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Proinsulin-Transferrin Fusion Protein as a Novel Long-Acting Insulin Analog for the Inhibition of Hepatic Glucose Production

Proinsulin-transferrin (ProINS-Tf) fusion protein was evaluated for its in vivo pharmacokinetics, efficacy, and mechanism. Our previous studies have shown that ProINS-Tf was converted to active insulin-transferrin (INS-Tf) via the transferrin (Tf)-receptor–mediated pathway in hepatoma cells. We hypothesized that this fusion protein can be administered as a prodrug and be converted to a biologically active protein with specificity for the liver versus other insulin (INS)-sensitive tissues (muscle and adipose). Administration as an inactive prodrug with liver-specific action compared with other INS-sensitive tissues conceivably reduces negative side effects seen with other INS analogs. In this report, the data show that ProINS-Tf exhibited a slow, but sustained, in vivo hypoglycemic efficacy and long plasma half-life. The fusion protein showed activity in the liver, as evidenced by decreased expression of two key hepatic glucose production (HGP) enzymes, PEPCK and glucose-6-phosphatase, and increased glycogen levels under feeding conditions. Furthermore, the INS receptor (IR) phosphorylation (activation) in liver and muscle tissues was compared with postinjection of INS or ProINS-Tf. While INS activated IR in both the liver and muscle, ProINS-Tf only showed activation in the liver. Thus, ProINS-Tf fusion protein can potentially be administered as a prodrug with sustained Tf-mediated activation and selectivity in inhibiting HGP.

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Patients with type 1 or 2 diabetes are currently recommended basal-prandial insulin (INS) therapy to achieve optimized control of glycemia. This therapy requires a rapid-acting INS that simulates the physiological prandial INS levels and, additionally, a long-acting INS that replaces basal INS and targets the elevated basal glucose produced by the liver. Long-acting INS analogs provide sustained basal INS delivery and achieve extended hypoglycemic efficacy. Thus prolonging the time-action profiles for the glucose-lowering effects has been an important direction for development of INS therapeutics (1). The currently reported design of long-acting INS is based on either delaying absorption, such as INS glargine, or prolonging the half-life, such as INS degludec and single-chain INS-albumin fusion protein (2,3).

In addition to developing a long-acting INS analog, the targeting effect of INS analogs also needs to be considered. INS reduces hyperglycemia by inhibiting hepatic glucose production (HGP) and promoting peripheral glucose disposal (PGD). In normal physiology, the pancreas delivers endogenous INS directly into the hepatic portal vein, and approximately 50% of the INS is cleared in the liver before reaching the periphery (muscle and fat). Therefore, the liver is exposed to twofold to fourfold higher concentrations of INS than the periphery, which results in a greater effect in inhibiting HGP than promoting PGD (4). With exogenous therapeutic INS, on the other hand, INS and/or INS analogs are administered subcutaneously, thus leading to a relatively

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underinsulinized liver and hyperinsulinized periphery. The greater effects on PGD subsequently cause a higher risk of hypoglycemia as well as metabolic abnormalities, including excessive glycemic fluctuations, weight gain, and dyslipidemia (5). These associated side effects are the major disadvantages of current INS therapeutics.

Therefore, an INS analog with a preferential effect on HGP would better mimic the portal delivery of INS and thus offer a greater advantage over conventional INS therapeutics. Recently, we have designed a proinsulin-transferrin (ProINS-Tf) recombinant fusion protein that can be converted to an active form of INS-transferrin (INS-Tf) through a transferrin (Tf)-receptor–mediated endocytosis and recycling process in hepatoma cells (6). We report here that ProINS-Tf exhibited an extended and sustained in vivo hypoglycemic effect that was active in inhibiting HGP. Our results suggest that ProINS-Tf can be potentially developed into an effective drug for basal INS replacement therapy.

RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6J mice (6–7 weeks old, 20–25 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a University of Southern California animal facility on a 12-h day and 12-h night cycle, with room temperature maintained at 22 ± 3°C and relative humidity at 50 ± 20%. All mice had access to water and regular rodent diet (Labdiet, Richmond, IN) ad libitum. Under fasting conditions, mice were restrained from food and only provided with water. All animal studies were conducted according to National Institutes of Health guidelines. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Southern California.

Streptozotocin (STZ; Sigma, St. Louis, MO) solution was freshly prepared in 100 mmol/L sodium citrate buffer, pH 4.5. Following 4–6 h of fasting, mice were injected intraperitoneally with 150 mg/kg STZ to induce diabetes. Mice were then allowed to recover for 5 days to develop diabetes. Blood glucose (BG) levels were measured via tail vein using OneTouch glucose meter (LifeScan, Milpitas, CA) with a detection range between 20 and 600 mg/dL. Mice with BG levels ~500 mg/dL were used for experiments.

Recombinant Fusion Protein Production

ProINS-Tf recombinant fusion protein was produced as previously described (6). Briefly, human preproinsulin sequence was ligated in frame with C-terminally his-tagged full-length human Tf. Plasmids containing the fusion gene were transfected to HEK 293 cells (ATCC, Manassas, VA). Conditioned media were collected after 8-day cultures. Fusion protein was concentrated using tangential flow filtration (Millipore) and further purified by nickel nitrilotriacetic acid agarose (Qiagen, Valencia, CA). The fusion protein was characterized and quantified by SDS-PAGE followed by Coomassie blue staining and anti-Tf and antiproinsulin Western blot. Recombinant human INS from *Escherichia coli* (Sigma) was dissolved in 100 mmol/L HCl (pH 3.0) to 15 mg/mL and then further diluted in PBS to a stock of 15 μg/mL. Recombinant human proinsulin (ProINS; R&D Systems, Minneapolis, MN) was dissolved in PBS to a stock of 100 μg/mL. Recombinant INS glargine (Lantus) was diluted up to 13-fold in distilled water while maintaining the proper pH and zinc:INS ratio prior to in vivo use.

Trypsin Digestion

ProINS-Tf was mixed with trypsin in different w/w ratios from 1:0.045 to 1:1.51. The mixture was incubated at 37°C for 15 min to allow trypsin digestion. Bowman–Birk soybean protease inhibitors were subsequently added to the mixture in the equivalent amount to trypsin (w/w) to inhibit excess tryptic activity. The final mixtures were directly applied to nickel nitrilotriacetic acid column for purification. The eluted trypsin digested (TD) product was designated as ProINS-Tf-TD.

Glucose Uptake Assays in Adipocytes

Adipocytes were differentiated from 3T3-L1 murine fibroblasts (ATCC) as previously described (7). Prior to the treatment, adipocytes were serum-starved in Dulbecco's modified Eagle's medium supplemented with 0.5% BSA for 16 h. Cells were incubated with different proteins in Krebs–Ringer phosphate buffer containing 0.1% BSA at 37°C for 30 min. Following drug treatment, 0.5 μCi/mL of 2-deoxy-D-[2,6-^{3}H]glucose (Perkin Elmer, Waltham, MA) was added to the medium and incubated for 10 min. Glucose uptake was stopped by aspirating the medium and immediately washing the cells four times with ice-cold Krebs–Ringer phosphate buffer. Cells were lysed with 0.1 mol/L NaOH/0.1% SDS, followed by measurement of the internalized radiolabeled glucose using a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (Perkin Elmer) and protein quantification using the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA).

Measurement of In Vivo Hypoglycemic Efficacy

For experiments carried out under fasting conditions, mice were given a single intravenous or subcutaneous injection of protein or buffer only (vehicle group) on day 5 following STZ induction. Mice were fasted for 2 h prior to protein injection and remain fasted until 12 h post-injection. For experiments under free-feeding conditions, protein injections were executed on day 3 to avoid BG levels beyond the detection range of the glucose meter. Mice were given food ad libitum during the recording period. BG was monitored at the indicated time points postadministration.

Measurement of In Vivo Pharmacokinetics

Blood (20 μL) was collected from the saphenous vein of mice at different time points following protein injection.

Blood samples were mixed with heparin and centrifuged at 1,000*g* for 30 min to obtain plasma. Human ProINS-specific radioimmunoassay (Millipore, Billerica, MA) was applied to quantify the concentration of ProINS or ProINS-Tf in the plasma samples using various concentrations of ProINS or ProINS-Tf as standard curves, respectively. Pharmacokinetic parameters were calculated as previously described (8).

Measurement of mRNA Expression Levels

Liver glucose-6-phosphatase (G6Pase) and PEPCK mRNA expression was measured using real-time PCR. Briefly, following protein injection, mouse livers were excised and perfused with PBS and then immediately frozen in liquid nitrogen. Total RNA from frozen liver tissues was extracted using TRIzol reagent (Invitrogen) followed by DNase treatment using TURBO DNase (Invitrogen, Carlsbad, CA) to remove contaminating DNA. DNase-treated RNA was further reverse transcribed into cDNA, and target cDNA was subsequently quantified by TaqMan real-time PCR using specific oligo probes and primers from Applied Biosciences and normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The assay numbers for the probes and primers of G6Pase, PEPCK, and GAPDH were Mm00839363_m, Mm00440636_m1, and Mm99999915_g1, respectively. Relative expression levels compared with the vehicle-treated group were analyzed using the comparative C_T method (9).

Measurement of Glycogen

Free-fed mice were injected subcutaneously with either PBS, INS, or ProINS-Tf, and the liver tissue was isolated at 5 or 17 h postinjection. The liver tissue was perfused and homogenized and the glycogen content was measured as previously described (10). Briefly, tissues were hydrolyzed with hot alkaline solution (33% KOH), and the glycogen was precipitated with ethanol. The glycogen was then redissolved in distilled water and reacted with 0.2% anthrone in 96% sulfuric acid to give a blue-colored compound. The absorbance of the solutions was measured at 620 nm. The glycogen concentration was calculated against a D-glucose standard curve.

INS Receptor Phosphorylation Assays

Mice were fasted for 2 h prior to subcutaneous injection with PBS, ProINS-Tf, or INS. Liver and muscle tissues were collected at 1 and 8 h postinjection and homogenized. INS receptor (IR) was immunoprecipitated by anti-IR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to 10% SDS-PAGE followed by Western blot against antiphosphotyrosine (PY20; Millipore) or anti-IR antibodies. Band densities were measured using Quantity One (Bio-Rad, Hercules, CA) software.

Statistical Analysis

The data are presented as average plus SD for all experiments. The Student *t* test was used to compare data sets, where differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

ProINS-Tf Fusion Protein Exhibited Prolonged and Enhanced In Vivo Exposure

The in vivo pharmacokinetic profiles of ProINS and ProINS-Tf were compared in STZ-induced diabetic mice. Plasma concentration of equimolar ProINS and ProINS-Tf (22.5 nmol/kg) following subcutaneous injections in free-fed diabetic mice is shown in Fig. 1. The calculated pharmacokinetic parameters are presented in Table 1. No difference in the pharmacokinetics of ProINS-Tf was observed between fasting and free-feeding conditions (data not shown). A 22.5 nmol/kg dose of ProINS was detectable within 2 h postinjection, while samples collected beyond that time point were nondetectable. A 22.5 nmol/kg dose of ProINS-Tf, however, could be detected up to 48 h postinjection. Subcutaneous administration of ProINS-Tf showed a protracted absorption with a t_{max} of 5.5 h, compared with ProINS, which has a t_{max} of 0.3 h (Fig. 1 and Table 1). In addition, ProINS-Tf exhibited an elimination half-life of 7.29 h and rate constant of 0.095 h^{-1}, whereas these were only 0.5 h and 1.42 h^{-1} for ProINS, respectively. The plasma concentration of ProINS-Tf showed a plateau region between 4 and 8 h, indicating that the absorption and elimination reached equilibrium during this period. Overall, ProINS-Tf showed enhanced exposure to the mice with a significantly higher area under the curve (AUC) value than ProINS (77,950.70 $h \cdot ng \cdot ml^{-1}$ for ProINS-Tf compared with 426.59 $h \cdot ng \cdot ml^{-1}$ for ProINS).

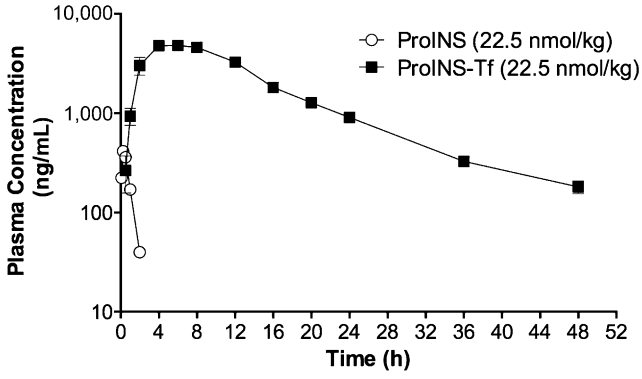

Figure 1—In vivo pharmacokinetic profiles of ProINS and ProINS-Tf. STZ-induced diabetic mice were subcutaneously administered with either 0.21 mg/kg ProINS or 2 mg/kg ProINS-Tf (equivalent to a dose of 22.5 nmol/kg). For ProINS group, blood was collected at 5, 15, and 30 min and 1, 2, 4, and 6 h postinjection. For ProINS-Tf group, blood was collected at 5, 15, and 30 min and 1, 2, 4, 6, 8, 12, 16, 20, 24, and 48 h postinjection. The concentration of ProINS or ProINS-Tf from collected plasma samples at each time point was measured using human ProINS-specific radioimmunoassay. Data were presented as average, with error bars indicating the SD (n = 4 per group).

Table 1—Pharmacokinetic analyses of ProINS and ProINS-Tf in diabetic mice following subcutaneous administration

	ProINS (0.21 mg/kg, 22.5 nmol/kg)	ProINS-Tf (2 mg/kg, 22.5 nmol/kg)
C_{max} (ng · mL^{-1})	432.52 ± 84.19	5,032.67 ± 161.91
t_{max} (h)	0.31 ± 0.11	5.50 ± 1.91
$t_{1/2elim}$ (h)	0.50 ± 0.09	7.29 ± 0.15
K_{el} (h^{-1})	1.42 ± 0.23	0.095 ± 0.002
AUC_{0-inf} (h · ng · mL^{-1})	426.59 ± 56.51	77,950.70 ± 2,947.35

Data are represented as average ± SD (n = 4 per group). C_{max}, maximal plasma concentration; t_{max}, time to maximal plasma concentration; $t_{1/2elim}$, elimination half-life; K_{el}, elimination rate constant; AUC_{0-inf}, area under the plasma concentration curve extrapolated to infinity.

ProINS-Tf Fusion Protein Elicited a Delayed but Sustained In Vivo Hypoglycemic Effect Under Fasting Condition Following Subcutaneous Injection

The ability of ProINS-Tf to reduce hyperglycemia in STZ-induced diabetic mice was evaluated through subcutaneous injection (Fig. 2*A*). When mice were fasted for 14 h (2 h preinjection and 12 h postinjection), BG levels in the vehicle-treated mice gradually declined during the early time points to ~300 mg/dL (~60% of initial levels) recorded at 6 h postinjection. However, the BG levels elevated and returned to ~400 mg/dL (~80%) by 12 h postinjection. The hypoglycemic effect of 22.5 nmol/kg ProINS-Tf slowly increased, and a maximum effect was achieved starting at 4 h and sustained until at least 12 h postinjection. During this period, a 22.5 nmol/kg dose of ProINS-Tf reduced 73–81% of the BG relative to their initial levels (Fig. 2*B*). This resulted in a decrease of BG to approximately 100 mg/dL, which was within the normoglycemic range and similar to non-STZ-induced mice (11).

On the other hand, when mice were administered with equimolar INS or ProINS as ProINS-Tf, BG levels rapidly decreased within the first 2 h and then quickly returned to the levels similar to the vehicle-treated group at 6 h postinjection. The lowest BG levels in INS and ProINS groups were ~100 mg/dL (78% reduction) and ~150 mg/dL (62% reduction), respectively (Fig. 2*B*). Interestingly, the activity of ProINS-Tf at 12 h was comparable to that of INS at 1 and 2 h.

The hypoglycemic effect of ProINS-Tf was dose dependent (Fig. 2*C*). Compared with a 22.5 nmol/kg dose, a lower dose of ProINS-Tf at 7.7 nmol/kg exhibited a weaker effect, showing BG levels of ~150 mg/dL (a 60–70% decrease relative to the initial levels). The effect of the low-dose ProINS-Tf was maintained for a shorter time period (~8 h postinjection) compared with the high dose.

Furthermore, the hypoglycemic effect of ProINS-Tf was also compared with the long-acting INS analog, INS glargine. Similar to previous studies in STZ-induced diabetic rats (12), a low dose of INS glargine (22.5 nmol/kg) showed a fast onset with a short-term response that was statistically similar to the PBS control after ~6 h postinjection (Fig. 2*D*). At a sixfold higher dose of 135 nmol/kg, INS glargine exhibited a more prolonged hypoglycemic effect; however, the mice suffered from severe hypoglycemia (13), with BG levels <30 mg/dL, especially in the initial phase. Meanwhile, ProINS-Tf exhibited a late onset and sustained hypoglycemic effect at both 22.5 and 135 nmol/kg doses, with BG levels maintained above the severe hypoglycemia threshold of 30 mg/dL (Fig. 2*D*).

ProINS-Tf Fusion Protein Showed a Delayed Rather Than Immediate Response Following Intravenous Injection

Previous studies have shown that ProINS-Tf lacks immediate short-term activity in adipocytes and requires conversion to bioactive INS-Tf (6). Trypsin digestion has been used to remove C-peptide from ProINS and generate an INS-like molecule (14). The ProINS-Tf-TD band on anti-Tf Western blot (Fig. 3*A*) was shifted to the position between Tf (80 kDa) and ProINS-Tf (89 kDa), indicating that ProINS-Tf was converted to an INS-like Tf, and the Tf moiety remained intact and attached. Next, the ProINS-Tf-TD activation was verified by measuring the in vitro promotion of glucose uptake in adipocytes. Compared with ProINS-Tf by itself, ProINS-Tf-TD showed significantly increased ability to promote glucose uptake. Moreover, the activation was in a trypsin-concentration-dependent manner (Fig. 3*B*).

In order to determine whether the delayed effect of ProINS-Tf following subcutaneous administration (Fig. 2*B*) was due to a delayed absorption (Fig. 1) or possibly an in vivo activation to INS-like-Tf, the activity of ProINS-Tf and ProINS-Tf-TD was compared following intravenous administration in fasted diabetic mice. As expected, INS (4.5 nmol/kg) exhibited a sharp and fast BG decrease from 400 to 140 mg/dL (65% decrease) at 1 h, and the BG subsequently recovered to the levels similar to the vehicle group at 4 and 12 h postinjection (Fig. 3*C* and *D*). Intravenous injection of equimolar ProINS-Tf only resulted in a minimal decrease in BG at the initial 1 and 2 h postinjection; however, BG dropped to 181 mg/dL (61% decrease) at 4 h and was maintained at ~150 mg/dL (65% decrease) at 12 h postinjection (Fig. 3*C* and *D*). On the other hand, ProINS-Tf-TD exhibited immediate hypoglycemic activity, with BG levels decreasing to 282 and 220 mg/dL (39 and 55% decrease) at 1 and 2 h, respectively. This immediate response was similar to INS and was considerably more potent than ProINS-Tf itself (Fig. 3*C*). Furthermore, ProINS-Tf-TD also demonstrated an extended pattern, with BG levels dropping to 116 and 224 mg/dL (76 and 57% decrease) at 4 and 12 h, respectively (Fig. 3*D*).

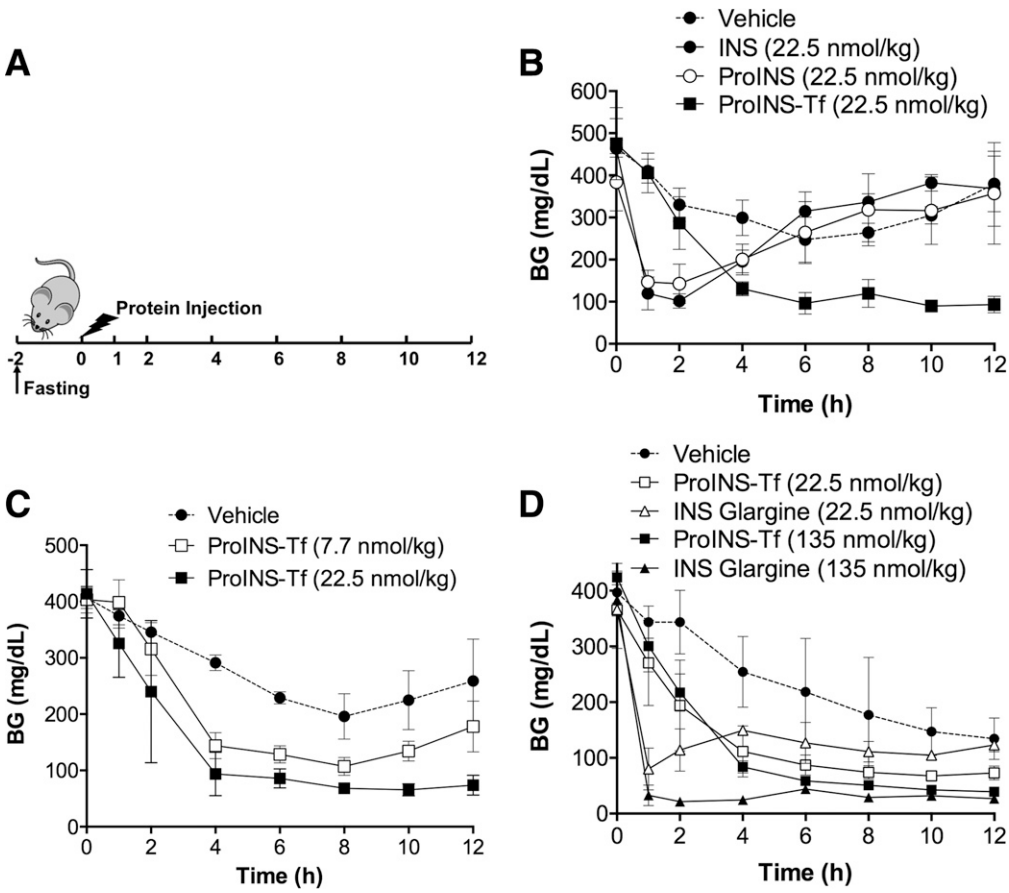

Figure 2—ProINS-Tf fusion protein exhibited a delayed but sustained in vivo hypoglycemic effect following subcutaneous injection in STZ-induced diabetic mice under fasting conditions. *A*: Experimental procedure. STZ-induced diabetic mice were fasted for 2 h prior to protein injection. Mice remained fasted until 12 h postinjection. BG was monitored at indicated time points. *B*: Mice were administered with buffer (vehicle) or protein (22.5 nmol/kg) through subcutaneous injection. *C*: Dose-dependent response of ProINS-Tf, where 7.5 or 22.5 nmol/kg of ProINS-Tf was subcutaneously injected in mice. *D*: A comparison with INS glargine was made following subcutaneous injection of a low (22.5 nmol/kg) or high (135 nmol/kg) dose. Data obtained from four mice per group were shown as average with error bars indicating the SD.

Hypoglycemic Effect of ProINS-Tf Fusion Protein Under Fasting Conditions Resulted From the Suppression of HGP

G6Pase and PEPCK are two key enzymes controlling the HGP in the liver. G6Pase catalyzes the terminal step in both gluconeogenic and glycogenolytic pathways, and PEPCK synthesizes phosphoenolpyruvate from oxalacetate, which is the rate-controlling step of gluconeogenesis. In diabetic conditions, the mRNA levels of these two enzymes are elevated, and they can be reduced by administration of INS (15,16). Therefore, the mRNA expression of G6Pase and PEPCK in liver tissues was measured to evaluate the suppression of HGP. Under a 12-h fasting period, ProINS-Tf (22.5 nmol/kg) remarkably suppressed 89% of the G6Pase mRNA expression at 12 h postinjection, whereas the inhibitory effects by equimolar ProINS and INS were 59% and 16%, respectively. Furthermore, ProINS-Tf inhibited 22% of the PEPCK mRNA expression at 12 h postinjection, which was much less compared with G6Pase. ProINS and INS both showed minimal effects in inhibiting PEPCK transcription (Fig. 4). These data indicated that ProINS-Tf efficaciously suppressed the HGP pathway in the liver when mice were fasted for a 12-h duration.

Prolonged Hypoglycemic Activity of ProINS-Tf Fusion Protein Is Liver Specific

The activation (i.e., phosphorylation) of the IR was compared in the liver versus muscle tissue where the majority (80–85%) of the peripheral effects of INS occur (17). Consistent with the hypoglycemic efficacy, IR phosphorylation was detected in both liver and muscle tissue only at 1 h, but not 8 h, postinjection in INS-treated mice. ProINS-Tf–treated mice, on the other hand, showed a prolonged IR phosphorylation at 1 and 8 h, and this effect was only detected in the liver and not in muscle (Fig. 5*A–C*).

ProINS-Tf Fusion Protein Promoted Liver Glycogen Synthesis Under Free-Feeding Conditions

The hypoglycemic effect of ProINS-Tf was evaluated in free-fed diabetic mice within 12 h following

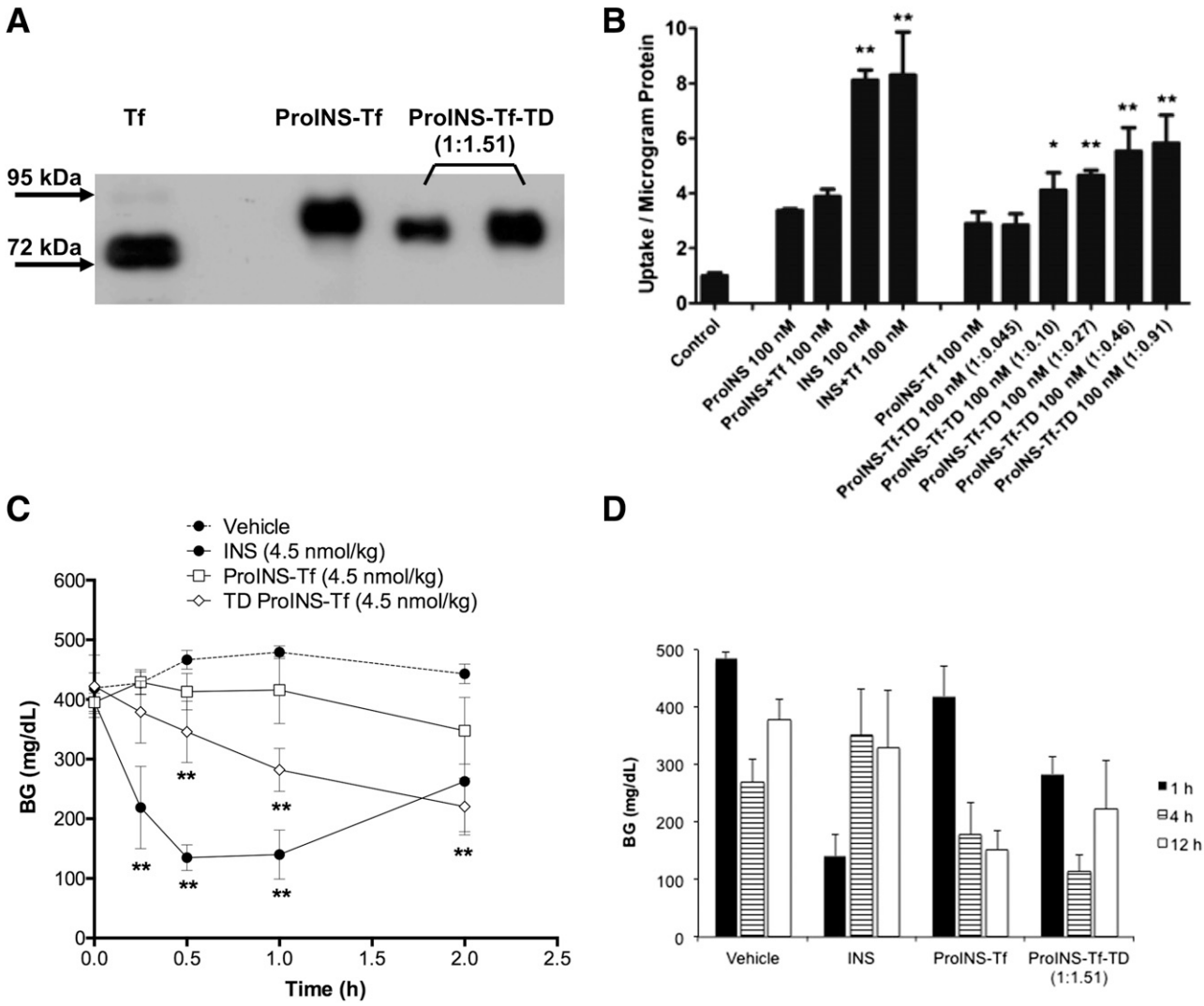

Figure 3—ProINS-Tf was activated in vivo following administration to STZ-induced diabetic mice. *A*: Profiles of ProINS-Tf and ProINS-Tf-TD (1:1.51) were analyzed by anti-Tf Western blot. ProINS-Tf was mixed with trypsin in the ratio of 1:1.51 (w/w) and incubated at 37°C for 15 min. After the reaction, Bowman–Birk soybean protease inhibitor was added to inhibit excess trypsin activity. The mixture was further purified by nickel column, and ProINS-Tf-TD was eluted and dialyzed. Aliquots of ProINS-Tf and ProINS-Tf were resolved in 8% non-reducing SDS-PAGE followed by anti-Tf Western blot detection. *B*: Promotion of glucose uptake in cultured adipocytes by ProINS-Tf and by various ProINS-Tf:TD at various fusion protein:trypsin ratios shown in parentheses on the *x*-axis label. Differentiated adipocytes were treated with either buffer only or 100 nmol/L of different proteins for 30 min followed by a 10 min uptake of 2-deoxy-D-[2,6-^{3}H]glucose. The amount of radiolabeled glucose was determined by a β-counter. Results were calculated as count per minute normalized to total cellular protein amount. Data were presented as the average fold of uptake compared with control (buffer only), with error bars indicating the SD (n = 3). Data marked with an asterisk indicated statistically significant differences compared with control group using Student t test. *C* and *D*: Hypoglycemic efficacy of INS, ProINS-Tf, and ProINS-Tf-TD (1:1.51) through intravenous injection to STZ-induced diabetic mice under fasting conditions. Mice were administered with either buffer (vehicle) or three proteins in the equimolar dose equivalent to 4.5 nmol/kg, and the BG was measured at various time points postinjection. Results were expressed as percentage of BG relevant to the vehicle group at each time point. Data were presented as average, with error bars indicating the SD (n = 4 per group). *P < 0.05; **P < 0.01.

subcutaneous injection. The BG level of vehicle-treated (PBS) mice remained at ~500 mg/dL during the time course. INS-treated mice showed a rapid initial reduction in BG levels to 100 mg/dL, which returned to ~400 mg/dL at the next measure time point (8 h). ProINS-Tf induced a decrease of BG levels from ~500 to ~400 mg/dL after 2 h and remained steady between 350 to 400 mg/dL from 2 to 12 h postinjection (Fig. 6).

The effect of ProINS-Tf on stimulating glycogen synthesis was also measured under free-feeding conditions. Following subcutaneous injection of either PBS, INS, or ProINS-Tf, liver glycogen levels were measured at time points with similar BG levels (i.e., at 5 and 17 h postinjection). As shown in Fig. 7*A* and *B*, there was no significant difference in glycogen levels at 5 vs. 17 h postinjection for either the PBS or INS-treated groups.

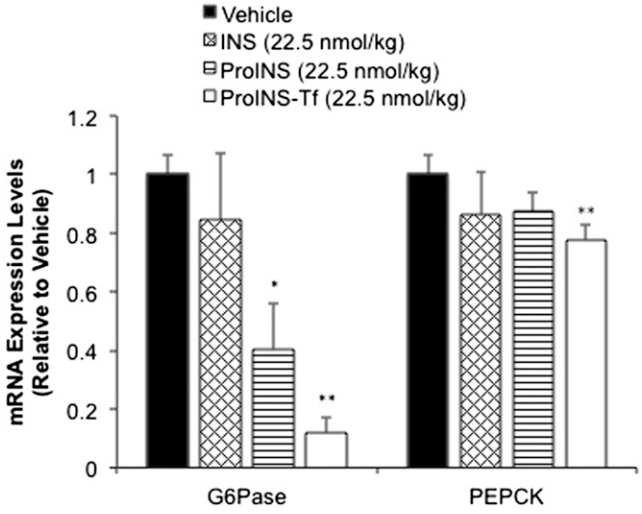

Figure 4—Inhibition of hepatic G6Pase and PEPCK mRNA expression levels by subcutaneously injected PBS (vehicle), ProINS-Tf, ProINS, or INS at 12 h postinjection. Equimolar dose of INS (0.13 mg/kg), ProINS (0.21 mg/kg), or ProINS-Tf (2 mg/kg) was administered to STZ-induced diabetic mice through subcutaneous injection. At 12 h postinjection, liver was excised, perfused with PBS, and immediately frozen in liquid nitrogen. Total RNA was extracted and reversely transcribed to cDNA, from which the expression of G6Pase, PEPCK, and GAPDH were measured using real-time PCR. The mRNA levels of G6Pase and PEPCK were normalized to GAPDH expression. Data were presented as relative expression in protein-treated group compared with vehicle (buffer-treated) group using the comparative C_T method. Data marked with asterisks indicate statistically significant differences compared with vehicle group using Student *t* test. $^*P < 0.05$; $^{**}P < 0.01$.

However, in the ProINS-Tf group, glycogen levels significantly increased at 17 h compared with 5 h postinjection (Fig. 7*C*).

DISCUSSION

The extended efficacy of ProINS-Tf fusion protein in reducing hyperglycemia suggested its potential advantages as a long-acting INS analog. Due to its stability and receptor recycling mechanism (18,19), Tf has been applied to prolong half-life of small peptides and proteins, such as glucagon-like peptide 1 (20) and human growth hormone (8). As expected, a 15-fold increase of elimination half-life in ProINS-Tf was observed when compared with ProINS alone in diabetic mice. Accordingly, ProINS-Tf provided a significantly greater in vivo exposure than ProINS alone as calculated by the AUC values (Table 1). The augmented pharmacokinetics of ProINS-Tf appeared to correspond with its extended hypoglycemic effects starting from 4 to 12 h postinjection (Fig. 2*B*), although the efficacy was only recorded until 12 h to avoid the long period of fasting for mice. The ProINS-Tf also exhibited superior biological activity to the long-acting INS analog, INS glargine (Fig. 2*D*). At a low dose, the BG levels were maintained at normoglycemic levels for a prolonged period following injection with ProINS-Tf, while INS glargine was similar to INS at this dose. At a sixfold higher dose, the effect of INS glargine was drastically different, where the BG levels were below the severe hypoglycemia threshold, especially at the initial time points as shown between 2 and 4 h after injection. The ProINS-Tf fusion protein, on the other hand, was devoid of the initial INS shock effect and still maintained BG above the severe hypoglycemia threshold at all time points at both doses, indicating a better therapeutic window and safety profile compared with that of INS glargine. These features indicate ProINS-Tf as a promising candidate for a long-acting INS (21,22).

Upon first inspection, there seemed to be good accordance of the slow absorption process with the delayed onset of the hypoglycemic effect following subcutaneous administration (Figs. 1 and 2*B*). The hypoglycemic effect of ProINS-Tf through intravenous administration was then determined to evaluate the involvement of the absorption phase with the delayed occurrence of in vivo efficacy. Although all the ProINS-Tf enters the blood circulation immediately following intravenous injection, it only resulted in a minimal decrease in BG at 1 h postinjection, as opposed to the acute and considerable reduction of 65% in BG by INS administered at the same molar dose (Fig. 3*C*). This result indicated that ProINS-Tf itself had weak activity in reducing in vivo hyperglycemia. However, the activity of ProINS-Tf gradually improved and demonstrated a 65% decrease at 12 h postinjection (Fig. 3*D*), similar to that of subcutaneous injection (Fig. 2*B*). Without interference from absorption, this delayed rather than immediate occurrence of activity for ProINS-Tf suggested an in vivo activation of this fusion protein. A Tf-receptor–mediated conversion of ProINS-Tf to an active form of INS-Tf was previously reported in hepatoma cells (6). Therefore, it is likely that ProINS-Tf was activated to INS-like Tf in vivo. Furthermore, the activity of INS-like Tf was demonstrated by ProINS-Tf-TD, which elicited an immediate response, with a 39% decrease in BG at 1 h following intravenous injection (Fig. 3*C*). ProINS-Tf-TD was weaker than INS but considerably stronger than ProINS-Tf, which was consistent with results detected in cultured adipocytes (Fig. 3*B*).

The hypoglycemic efficacy of ProINS-Tf at 12 h postinjection correlated with its suppression in liver G6Pase and PEPCK mRNA expression (Fig. 4). The higher effect on suppressing G6Pase than PEPCK was likely due to the intermediate fasting time of 12 h, where the major pathway of HGP is mainly via glycogenolysis (affected by G6Pase) rather than gluconeogenesis (affected by both G6Pase and PEPCK) (15,16). In order to directly compare the effect of ProINS-Tf in the liver versus peripheral tissues, IR activation (phosphorylation) in both tissues stimulated by ProINS-Tf was evaluated. The results showed that, at 8 h postinjection, ProINS-Tf promoted IR phosphorylation only in the liver but not in the muscle (Fig. 5*A–C*). These data strongly support the prolonged activity of ProINS-Tf in the liver and also indicate that

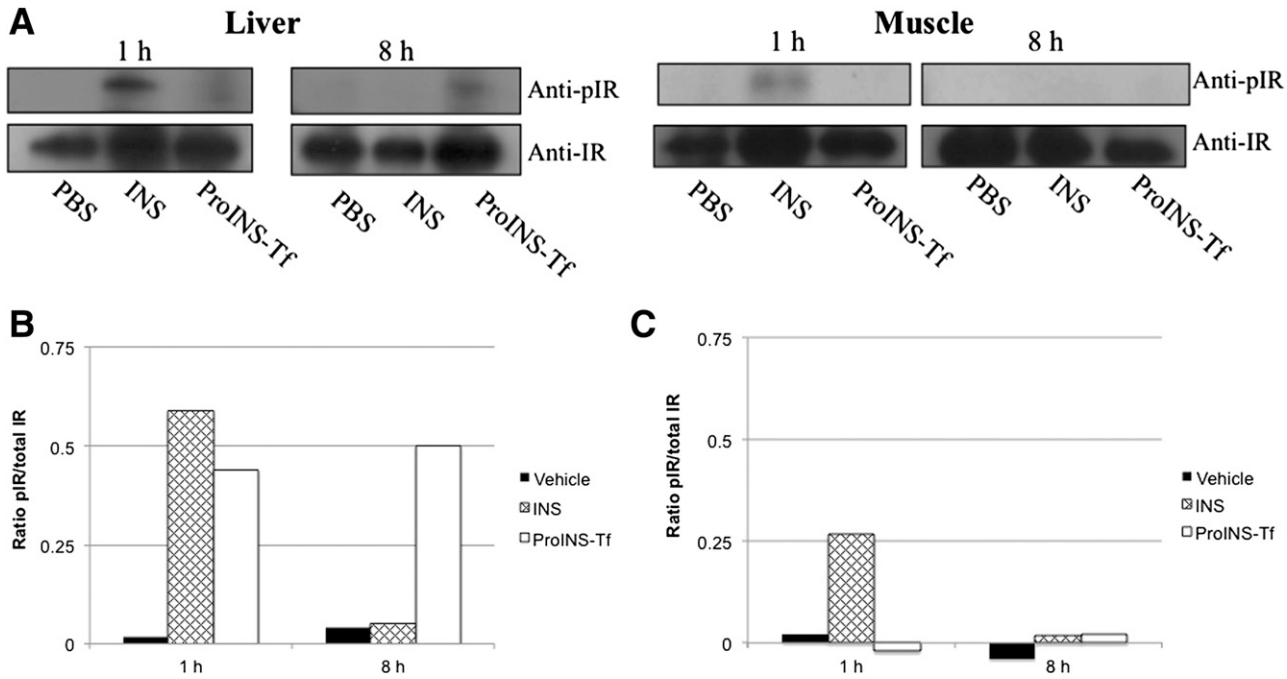

Figure 5—IR phosphorylation in the liver versus muscle. Mice were fasted for 2 h prior to subcutaneous injection with PBS (vehicle), ProINS-Tf, or INS. Liver and muscle tissues were collected and homogenized at 1 and 8 h postinjection. *A*: IR was isolated by immunoprecipitation using anti-IR antibody and subjected to 10% SDS-PAGE followed by Western blot against antiphosphotyrosine or anti-IR antibodies. The bar graphs represent the quantified band densities from the Western blot analysis of (*B*) liver and (*C*) muscle samples. pIR, IR phosphorylation.

this effect is hepatospecific, with little activity in the periphery.

In addition, the drastic difference in the efficacy of ProINS-Tf between prolonged fasted (BG decreased from ~500 to ~100 mg/dL) (Fig. 2*B*) and free-fed (BG decreased from ~500 to 350–400 mg/dL) (Fig. 6) diabetic mice further indicated a connection of the fusion protein's efficacy with the liver. Under a duration of fasting longer than 6 h, HGP from the liver becomes the exclusive supplier to the BG. Under feeding conditions, reduced BG results mainly from promoting glucose disposal in the periphery (4). Thus the more potent efficacy of ProINS-Tf observed during long fasting than free-feeding indicated a preferential effect of ProINS-Tf in inhibiting HGP in the liver compared with promoting PGD in the periphery. ProINS-Tf is able to promote glycogen synthesis under feeding conditions (Fig. 7), which could account for the observed 20% decrease in BG in fed mice (Fig. 6). This explanation is consistent with studies showing that, under feeding conditions, ~20–30% of the oral glucose load is stored in the liver (23–25).

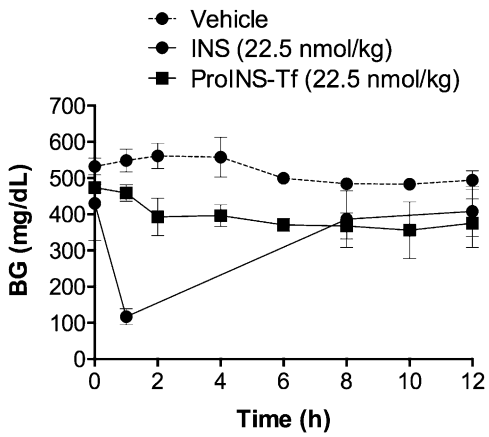

Figure 6—In vivo hypoglycemic effect in free-fed STZ-induced diabetic mice. Experiments were carried out on day 3 following STZ induction, and mice were kept in free-feeding condition. Mice were injected with either PBS (vehicle) or 22.5 nmol/kg of INS or ProINS-Tf through subcutaneous administration. Results were calculated as percentage of initial BG level at the time of injection. Data were shown as average, with error bars indicating the SD (n = 4 per group).

Furthermore, the relatively low activity of ProINS-Tf in the peripheral tissues was also supported by the low in vitro activity measured in cultured adipocytes (Fig. 3*B*) and the lack of IR phosphorylation in muscles at both 1 and 8 h postinjection (Fig. 5*A* and *C*). This conclusion is further supported by the observation that no acute severe hypoglycemia was detected in ProINS-Tf–treated diabetic mice even at an extremely high dose (Fig. 2*D*). This observed liver-preferential effect of ProINS-Tf could be attributed to the in vivo activation process. Liver cells express high levels of Tf receptor, which may facilitate

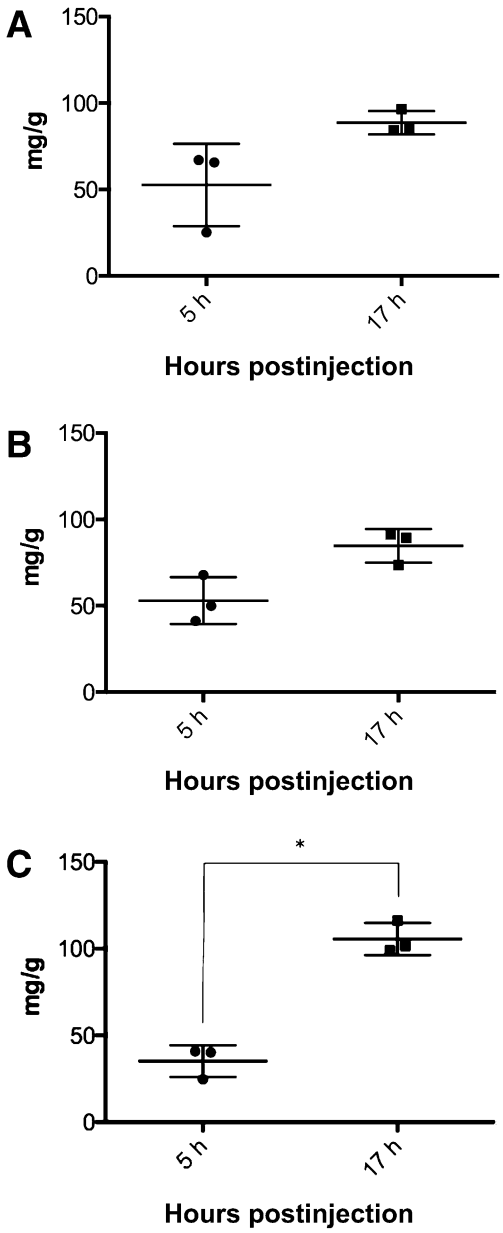

Figure 7—Measurement of glycogen levels in the liver. Free-fed mice were injected subcutaneously with either (*A*) PBS, (*B*) INS, or (*C*) ProINS-Tf (22.5 nmol/kg), and the liver tissue was isolated at 5 or 17 h postinjection. The liver tissue was perfused and homogenized, and the glycogen content was measured. Individual data points (n = 3) are represented as closed circles, along with the mean ± SD (horizontal lines). Data marked with an asterisk indicate statistically significant differences comparing different time points using Student t test. *P < 0.01.

this receptor-mediated intracellular activation process (26). Since ProINS-Tf itself was not effective, its potential in vivo activation, if specifically occurring in the liver, could lead to a more potent effect locally in the liver.

A long-acting INS analog with a liver-preferential effect or liver specificity is considered as an ideal replacement for basal INS (21,22). Our data indicated that ProINS-Tf fusion protein exhibited a prolonged pharmacokinetics as well as extended duration of action with a preference to inhibiting HGP in diabetic mice. Therefore, ProINS-Tf shows promising potentials as a basal INS replacement for reducing the elevated fasting BG in diabetes.

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