Novel Hepatoselective Insulin Analog

Studies with a covalently linked thyroxyl-insulin complex in humans

FARIBA SHOJAEE-MORADIE, PHD
JAMES K. POWRIE, MD, FRCP
ERIK SUNDERMANN, MSC
MARK W. SPRING, DR, MRCP
ACHIM SCHÜTTLER, MD, PHD
DIETRICH BRANDENBURG, DR. RER. NAT
PETER H. SÖNKSEN, MD, FRCP
RICHARD H. JONES, MA, FRCP

OBJECTIVE — To test whether a thyroxyl-insulin analog with restricted access to receptor sites in peripheral tissues displays relative hepatoselectivity in humans.

RESEARCH DESIGN AND METHODS — Five normal human subjects received a subcutaneous bolus injection of either $N^{\alpha B1}$L-thyroxyl-insulin (B1-T4-Ins) or NPH insulin in random order. Insulin kinetics, relative effects on hepatic glucose production, and peripheral glucose uptake were studied using euglycemic clamp and stable isotope [D-6,6-$^2H_2$]glucose) dilution techniques. Blood samples were taken for the determination of total immunoreactive insulin/analog concentrations and for liquid chromatography to assess the protein binding of the analog in the circulation.

RESULTS — After subcutaneous administration, B1-T4-Ins was well tolerated and rapidly absorbed. The analog had a long serum half-life and was highly protein bound (~86%). Its duration of action, as judged by the duration of infusion of exogenous glucose to maintain euglycemia, was similar to that of NPH insulin. The effect of the analogs on hepatic glucose production was similar to that of NPH insulin, indicating equivalent hepatic potency. The analog demonstrated less effect on peripheral glucose uptake than NPH insulin ($P = 0.025$), had no effect on metabolic clearance rate of glucose, and exhibited a reduced capacity to inhibit lipolysis ($P < 0.05$).

CONCLUSIONS — When injected subcutaneously into normal human subjects, B1-T4-Ins is well tolerated, quickly absorbed, and highly protein bound, resulting in a long plasma half-life. This analog appears to have a hepatoselective action, and, therefore, has the potential to provide more physiological insulin action than the insulin preparations currently used.

Diabetes Care 23:1124–1129, 2000

Most patients with type 1 diabetes continue to be treated by means of multiple daily boluses of subcutaneous insulin. These conventional regimens do not mimic normal insulin physiology. Patients are unable to achieve sustained normoglycemia and are prone to develop hypoglycemia.

The normal pancreas delivers insulin into the hepatic portal vein. Therefore, the liver is subject to a relatively high concentration of insulin of which it extracts up to 60%, depending on the prevailing circumstances (1). With subcutaneous insulin delivery in patients with type 1 diabetes, the portal/peripheral insulin gradient is lost and the liver is relatively underinsulinized. There is relative peripheral hyperinsulinemia and whole-body insulin resistance (2–4). Patients treated this way, even those who manage to achieve near-normoglycemia, continue to exhibit a variety of metabolic abnormalities including excessive glycemic fluctuations, dyslipidemia, and a reduction in plasma IGF-1 with elevated plasma levels of growth hormone. These persistent metabolic derangements have been implicated in the etiology of the long-term micro- and macrovascular complications of diabetes (5) and may be improved by intraperitoneal pump insulin delivery, where the majority of insulin is delivered into the portal circulation (6).

Therefore, there are good theoretical reasons why portal insulin delivery rather than peripheral insulin delivery may be desirable. Currently, insulin can be delivered into the portal vein only by intraperitoneal insulin pumps (6), by pancreatic transplantation with enteric drainage, or by islet cell transplantation (7,8). At present, these methods all have significant drawbacks that preclude their use as treatment for the vast majority of patients with type 1 diabetes.

Another potential solution to this problem is the development of an insulin analog that has a greater effect on the liver than the periphery. Previous studies with insulin analogs have led us to suggest that the molecular size of covalent insulin dimers and proinsulin could influence access of these substances to peripheral tissues when compared with insulin (9,10). In contrast, the open sinusoids in the liver allow free access of all plasma constituents to the hepatocyte cell surface (11). We propose that exploitation of the peripheral capillary endothelial barrier can allow the design of insulin analogs that have the potential to restore the physiological balance of insulin action even when peripherally administered (12). We describe here the first administration of such an analog in humans. We

From the Department of Diabetes (F.S.-M., J.K.P., M.W.S., P.H.S., R.H.J.), Endocrinology and Internal Medicine, Guy's, King's and St. Thomas' School of Medicine, King's College London, St. Thomas' Hospital, London, U.K.; and the Deutsches Wollforschungsinstitut (E.S., A.S., D.B.), Aachen, Germany.

Address correspondence and reprint requests to F. Shojaee-Moradie, PhD, GKT School of Medicine, Department of Diabetes, Endocrinology and Internal Medicine, 4th Floor, N. Wing, St. Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, U.K.

Received for publication 2 November 1999 and accepted in revised form 17 March 2000.

D.B. and R.H.J. have received research support from the Institute for Pharmaceutical Discovery, and F.S.-M.'s salary is funded by the Special Trustee's of St. Thomas' Hospital, London.

Abbreviations: AUC, area under the curve; AUCR_a, area under the curve calculated for glucose R_a; AUCR_d, area under the curve calculated for glucose R_d; B1-T4-Ins, $N^{\alpha B}$L-thyroxyl-insulin; CV, coefficient of variation; FPLC, fast protein liquid chromatography; GINF, exogenous glucose infusion; IRI, immunoreactive insulin; MCR, metabolic clearance rate; NEFA, nonesterified free fatty acid; R_a, glucose production; R_d, glucose disposal; RIA, radioimmunoassay; TBG, thyroxine binding protein; THBP, thyroid hormone binding protein; TSH, thyroid-stimulating hormone.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

report its kinetics and potential hepatoselectivity in nondiabetic subjects.

RESEARCH DESIGN AND METHODS

Material

We have designed and synthesized an insulin-like protein consisting of insulin covalently linked to thyroxine: $N^{\alpha B1}$ L-thyroxyl-insulin (B1-T4-Ins). This analog has the capacity to bind both to the insulin receptor and, via the thyroxyl moiety, to thyroid hormone binding proteins (THBPs) (i.e., thyroxine binding protein [TBG], transthyretin, and human serum albumin), present in plasma. The size of the bound complex (i.e., THBP-bound B1-T4-Ins) may confer relative hepatoselectivity compared with insulin by inhibiting access to peripheral tissues but not to the more exposed hepatocytes. B1-T4-Ins was synthesized as reported previously (12) and the lyophilized powder reconstituted (0.688 nmol/l) at Guy's, King's and St. Thomas' Hospitals' Pharmacy under aseptic conditions in $NaHCO_3$ (1.5% wt/vol) as an isotonic solution for injection.

Subjects

The subjects were 5 healthy men 31.6 ± 7.2 years of age with BMI 25.4 ± 3.3 recruited from the staff of Guy's, King's and St. Thomas' Hospitals or King's College London. None of the subjects were taking regular medication or had any medical condition likely to interfere with the study.

Experimental protocol

We examined the effects of a subcutaneous bolus injection of B1-T4-Ins in comparison with NPH insulin. Insulin kinetics and their relative effects on hepatic glucose production (R_a) and glucose disposal (R_d) were studied using euglycemic clamp and stable isotope ([D-6,6-$^2H_2$]glucose) dilution techniques (13). The ethics committee of the West Lambeth Health Authority approved the protocols, and all of the subjects gave informed written consent.

The subjects were admitted to the metabolic ward at 7:00 A.M. after an overnight fast. They were studied in a supine position and allowed only water to drink for the duration of the study. After a baseline blood sample for glucose isotope ratio measurement, a primed (170 mg) constant infusion (1.7 mg/min) of [D-6,6-$^2H_2$]glucose (Mass Trace, Somerville, MA) was administered for 17.5 h or less if the exogenous glucose requirements had returned to 0 (13). The subjects were studied twice, receiving either NPH insulin or B1-T4-Ins in randomized order with an interval of 2 weeks' washout period between each treatment. The [D-6,6-$^2H_2$]glucose infusion was allowed to equilibrate over 2 h followed by steady-state baseline blood sampling every 10 min for another 30 min. Subjects were then given a subcutaneous bolus injection of either NPH insulin (2.06 nmol/kg [0.3 U/kg]) or B1-T4-Ins 3.44 nmol/kg) at 2.5 h into a D-[6,6-$^2H_2$]glucose infusion to the anterior abdominal wall.

After the bolus injection of the insulin or analog, blood samples were drawn at 15-min intervals for glucose determination and a variable infusion of dextrose (20%) was administered to maintain blood glucose at the basal concentration of each subject. The protocol was limited to a maximum of 15 h. Dextrose (20%) was premixed with D-[6,6-$^2H_2$]glucose (4 mg [D-6,6-$^2H_2$]glucose/g of dextrose) to prevent a fall in tracer enrichment and consequent errors in glucose turnover calculations that otherwise occur (13,14). The study was terminated when the exogenous glucose requirements returned to 0 or at 15 h after the insulin administration, whichever was shorter.

Four samples were collected for basal measurements of insulin, C-peptide, and glucose concentrations and for glucose enrichment. A sample was also taken for basal measurements of IGF-I, glucagon, thyroid hormones, thyroid-stimulating hormone (TSH), and nonesterified free fatty acid (NEFA). After administration of NPH insulin or B1-T4-Ins, samples were taken every 30 min for the determination of insulin or analog, C-peptide, and glucose concentrations and for glucose enrichment. Samples were taken every 3 h for the measurement of NEFA and glucagon and every 5 h for the measurement of IGF-I, free T4, free T3, and TSH concentrations. A sample was also taken after 24 h (after breakfast) for the determination of insulin or analog and thyroid hormones concentrations.

Serum samples also were taken at 0, 3, 8, and 24 h for fast protein liquid chromatography (FPLC) analysis. Serum and plasma samples were separated immediately and stored at −20°C until assayed.

Analytical procedures

Immunoreactive insulin (IRI) and IGF-I concentrations in serum samples and C-peptide in plasma were determined by double-antibody radioimmunoassay (RIA) as previously described (15). The within-assay coefficients of variation (CVs) were 7, 7, and 6%, respectively. Thyroxyl-insulin analog concentrations (IRI) in serum samples and in the FPLC fractions were determined by a double-antibody RIA with some modifications as previously described (CV 8%) (12). Glucagon concentrations in plasma samples were measured by RIA using a commercially available kit from Linco Research (St. Louis, MO).

Glucose concentrations in the plasma samples were determined by a glucose oxidase method using a Clandon Scientific Glucose Analyzer (Yellow Springs Instruments, Yellow Springs, OH).

NEFA concentrations in serum samples were determined using a commercially available kit from Wako Chemicals (Neuss, Germany).

Glucose isotopic enrichment was determined by gas chromatography mass spectrometry on a VG Trio-2 5890 (VG Micromas, Manchester, U.K.) using selected ion monitoring of a glucose penta-acetate derivative (16). The ions monitored were of molecular mass 242 and 244 representing $[M–C_5O_5H_8]^+$ and the corresponding fragment enriched with 2 deuterium atoms, respectively. The within-assay CV was <2%.

To determine the status of B1-T4-Ins (bound or free) in the serum samples, samples were subjected to FPLC separation as previously described (12).

Calculations

R_a and R_d at all points were calculated using the 2-compartment model proposed by Mari (17), which was modified for the inclusion of [D-6,6-$^2H_2$]glucose in the dextrose infusion. Before the calculation of glucose turnover, plasma glucose concentrations and glucose enrichment time courses were smoothed using the Optimal Segments Technique Analysis (18). Calculation of glucose metabolic clearance rate (MCR) ($ml \cdot min^{-1} \cdot kg^{-1}$) is model independent (glucose tracer infusion rate/glucose tracer concentration).

For each experiment, the mass reduction in endogenous glucose appearance (AUCR_a) and mass increase in glucose uptake (AUCR_d) were calculated from the R_a and R_d time course values, respectively.

Statistical analysis

Measurements of glucose MCR, R_d, R_a, and exogenous glucose infusion (GINF) achieved after the NPH insulin and the ana-

log injections were converted to areas under the curves separately for each patient at each visit. The mean difference between treatments, corrected for the order of administration, was estimated using multiple regression. Repeated measurement per patient was allowed for by the robust variance estimator (19,20). The statistical significance of changes occurring during the NPH insulin and the analog injections were determined by 2-way analysis of variance. The significance of differences between 2 means was tested using Fisher's least-squares method with Number Cruncher Statistical System software (Hintze, Kaysville, UT) and Stata software (Stata, College Station, TX).

Descriptive data are expressed as mean value with 95% CI where appropriate, and P values <0.05 were considered statistically significant.

RESULTS

Tolerability

B1-T4-Ins was extremely well tolerated. None of the subjects suffered any significant side effects.

After a bolus injection of B1-T4-Ins, the total IRI/analog concentration in serum increased from a basal value of 0.08 nmol/l (95% CI, 0.06–1.01) to 0.97 nmol/l (0.61–1.33) and 4.02 nmol/l (2.84–5.20) at 30 min and 6 h, respectively ($P < 0.01$). These elevated IRI concentrations persisted throughout the study (Fig. 1*A*).

After a bolus injection of NPH insulin, the serum IRI concentration increased from a basal value of 0.06 nmol/l (0.03 and 0.09) to 0.07 nmol/l (0.02 and 0.09) and 0.15 nmol/l (0.1 and 0.2) at 30 min and 6 h, respectively ($P < 0.01$).

The increase in IRI/analog concentrations from basal level with B1-T4-Ins (0.89 nmol/l [1.22–0.76]) was significantly different from that of NPH insulin (0.014 nmol/l [0.01 and 0.03]).

IRI concentrations postbreakfast at 24 h after B1-T4-Ins were 4.18 nmol/l (3.31 and 5.05), significantly higher than those after NPH insulin (0.24 nmol/l [0.02 and 0.48]) ($P < 0.05$).

C-peptide concentrations did not change from the basal level of 0.44 nmol/l (0.38 and 0.50) during the study with B1-T4-Ins, but fell significantly from its basal levels of 0.40 nmol/l (0.28 and 0.54) to 0.30 nmol/l (0.27 and 0.33) toward the end of the study with NPH insulin (11 h, $P < 0.05$) (Fig. 1*B*).

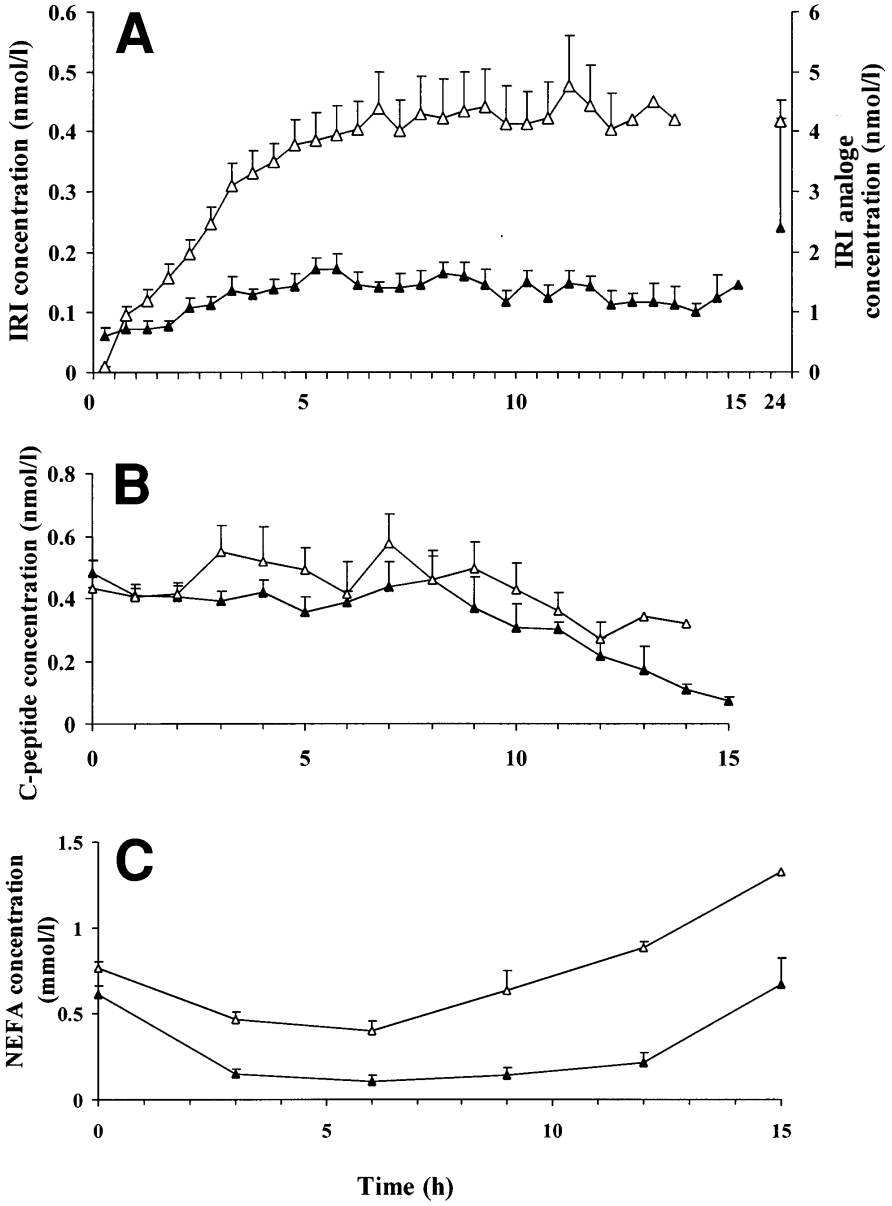

Figure 1—*Immunoreactive serum insulin or total analog concentrations (*A*), C-peptide concentrations (*B*), and NEFA concentrations (*C*) after a subcutaneous injection of NPH insulin (▲) and B1-T4-Ins (△) during clamp studies, (*n = 5*). Values are means ± SEM.*

IRI, after injection of NPH insulin, eluted at 15–20 ml, corresponding to the native H-Ins peak (i.e., <12,000 Da). Immunoreactive analog, after injection of B1-T4-Ins (at times 3, 8, and 24 h), eluted mostly (86, 86, and 86%) between 5 and 15 ml, corresponding to the high molecular weight IRI (i.e., bound form ~60,000 Da). The remaining IRI in the sample eluted between 15 and 20 ml (i.e., free form <12,000 Da).

After the B1-T4-Ins injection at 6 h, the total concentration of B1-T4-Ins was 4.02 nmol/l (i.e., a free concentration of ~0.56 nmol/l, which is ~3.8 times higher than that after the NPH insulin injection).

Fasting plasma glucose concentration was 5.3 mmol/l (5.0 and 5.6) and euglycemia was maintained during the clamp (glucose concentration of 5.2 mmol/l [4.5 and 5.9]).

The mass of glucose required to maintain euglycemia throughout each experiment was calculated from the record of the glucose infusion rates (area under curve [AUC] GINF). AUC GINF to maintain

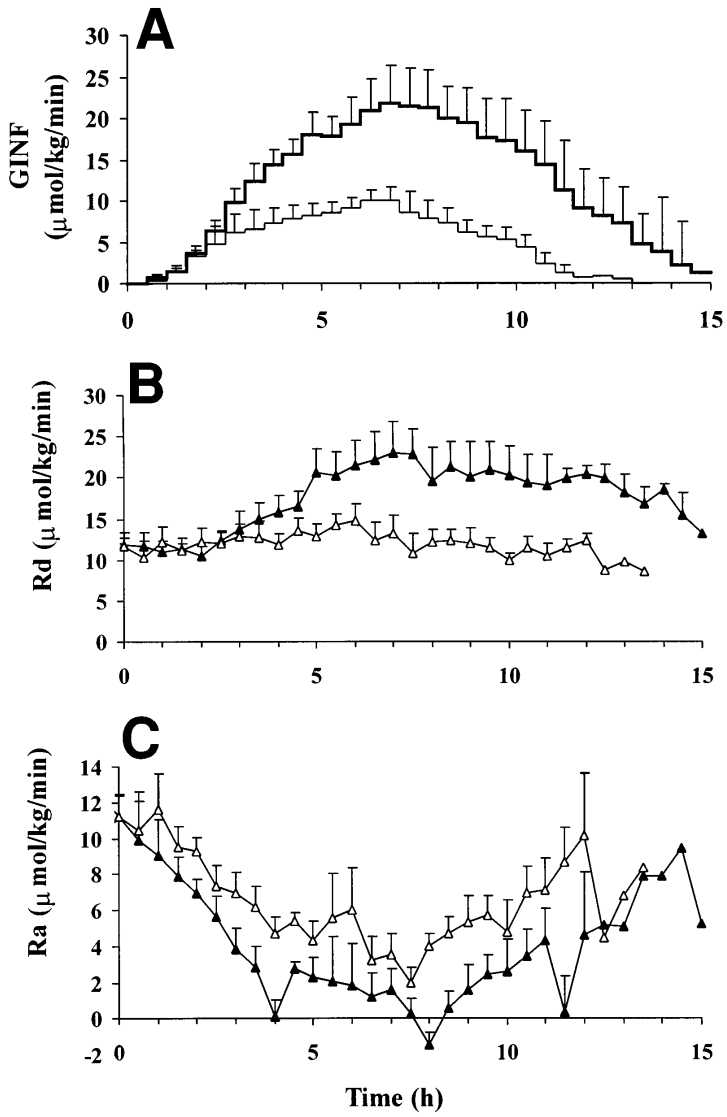

Figure 2—*Exogenous glucose infusion rates (*A*),* R_d *(*B*), and* R_a *(*C*) achieved during euglycemic clamp studies after a subcutaneous injection of NPH insulin (thick line; ▲) and B1-T4-Ins (thin line; △), (*n = 5*). Values are means ± SEM.*

euglycemia after injection of NPH insulin (10.73 mmol/kg [5.77 and 15.69]) was significantly greater than the AUC calculated after the injection of B1-T4-Ins (4.04 mmol/kg [1.73 and 6.35]) ($P = 0.027$) (Fig. 2*A*). The duration of action for B1-T4-Ins (672 min [579 and 765]) was not different from that of NPH insulin (765 min [631 and 899]). The starting times for exogenous glucose infusion for B1-T4-Ins and NPH insulin were also similar 45 min (28 and 62) compared with 67 min (19 and 115), respectively.

The glucose AUCR_a values, after injection of either NPH insulin (−5.63 mmol/kg [–7.3 and −3.96]) or B1-T4-Ins (−3.32 mmol/kg [–5.89 and −0.75]), were not different (Fig. 2*C*), suggesting there were similar effects on the liver at the dose used.

Glucose disposal rates significantly increased from basal level 11.07 μmol · kg^{-1} · min^{-1} (8.64 and 13.50) after NPH insulin injection ($P = 0.01$) but not after B1-T4-Ins. AUCR_d, after the injection of NPH insulin (4.95 mmol/kg [1.17 and 8.73]) was greater than that of AUCR_d after the B1-T4-Ins injection (0.49 mmol/kg [–0.97 and 1.95], $P = 0.025$) (Fig. 2*B*).

Glucose MCR significantly increased from basal levels after NPH insulin injection ($P = 0.01$) but not after B1-T4-Ins. The AUC for glucose MCR, after the injection of NPH insulin (1.03 ml/kg [0.41 and 1.65]), was significantly different from the AUC for glucose MCR after the B1-T4-Ins injection (0.15 ml/kg [0 and 1.95], $P = 0.002$).

NEFA concentrations were suppressed significantly from basal levels (0.69 μmol/l [0.56 and 0.82]) with both B1-T4-Ins and NPH insulin at 3 h to 0.46 μmol/l (0.36 and 0.56) and 0.15 μmol/l (0.07 and 0.22), respectively. The reduction of NEFA concentrations, after the administration of NPH insulin, was greater at every time point than after the B1-T4-Ins administration ($P < 0.05$) (Fig. 1*C*). Concentrations of IGF-I, glucagon, TSH, free T3, and free T4 did not change during either of the protocols.

CONCLUSIONS

— This study reports the first administration of a covalently linked thyroxyl-insulin analog to human subjects. We have shown that B1-T4-Ins is well tolerated, well absorbed after subcutaneous administration, has a long duration of action, and appears to confer hepatoselectivity when compared with NPH insulin.

Through in vitro and in vivo experiments, we have demonstrated that B1-T4-Ins binds to plasma THBPs (12,21). We have also demonstrated that in the absence of THBPs, B1-T4-Ins binds to isolated rat liver plasma membranes with the same affinity as native insulin (21). However, in the presence of THBPs, the binding affinity of the analog was reduced compared with insulin, suggesting that the receptor binding site on the insulin moiety may be partially obscured by the binding protein. These data are supported by our previous results from hyperinsulinemic-euglycemic clamp studies in normal dogs (12) in which the MCR of B1-T4-Ins was lower than that of human insulin and was further reduced by the infusion of human TBG. In studies performed in humans, recent experiments with the acylated insulin analogs (NN304) and [N^{ϵ}-palmitoyl Lys (B29)] human insulin, which bind to albumin in the circulation, also exhibit higher concentrations compared with NPH insulin when administered subcutaneously (22,23).

The design of insulin analogs, which can specifically target the liver after subcutaneous injection, is a novel therapeutic approach to mimic the actions of insulin secreted directly into the hepatic portal circulation. Here we have demonstrated that although B1-T4-Ins and NPH insulin have similar effects on the liver (inhibition of R_a),

the peripheral effects of B1-T4-Ins on glucose metabolism (i.e., R_d, MCR) and the effect on NEFA concentrations are significantly reduced compared with the effects of NPH insulin. These data are supported by our previous results from clamp studies in dogs (12), in which a relatively hepatoselective profile of action of the thyroxyl-insulin analogs was apparent in comparison with human insulin after intravenous administration. These findings further support the hypothesis that insulin-like moieties bound to plasma proteins may be prevented by the endothelial barrier from reaching receptor sites on cells of peripheral tissues.

Data obtained from infusion studies in normal dogs with NN304 are in agreement with this conclusion (24), again suggesting that transport across the peripheral capillary endothelial barrier is a rate-limiting step for insulin action when the hormone is protein bound. Our data show a change in the relationship over time between plasma analog concentrations and glucose infusion rates. Particularly striking is the persistence of a high level of immunoreactive but apparently biologically inactive analog at 24 h (i.e., ~10 h after the end of glucose infusion). TBG has a very high affinity for B1-T4-Ins in vivo, and it is likely that the sustained high levels represent TBG-bound B1-T4-Ins; this result is supported by the FPLC data. Furthermore, the fact that total serum B1-T4-Ins remains high for longer period of time than the effect on glucose metabolism supports the suggestion that the bound fraction is protected from receptor-mediated degradation, at least in part by this effect. Therefore, it is likely that the duration of action of such an analog will in part be determined by its affinity for the relevant binding proteins and by their capacity. Data obtained from studies in diabetic dogs after a subcutaneous injection of [N$^{\epsilon}$-palmitoyl Lys (B29)] human insulin (3.15 nmol/kg) compared with intermediate-acting insulin suspension Humulin L (1.8 nmol/kg) also exhibited a higher analog concentration and extended time-action profile compared with Humulin L (25).

The time-action profiles of both NPH insulin and B1-T4-Ins were similar and were characterized by a tendency toward steadily increasing effects for ~6 h after the injection followed by a progressive decline.

It can be argued that hypoglycemia may be less severe in the absence of overstimulation of glucose disposal (i.e., if induced primarily by a reduction in R_a). Counterregulatory responses to hypoglycemia act by counteracting the effects of insulin on R_a. Normoglycemia may possibly be restored and maintained more effectively by any given counterregulatory response if and when glucose utilization by muscle and adipose tissue is not in a state of insulin overstimulation.

Although in these experiments no change in TSH was observed, in studies using rats rendered hypothyroid by treatment with polythiouracil for 1 week (26), B1-T4-Ins administration by intraperitoneal injection was associated with a dose-dependent suppression of TSH concentration (F.S.-M., unpublished data). The B1-T4-Ins analog was chosen as the first example of the group to synthesize in sufficient quantity for human studies because T4 has a high affinity for THBPs and was therefore most likely to provide proof of concept. Other analogs of thyroxine (e.g., rT3-Ins [21]) exhibit altered THBP-binding characteristics associated with an absence of thyroid hormonal action. Linkage of these groups with insulin will lead to a new generation of insulin analogs, some of which may have potential for clinical use in diabetes. The primary use of these analogues is likely to be in type 1 diabetic patients as in type 2 diabetic patients treated by diet and/or tablets the insulin supply is endogenous and therefore its delivery remains intrahepatic. However, there is evidence that significant hepatic insulin resistance is a feature of type 2 diabetes exacerbating peripheral hyperinsulinemia with undesirable metabolic consequences (27). For this reason, it is possible that hepatoselective insulin analogs may also offer advantages in the treatment of type 2 diabetes.

We have shown that the administration of the insulin analog B1-T4-Ins to normal human subjects is safe and well tolerated. The analog is quickly absorbed from a subcutaneous site and results in high serum insulin concentration, the majority of which is protein bound. The analog has a long duration of action and similar efficacy to NPH insulin in inhibiting R_a but has a much reduced effect in the periphery. Therefore, B1-T4-Ins appears to be exhibiting hepatoselectivity. This principle is worthy of exploitation in an effort to produce hepatoselective insulin preparations for therapeutic use with a range of durations of action. Such analogs would overcome the metabolic abnormalities that result from current inadequacies of peripherally administered conventional insulins.

Acknowledgments — This work was supported in part by the Special Trustees of St. Thomas' Hospital, London, and the Institute for Pharmaceutical Discovery, Connecticut.

The authors wish to thank Dr. M.A. Boroujerdi for advice on the analysis of data and Mr. P. Seed for statistical analysis using STATA software. The authors also wish to thank P. Croos and A. Fofanah for their excellent laboratory assistance and Dr. E. Bingham and C. Russell for help during the clamp studies.

References

1. Chap Z, Ishida T, Chou J, Hartley CJ, Entman ML, Brandenburg D, Jones RH, Field JB: First pass hepatic extraction and metabolic effects of insulin and insulin analogues. *Am J Physiol* 252:E209–E217, 1987
2. Yki-Jarvinen H, Koivisto VA: Natural course of insulin resistance in type 1 diabetes. *N Engl J Med* 315:224–230, 1986
3. Pickup JC, Collins AC, Walker JD, Viberti GC, Pasic J: Patterns of hyperinsulinaemia in type 1 diabetic patients with and without nephropathy. *Diabet Med* 6:685–691, 1989
4. Reaven GM: Insulin resistance and compensatory hyperinsulinemia: role in hypertension, dylipidemia and coronary heart disease. *Am Heart J* 4:1283–1288, 1991
5. Sönksen PH, Russell-Jones D, Jones RH: Growth hormone and diabetes mellitus. *Horm Res* 40:68–79, 1993
6. Ruotolo G, Micossi P, Galimberti G, Librenti MC, Petrella G, Marcovina S, Pozza G, Howard BV: Effects of intraperitoneal versus subcutaneous insulin administration on lipoprotein metabolism in type 1 diabetes. *Metabolism* 38:598–604, 1990
7. Stratta RJ, Taylor RJ, Larsen JL, Cushing K: Pancreas transplantation (Review). *Ren Fail* 17:323–337, 1995
8. Stratta RJ, Taylor RJ, Sudan D, Sindhi R, Castaldo P, Cushing K, Frisbie K, Radio S: Solitary pancreas transplantation: experience with 50 consecutive cases. *Transplant Proc* 27:3022–3023, 1995
9. Shojaee-Moradie F, Jackson NC, Brandenburg D, Sönksen PH, Jones RH: Demonstration of a relatively hepatoselective effect of covalent insulin dimers on glucose metabolism in dogs. *Diabetologia* 38:1007–1013, 1995
10. Glaubler HS, Revers R, Henry R, Schmeiser L, Wallace P, Kolterman O, Cohen RM, Rubenstein AH, Galloway JA, Frank BH, Olefsky JM: In vivo inactivation of proinsulin action on glucose disposal and hepatic glucose production in normal man. *Diabetes* 35:311–317, 1986
11. Reichen J: The role of the sinsoidal endothelium in liver function. *News Physiol Sci* 14:117–121, 1999
12. Shojaee-Moradie F, Eckey H, Jackson NC, Schüttler A, Brandenburg D, Sönksen PH,

Jones RH: Novel hepatoselective insulin analogues: studies with covalently linked thyroxyl insulin complexes. *Diabet Med* 15: 928–936, 1998
13. Powrie JK, Smith GD, Shojaee-Moradie F, Sönksen PH, Jones RH: Mode of action of chloroquine in patients with non-insulin dependent diabetes mellitus. *Am J Physiol* 260:E897–E904, 1991
14. Finegood DT, Bergman RN, Vranic M: Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps: comparison of unlabeled and labeled exogenous glucose infusates. *Diabetes* 36:914–924, 1987
15. Sönksen PH: Double antibody technique for the simultaneous assay of insulin and growth hormone. In *Hormones in Human Blood: Detection and Assay*. Antoniades HN, Ed. Cambridge, MA, Harvard University Press, 1976, p. 176–199
16. Wolf RR: *Radioactive and Stable Isotope Tracers in Biomedicine*. New York, Wiley, 1992
17. Mari A: Estimation of the rate of appearance in the non-steady state with a two-compartment model. *Am J Physiol* 263: E400–E415, 1992
18. Finegood DT, Thomaseth K, Pacini G, Bergman RN: OPSEG: a general routine for smoothing and interpolating discrete biological data. *Comput Methods Programs Biomed* 26:289–300, 1988
19. Binder DA: On the variances of asymptotically normal estimators from complex surveys. *International Statistical Review* 51:279–292, 1983
20. Huber PJ: The behaviour of maximum likelihood estimates under non-standard conditions. In *Proceedings of the Fifth Berkeley Symposium in Mathematical Statistics and Probability*. Berkeley, CA, University of California Press, 1967, p. 221–233
21. Telfer MJ, Shojaee-Moradie F, Sundermann E, Schüttler A, Brandenburg D, Jones RH: The effects of thyroid hormone binding proteins on insulin receptor binding of thyroxyl-insulin analogues in vitro. *Diabet Med* 15 (Suppl. 2):55, 1998
22. Radziuk J, Pye S, Bradley B, Braaten L, Vignati L, Roach P, Bowsher R, DiMarchi R, Chance R: Basal activity profiles of NPH insulin and [N$^{\epsilon}$-palmitoyl Lys (B29)] human insulins in subjects with IDDM. *Diabetologia* 41:116–120, 1998
23. Heinemann L, Sinha K, Werer C, Loftager M, Hirschberger S, Heise T: Time-action profile of the soluble, fatty acid acylated, long-acting insulin analogue NN304. *Diabet Med* 16:332–338, 1999
24. Myers SR, Yakubu-Madus F, Johnson T, Baker JE, Cusisk TS, Williams VK, Tinsley FC, Kriauciunas A, Manetta J, Chen VJ: Acylation of human insulin with palmitic acid extends the time action of human insulin in diabetic dogs. *Diabetes* 46:637–642, 1997
25. Hamilton-Wessler M, Ader M, Dea M, Moore D, Markussen J, Bergman RN: Long-acting insulin analog NN304 is transported independent of native insulin (Abstract). *Diabetes* 46 (Suppl. 1):164A, 1997
26. Lemarchand-Beraud T, Berthier C: Effects of graded doses of triiodothyronine on TSH synthesis and secretion rates in hypothyroid rats. *Acta Endocrinol* 97: 74–84, 1981
27. Lewis GF, Carpentier A, Vranic M, Giacca A: Resistance to insulin's acute direct hepatic effect in suppressing steady-state glucose production in individuals with type 2 diabetes. *Diabetes* 48:570–576, 1999