. Over the next 3 hours, further blood samples were drawn at 15-minute intervals for the first hour after GLP-2 or placebo administration and then half hourly until the conclusion of the study at 5 PM. All blood samples were collected in tubes covered with aluminum foil to avoid light-induced denaturation of retinyl palmitate. Plasma glucose, plasma TG, TRL TG, and TRL apoB-48 were measured as described earlier. In addition, the chylomicron ($S_f > 400$) fraction was isolated from TRL by ultracentrifugation (12,000 rpm for 30 minutes at 15°C) at 3 time points: at 2 PM immediately before administration of GLP-2 or placebo, and 30 minutes and 60 minutes thereafter.[7] The retinyl palmitate concentration in the TRL and chylomicron fraction was measured using high-performance liquid chromatography as previously described.[8] Because of degradation by light and limited sample volume, it was not possible to analyze chylomicron retinyl palmitate concentration in 1 volunteer and hence data for the remaining 5 participants has been presented for this parameter.

Statistics

Results are presented as means ± SEM. A paired *t* test was used to compare TG, FFA, apoB-100, and apoB-48 concentrations, and FCR and PR (for TRL apoB-100) between the 2 treatments. For study A, the area under the curve in the first 3 hours (AUC_{0-3}) after GLP-2

administration as well as ANOVA was calculated to compare the transient effect of GLP-2 on plasma and TRL triglyceride as well as TRL apoB-48 concentration. For study B, the $iAUC_{0-3}$ as well as ANOVA of the increment in plasma and TRL TG, TRL retinyl palmitate, and TRL apoB-48 were calculated. All statistics were performed with SAS (version 9; Cary, NC). A *P* value less than .05 was considered significant.

The primary end point for study A was the change in TRL apoB-48 concentration with GLP-2 treatment. Post hoc calculations showed a power of 92% and 88% based on mean values and standard deviation of peak values and AUC, respectively, in the first 3 hours after GLP-2 treatment compared with placebo.

The primary end point for study B was the change in TRL apoB-48 and TRL RP after GLP-2 administration. For TRL apoB-48, post hoc analysis based on mean values and standard deviation of peak concentration and AUC showed a power of 88% and 95%, respectively. For TRL RP, post hoc analysis based on mean values and standard deviation of peak concentration and AUC showed a power of 88% and 95%, respectively.

References

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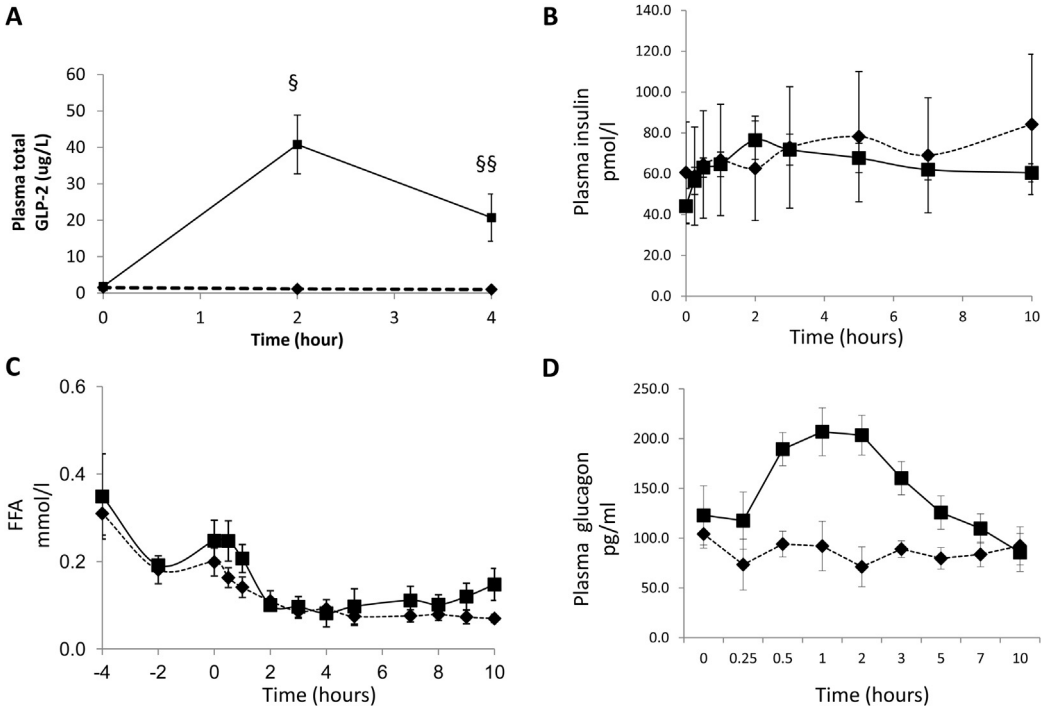
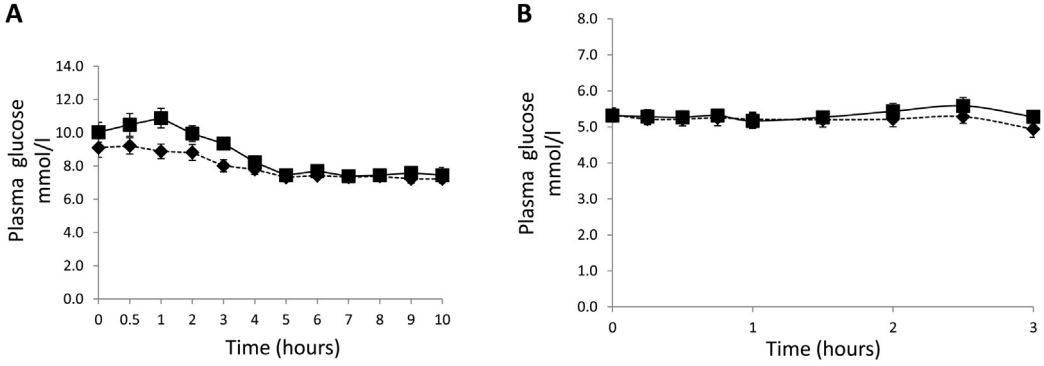


Supplementary Figure 1. Mean plasma total GLP-2 concentration (A) after administration of GLP-2 and placebo (placebo, *black diamond* and *dotted line*; GLP-2, *black square* and *solid line*). Total GLP-2 concentration was significantly higher 2 and 4 hours after GLP-2 administration compared with placebo. $^{\S}P < .005$, $^{\S\S}P < .05$. Plasma insulin (*B*), FFAs (*C*), and glucagon (*D*) (placebo, *black diamond* and *dotted line*; GLP-2, *black square* and *solid line*) were assessed during the course of the kinetic study (study A). There was no significant difference in concentration of insulin or FFA between treatments whereas glucagon was significantly higher with GLP-2 treatment vs placebo (mean concentration: placebo, 85.3 ± 15.9 vs GLP-2, 143.3 ± 14.2 pg/mL; $P = .01$).



Supplementary Figure 2. Plasma glucose concentrations (placebo, *black diamond* and *dotted line*; GLP-2, *black square* and *solid line*) were assessed during the course of study A (*A*) and study B (*B*). Glucose concentration was modestly higher with GLP-2 treatment (mean concentration: placebo, 8.0 ± 0.3 vs GLP-2, 8.7 ± 0.3 mmol/L; $P = .049$) in study A (*A*). There was no significant difference in glucose concentration between treatments in study B (*B*).