Acylation of Human Insulin With Palmitic Acid Extends the Time Action of Human Insulin in Diabetic Dogs

Sharon R. Myers, Fatima E. Yakubu-Madus, William T. Johnson, James E. Baker, Tania S. Cusick, Valerie K. Williams, Frank C. Tinsley, Aidas Kriauciunas, Joseph Manetta, and Victor J. Chen

To test whether the binding of insulin to an endogenous serum protein can be used to extend the time action of insulin, human insulin was acylated at the epsilon-amino group of Lys(B_{29}) with palmitic acid to promote binding to serum albumin. Size-exclusion chromatography was used to demonstrate specific binding of the resulting analog, [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin, to serum albumin in vitro, and the time action and activity of the analog were determined in vivo using overnight-fasted, insulin-withdrawn diabetic dogs. In the diabetic animal model, the duration of action of [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin administered intravenously was nearly twice that of unmodified human insulin, and the plasma half-life was nearly sevenfold that of the unmodified protein. Administered subcutaneously, [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin had a longer duration of action; a flatter more basal plasma insulin profile; and a lower intersubject variability of response than the intermediate-acting insulin suspension Humulin L (Lilly, Indianapolis, IN). These studies support the concept that modification of insulin to promote binding to an existing serum protein can be used to extend the time action of human insulin. In addition, the time action, pattern, and decreased variability of response to [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin support the development and further testing of this soluble insulin analog as a basal insulin to increase the safety of intensive insulin therapy. *Diabetes* 46:637–642, 1997

Although intensive insulin therapy can delay the onset and slow the progression of diabetic complications in IDDM patients, it can also triple the risk of developing severe hypoglycemia (1). Part of this risk can be attributed to the nonphysiological pattern of insulinemia and excessive variability in absorption associated with current basal insulins (2–4).

At least a portion of the variability associated with current basal insulins appears to be due to the insoluble nature of the preparations; because they are suspensions, variability in dosing can occur if samples are improperly mixed prior to withdrawal into the syringe. In addition, because these formulations derive their protracted time action through slow dissolution of the crystalline material at the site of injection, subtle changes in the physical milieu or the distribution of the material at the subcutaneous site may influence the dissolution process and further increase day-to-day variability. Finally, because absorption of insulin depends on blood flow (5), normal changes in blood flow throughout the day have the potential to significantly affect the absorption profile. Variability based on all these factors could be decreased with a soluble form of insulin that has an extension of time action independent of retention at the injection site.

Clinical experiences with two soluble, extended-acting insulin agonists—NovoSol Basal (Novo Nordisk, Bagsvaerd, Denmark) and human proinsulin—support the concept that changing from an insoluble to a soluble formulation can result in a marked decrease in the variability of response (6,7). Although development of these compounds has been discontinued (2), the information gained from their study has provided impetus for the continued search for a soluble basal insulin (8–11).

One possible means of obtaining a soluble, extended-acting insulin is by modifying insulin to promote binding to an existing serum protein. It is well established that the serum half-life of a number of endogenous hormones is extended through binding to specific serum-binding proteins (12–15). Studies in diabetic patients with high antibody titers to insulin have shown that the duration of insulin's action can be increased in a similar manner (16,17).

To test whether binding of insulin to an existing serum protein can be used to extend the time action of insulin, human insulin was acylated at the epsilon-amino group of Lys(B_{29}) with palmitic acid to promote binding to serum albumin. Serum albumin was chosen as the protein carrier because its circulatory levels are high and reasonably constant (18,19), its capacity for binding fatty acids is well characterized (20,21), and the chemistry for derivatizing insulin with fatty acids is well known (22–24). In this study, we describe the synthesis and pharmacology of this soluble insulin analog, [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin.

RESEARCH DESIGN AND METHODS

Synthesis and labeling of [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin. [N^{ϵ}-palmitoyl Lys(B_{29})] human insulin (Lilly, Indianapolis, IN) was prepared by rapid addition of *N*-hydroxylsuccinimidyl palmitate (Sigma, St. Louis, MO) in *N,N*-

From Lilly Research Laboratories, Indianapolis, Indiana.

Address correspondence and reprint requests to Dr. Sharon R. Myers, Lilly Research Laboratories, Lilly Corporate Center, D.C. 0540, Indianapolis, IN 46285.

Received for publication 24 May 1996 and accepted in revised form 14 November 1996.

BSA, bovine serum albumin; DMF, *N,N*-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; EWB, ELISA wash buffer; HPLC, high-performance liquid chromatography; NEFA, nonesterified fatty acid.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/555018/46-4-637.pdf by guest on 04 March 2024

dimethylformamide (DMF; EM Science, Gibbstown, NJ) to 20 µmol of insulin in 60% DMF, pH 10. After 30 min at room temperature, the reaction was stopped by adjusting the pH of the solution to 2.5 and then immediately filtering it through a 0.45 micron filter (Gelman Sciences, Ann Arbor, MI). Purification was performed at room temperature by reverse-phase high-performance liquid chromatography (HPLC; Beckman-System Gold, Fullerton, CA) using a Vydac C_4 column (Vydac, Hysperia, CA), 5 mm × 25 cm, 20 micron particle size, at an elution rate of 5 ml/min. The sample was applied to the column equilibrated in 50 mmol/l sodium phosphate (Mallindrodt Chemical, Paris, KY), 30% acetonitrile (pH 2.1) (Mallindrodt Chemical), then eluted with a linear gradient of increasing acetonitrile concentration (30–48%) over 3 h. Greater than 99% purity was achieved as assessed by analytical reverse-phase HPLC. The identity of [N^{ε}-palmitoyl Lys(B_{29})] human insulin was confirmed by ion-spray mass spectrometry (PESciex, API-III triple quadruple spectrometer; Thornhill, Ontario, Canada), which found M_r = 6,046 and NH_2-terminal sequencing, giving a similar Gly/Phe ratio in the first cycle as that found in unmodified insulin.

Tritiated [N^{ε}-palmitoyl Lys(B_{29})] human insulin was prepared similarly as described above by adding 3.7 µmol of tritiated N-hydroxylsuccinimidyl palmitate to 0.7 µmoles of insulin. Purification was performed at room temperature by reverse-phase HPLC using a Vydac C_4 column, 1 cm × 25 cm, 10 micron particle size, at an elution rate of 2 ml/min. Product identity was confirmed by comparison of retention time of authentic [N^{ε}-palmitoyl Lys(B_{29})] human insulin on the same column. Specific activity was determined to be 4.2 µCi/nmol.

Binding to serum albumin. Binding of [N^{ε}-palmitoyl Lys(B_{29})] human insulin to serum albumin was assessed using gel filtration chromatography (1 × 10 cm G-75 Sephadex column [Pharmacia, Uppsala, Sweden] equilibrated and eluted with 0.1 mol/l N-[2-hydroxyethyl]piperazine-N-[3-propanesulfonic acid] [EPPS; Sigma] buffer, pH 8). Elution profiles were obtained for tritiated [N^{ε}-palmitoyl Lys(B_{29})] human insulin and iodinated human insulin (both 0.16 nmol) in the absence and presence of bovine serum albumin (BSA; 6.7 nmol; radioimmunoassay (RIA) grade Fraction V; Sigma). Eluting peaks were detected by either absorbance at 280 nm (BSA) or radioactivity (iodinated human insulin and tritiated [N^{ε}-palmitoyl Lys(B_{29})] human insulin).

Determination of time action and activity in vivo. Experiments were carried out in conscious, overnight-fasted beagles (Marshall Farms, North Rose, NY) weighing 9–14 kg. At least 3 weeks prior to the day of study, surgery was performed under isoflurane anesthesia (Ohmeda PPD, Liberty Corner, NJ) during which each animal's entire pancreas was excised (25) and a Silastic (Dow Corning, Midland, MI) catheter was inserted in the femoral artery. The diabetic animals were maintained on a twice-daily feeding/insulin administration regimen (NPH Iletin II purified pork insulin, Lilly; dose adjusted to minimize glycosuria), with replacement of pancreatic enzymes (Viokase; A.H. Robins, Richmond, VA).

NPH insulin was withheld the day before the experiment. Food (plus Viokase) was administered as usual, and 4 U of Regular insulin (Regular Iletin II purified pork insulin; Lilly) were administered with the afternoon meal.

The afternoon before the experiment, the free end of the indwelling, arterial catheter was exteriorized through a small incision made under local anesthesia (2% lidocaine; Butler, Columbus, OH). In animals receiving an intravenous bolus of test substance, a 20-gauge over-the-needle Teflon catheter (Baxter, Deerfield, IL) was inserted percutaneously into a cephalic vein the morning of the experiment. After a 15-min rest, blood was drawn from the arterial catheter for the determination of baseline parameters, followed by administration of the test insulin. The fatty acid acylated insulin was dissolved in Humulin R (Lilly) unbuffered sterile diluent adjusted to pH 7.8 with a final concentration of 600 nmol/ml, the molar equivalent of a U-100 solution of Humulin. The test insulin was administered either intravenously through the previously inserted percutaneous catheter (1.2 nmol/kg [molar equivalent of 0.2 U human insulin/kg] of either Humulin R or analog diluted in 2 ml of saline containing 3% dog plasma) or subcutaneously in the dorsal aspect of the neck (1.8 nmol/kg [0.3 U/kg] Humulin L or 3.15 nmol/kg [molar equivalent of 0.525 U human insulin/kg] of the analog). Blood samples were drawn intermittently over the next 5 (in the intravenous studies) to 15 (in subcutaneous studies) hours, collected in vacuum blood collection tubes (Terumo, Elkton, MD) containing sodium heparin, and immediately placed on ice. After centrifugation, plasma was transferred to polypropylene test tubes and stored on ice for the duration of the study. At the conclusion of the experiment, the animals were anesthetized (isoflurane), and the free end of the catheter was placed subcutaneously as before.

Two groups of five animals were used, one for the intravenous studies and one for the subcutaneous administration studies. Each animal was studied twice—once with [N^{ε}-palmitoyl Lys(B_{29})] human insulin and once with the human insulin comparator—in a randomized fashion (intravenous studies) or sequentially (all subcutaneous studies with the analog were performed before the studies with the human insulin comparator) a minimum of 1 week apart to allow pairing of the database. Blood was drawn the day before each experiment to determine the health of the animals (Cell-Dyn 900, Sequoia-Turner, Mountain View, CA). All animals had hematocrits >38% and leukocyte counts <16,000/mm^3.

Sample analysis. Plasma glucose levels were determined the day of the study using a coupled hexokinase procedure in a clinical chemistry analyzer (Monarch, Instrumentation Laboratory, Lexington, MA). Samples for determination of nonesterified fatty acids (NEFAs) and insulin analog or insulin concentrations were stored at –70°C until analysis. Plasma NEFA concentrations were determined enzymatically using a kit available from Wako Pure Chemical Industries (Osaka, Japan) adapted for use on the Monarch clinical chemistry analyzer. Human insulin levels were determined using a commercial radioimmunoassay kit (Coat-a-Count, Diagnostic Products, Los Angeles, CA) with human insulin (Lilly) as the standard. Immunoreactive [N^{ε}-palmitoyl Lys(B_{29})] human insulin was analyzed by a competitive enzyme-linked immunosorbent assay (ELISA)-type radioimmunoassay with a polyclonal antibody that exhibits 100% reactivity with [N^{ε}-palmitoyl Lys(B_{29})] human insulin and insulin. The assay measures total (bound and free) [N^{ε}-palmitoyl Lys(B_{29})] human insulin with a sensitivity of approximately 50 pmol/l. $^{125}I(A_{14})$-Porcine insulin, prepared as previously described (26), was from either Lilly or New England Nuclear (Boston, MA). One hundred microliters of goat anti-guinea pig IgG (2.5 µg/ml in 0.05 mol/l sodium carbonate, pH 9.6–9.8, Jackson 106-006-008, Jackson Immunoresearch Labs, Westgrove, PA) were added to each well of an Immulon 1 breakaway plate (Dynatech Labs, Chantilly, VA) (12 wells per strip arranged in the holder to form a 96-well assay). The plate was incubated overnight at 4°C and then washed with a buffer containing 0.02 mol/l Tris (Mallindrodt Chemical), 0.15 mol/l NaCl, and 0.05% Tween 20 (Sigma), pH 7.4 (ELISA wash buffer [EWB]). To reduce nonspecific binding of [N^{ε}-palmitoyl Lys(B_{29})] human insulin, the plate was incubated at room temperature for 1 h with 160 µl/well of a 30 mg/ml gelatin solution. After washing twice with EWB, 70 µl of a 1:200,000 dilution of guinea pig anti-porcine insulin (Lilly, lot P5) were added to each well. The plate was incubated at room temperature for 2.5 h and then washed four times with EWB. Standards or unknowns were diluted with an equal volume of ^{125}I-insulin (7 pg insulin [~2.6 nCi] per well), and 50 µl of the diluted sample was transferred to each well and incubated at room temperature for 3 h. After incubation, each well was washed four times with EWB, punched out, and counted to measure the radioactivity captured.

Statistical analysis. Data are expressed as means ± the standard error. Coefficients of variation are included for a select group of parameters as an indication of intersubject variability of response. Differences between treatments were evaluated using paired Student's t tests. $P < 0.05$ was considered statistically significant.

RESULTS

Binding to serum albumin. The ability of [N^{ε}-palmitoyl Lys(B_{29})] human insulin to bind to serum albumin was tested in vitro using a G-75 Sephadex size exclusion column. Figure 1 illustrates the elution profiles of BSA alone (Fig. 1*A*), and human insulin and [N^{ε}-palmitoyl Lys(B_{29})] human insulin in the absence (Fig. 1*B*) and presence (Fig. 1*C*) of BSA. The elution position of human insulin was unaffected by the presence of albumin in the sample, whereas the elution position of [N^{ε}-palmitoyl Lys(B_{29})] human insulin changed from being nearly identical to that of unmodified insulin in the absence of albumin to co-elution with albumin when albumin was present.

Time action and activity. Figure 2 illustrates the average glucose and insulin analog or insulin concentrations measured during a 5-h period following the intravenous injection of either [N^{ε}-palmitoyl Lys(B_{29})] human insulin or Humulin R. Data from a group of six overnight-fasted, insulin-withdrawn diabetic dogs that received no treatment were included for comparison. Although the glucose nadir reached after injection of the acylated analog was similar to that reached after injection of regular insulin, the initial rate of glucose fall was significantly less for the former (Fig. 2*A*; Table 1). The duration of the maximum glucose-lowering effect (i.e., the time over which the glucose concentration remained within 5.5 mmol/l of the nadir) of [N^{ε}-palmitoyl Lys(B_{29})] human insulin in these experiments was nearly twice as great as that of unmodified human insulin (Fig. 2*A*; Table 1), and would have

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/555018/46-4-637.pdf by guest on 04 March 2024

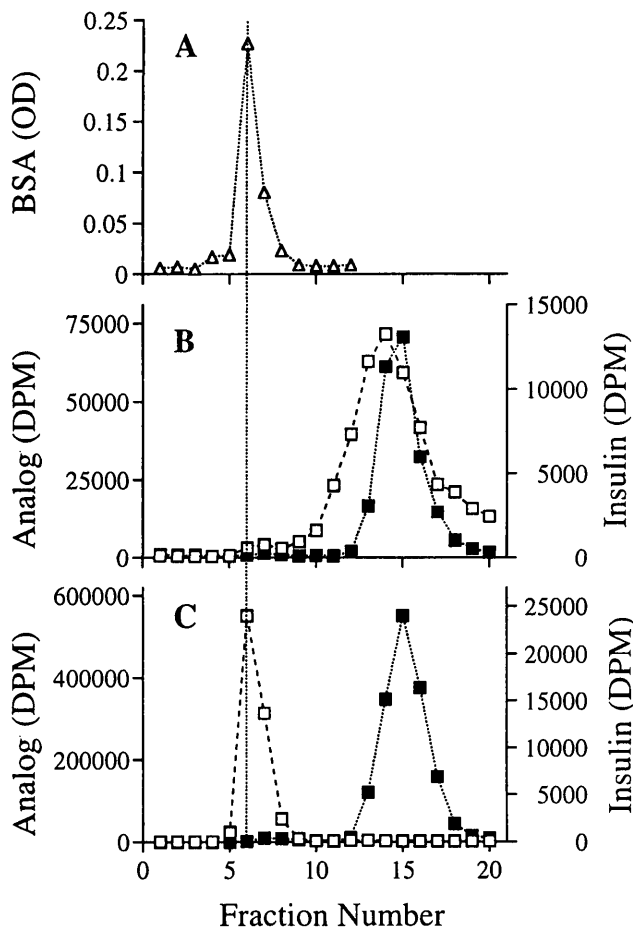

FIG. 1. Gel filtration chromatography assessment of binding of [N^{ε}-palmitoyl Lys(B_{29})] human insulin to bovine serum albumin (BSA). *A*: BSA (6.7 nmol) (△); *B*: tritiated [N^{ε}-palmitoyl Lys(B_{29})] human insulin (□) and iodinated human insulin (■) (both 0.16 nmol), run separately; *C*: tritiated [N^{ε}-palmitoyl Lys(B_{29})] human insulin (0.16 nmol) + BSA (6.7 nmol) (□) and iodinated human insulin (0.16 nmol) + BSA (6.7 nmol) (■), run separately.

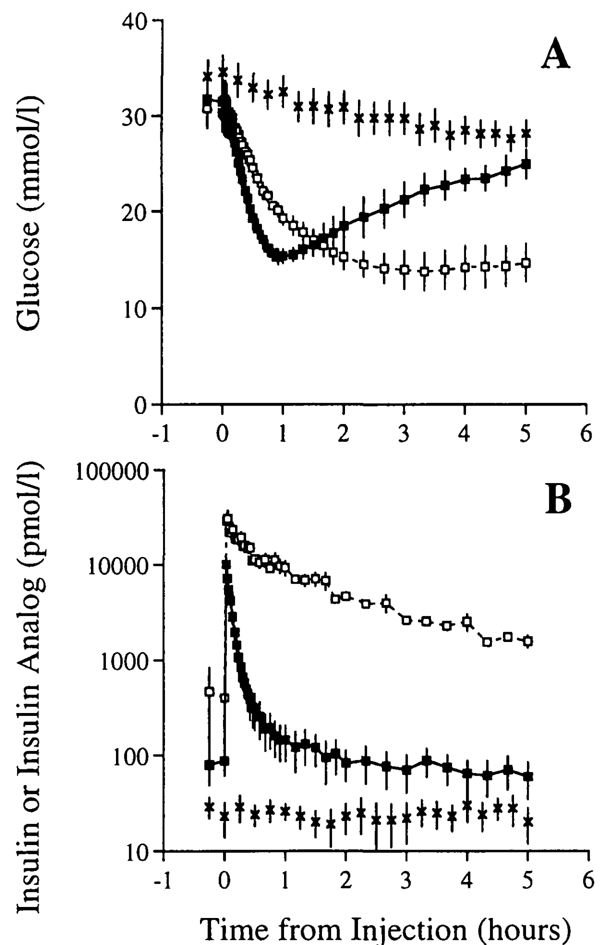

FIG. 2. Plasma glucose (*A*) and insulin or insulin analog concentrations (*B*) in untreated diabetic animals (×) and in diabetic animals after intravenous administration of 1.2 nmol/kg (molar equivalent of 0.2 U human insulin/kg) [N^{ε}-palmitoyl Lys(B_{29})] human insulin (□) or Humulin R (■).

been even greater had the study been continued long enough to allow the glucose concentration to return to control levels. The initial plasma distribution half-life of [N^{ε}-palmitoyl Lys(B_{29})] human insulin was nearly seven times that measured for unmodified human insulin, and the initial volume of distribution of the acylated analog was less than half that of Humulin R (Fig. 2*B*; Table 1). The pharmacokinetic values for Humulin R in these animals are consistent with those previously reported for native insulin (16,27).

Figure 3 illustrates the plasma glucose (Fig. 3*A*), NEFA (Fig. 3*B*), and insulin analog or insulin concentrations (Fig. 3*C*) measured over a 15-h period following the subcutaneous injection of either [N^{ε}-palmitoyl Lys(B_{29})] human insulin or the intermediate-acting insulin Humulin L. Although the rate of glucose fall and nadir reached were similar with the two treatments, the duration of the glucose-lowering effect was greater after treatment with the analog than after treatment with Humulin L (Fig. 3*A*; Table 2). Similarly, treatment with the analog decreased plasma NEFA concentrations to a similar extent as did treatment with the intermediate-acting human insulin formulation, but the duration of the effect was extended (Fig. 3*B*). Finally, the maximum insulin concentration, the area under the insulin curve, and the duration of the maximum insulin response (the time over which the insulin concentration remained within 50% of the maximum concentration reached) were all greater ($P < 0.05$) and the variability (as reflected in the coefficients of variation) tended to be less after treatment with [N^{ε}-palmitoyl Lys(B_{29})] human insulin than after treatment with Humulin L (Fig. 3*C*; Table 2).

DISCUSSION

Although intensive insulin therapy can diminish long-term complications in patients with IDDM (1), achieving tight glucose control safely is complicated by the less than optimal absorption patterns of currently available insulins (2). The more physiological insulin patterns achieved with use of [Lys(B_{28}), Pro(B_{29})]-human insulin (28) may solve the problems associated with mealtime insulins, but the inadequacies of the intermediate- and long-acting insulin suspensions currently used to replace basal insulin secretion have yet to be addressed. Changing to a soluble insulin formulation would likely decrease the variability of response associated with current extended-acting insulin suspensions (6,7); further, altering the mechanism for extension of time action could lead to an insulin analog that would provide for more physiological, basal glucose control. The present studies have demon-

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/555018/46-4-637.pdf by guest on 04 March 2024

TABLE 1
Pharmacodynamic and pharmacokinetic parameters describing glucose and insulin responses to intravenous administration of [N^{ε}-palmitoyl Lys(B_{29})] human insulin or Humulin R (1.2 nmol/kg; molar equivalent of 0.2 U human insulin/kg) in overnight-fasted diabetic dogs

	[N^{ε}-palmitoyl Lys(B_{29})] human insulin	Humulin R
Glucose nadir (mmol/l)	13.3 ± 1.7	14.9 ± 0.7
Rate of glucose fall (mmol · l^{-1} · min^{-1})	0.20 ± 0.04*	0.39 ± 0.04
Duration of maximum glucose-lowering effect (hours)	3.8 ± 0.2*	2.2 ± 0.6
Initial distribution half-life (min)	27.1 ± 6.1*	4.0 ± 0.3
Initial volume of distribution (ml/kg)	45 ± 5*	109 ± 15

Data are means ± SE of values determined for individual animals. *Significantly different from that of Humulin R ($P < 0.05$).

strated that modification of human insulin to promote binding to an existing serum protein can be used to create a soluble, extended-acting form of insulin with a novel mechanism for extension of time action. Also, in the present studies using an animal model of IDDM, the time action of [N^{ε}-palmitoyl Lys(B_{29})] human insulin was greater than that of the intermediate-acting human insulin suspension Humulin L, the intersubject variability of response was less, and the pattern of the insulin response was flatter. The combination of these attributes resulted in a prolonged stabilization of the fasting glucose concentrations in these insulin-dependent animals.

As expected (29), the in vitro binding studies described in the present report demonstrated a lack of detectable interaction between unmodified human insulin and serum albumin. In contrast, the elution position of [N^{ε}-palmitoyl Lys(B_{29})] human insulin changed from being nearly identical to that of unmodified insulin in the absence of albumin to co-elution with albumin when albumin was present, suggesting the formation of a stable [N^{ε}-palmitoyl Lys(B_{29})] human insulin–albumin complex.

In the present study, the time action and activity of [N^{ε}-palmitoyl Lys(B_{29})] human insulin was determined in vivo in conscious, overnight-fasted, insulin-withdrawn, diabetic dogs. Because the time action and activity of subcutaneously administered insulin can be affected by events occurring within the subcutaneous tissue, the time action and activity of the acylated analog were examined *1*) after intravenous administration, to determine the time action and activity of the analog independent of injection site effects; and *2*) after subcutaneous administration, to determine if the duration of action of the analog administered via the more therapeutic route was sufficient to support further development of the analog as a soluble, extended-acting insulin.

In the paired studies with Humulin R described here, [N^{ε}-palmitoyl Lys(B_{29})] human insulin administered intravenously to overnight-fasted, insulin-withdrawn, diabetic dogs demonstrated an overall potency at least as great as that of unmodified human insulin, and a duration of action at least 70% longer than that of human insulin. The longer time action of the analog appeared to be due to the greater than sixfold increase in its plasma half-life compared with that of human insulin. The slowed biological response, increased duration of action, and increased residence time in the plasma compartment demonstrated in these studies are consistent with a binding of the analog to serum albumin in vivo.

In the paired studies with Humulin L described in this manuscript, subcutaneous administration of 3.15 nmol/kg (molar equivalent of 0.525 U human insulin/kg) of [N^{ε}-palmitoyl Lys(B_{29})] human insulin resulted in a decrease in plasma glucose equivalent to that achieved after subcutaneous administration of 1.8 nmol/kg (0.3 U/kg) Humulin L, despite the difference in dosages used. This suggests that the potency of [N^{ε}-palmitoyl Lys(B_{29})] human insulin given subcutaneously is substantially less (just over 50%) than that of

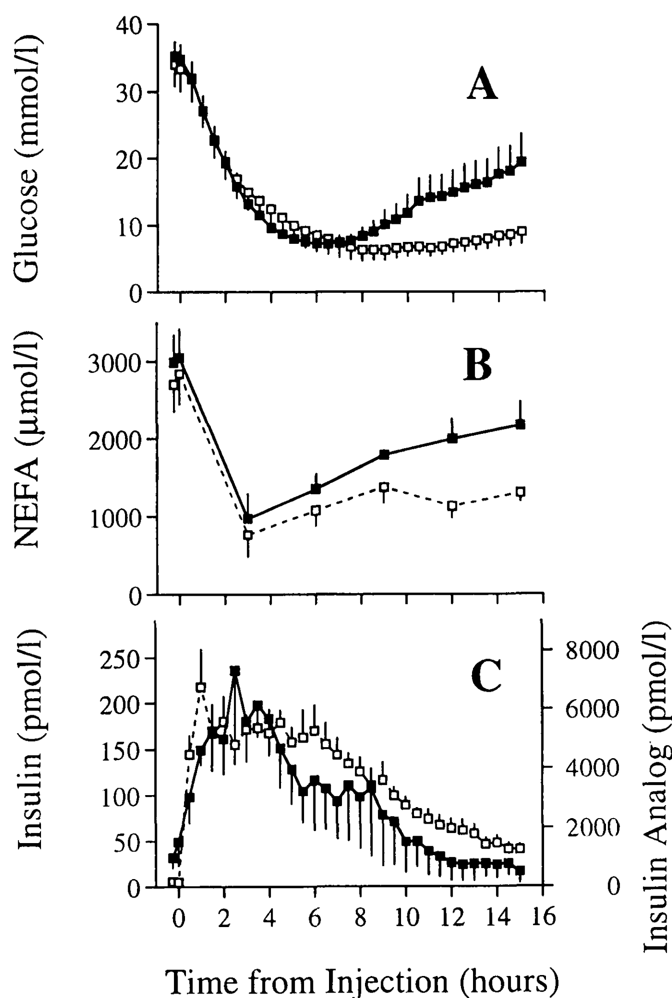

FIG. 3. Plasma glucose (*A*), non-esterified fatty acid (*B*), and insulin or insulin analog concentrations (*C*) after subcutaneous administration of either 3.15 nmol/kg (molar equivalent of 0.525 U human insulin/kg) [N^{ε}-palmitoyl Lys(B_{29})] human insulin (□) or 1.8 nmol/kg (0.3 U/kg) Humulin® L (■) to overnight-fasted, insulin-withdrawn, diabetic dogs.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/555018/46-4-637.pdf by guest on 04 March 2024

TABLE 2
Pharmacodynamic and pharmacokinetic parameters describing glucose and insulin responses to subcutaneous administration of 3.15 nmol/kg (molar equivalent of 0.525 U human insulin/kg) [N^{ε}-palmitoyl Lys(B_{29})] human insulin or 1.8 nmol/kg (0.3 U/kg) Humulin L in overnight-fasted diabetic dogs

	[N^{ε}-palmitoyl Lys(B_{29})] human insulin	CV	Humulin L	CV
Glucose nadir (mmol/l)	5.5 ± 1.3	54%	5.5 ± 1.4	56%
Time to glucose nadir (hours)	10.2 ± 1.1	24%	6.8 ± 1.0	34%
Duration of maximum glucose-lowering effect (hours)	9.5 ± 0.6*	15%	7.0 ± 1.4	45%
Maximum plasma insulin concentration (pmol/l)	7,678 ± 1,098*	32%	316 ± 92	65%
Area under insulin curve (nmol · l^{-1} · min^{-1})	3,334 ± 232*	16%	87 ± 22	58%
Duration of maximum insulin response (hours)	7.0 ± 1.0*	31%	4.0 ± 1.2	68%

Data are means ± SE of values determined for individual animals. *Significantly different from that of Humulin L ($P < 0.05$).

human insulin. The cause for the apparent decrease in potency when given subcutaneously has yet to be examined. Although potency appeared to be less than that of the intermediate-acting insulin formulation Humulin L, the plasma insulin analog concentrations were maintained at a more constant level for a longer period of time after treatment with the acylated analog compared with Humulin L. This flatter (more basal) pattern of the insulin response, together with the extended time action and decreased intersubject variability of response of the analog, resulted in a prolonged stabilization of the fasting glucose concentrations in these insulin-dependent animals.

Although the [N^{ε}-palmitoyl Lys(B_{29})] human insulin concentration curve showed a pattern desirable of a basal insulin, the absolute concentrations achieved were 20–30 times higher than those measured after administration of an equivalent biological dose of human insulin. These results were consistent with the much lower plasma clearance rate of the analog compared with unmodified human insulin, and were not unexpected for an analog with a major depot site in the plasma compartment. It should be noted that the majority of the drug measured was most likely in the bound form and, as such, was likely to have been virtually inactive because [N^{ε}-palmitoyl Lys(B_{29})] human insulin bound to serum albumin has a very low binding affinity to the insulin receptor (receptor binding affinity in the presence of 4 mg/ml serum albumin was estimated to be <1% that of unmodified human insulin in a human placental insulin receptor preparation; data not shown).

Although the pharmacokinetics and pharmacodynamics of intravenously delivered [N^{ε}-palmitoyl Lys(B_{29})] human insulin clearly indicate that acylation of insulin can significantly decrease its plasma clearance and, in turn, increase its time action, the difference in its pharmacokinetics (peak analog levels and pattern of the plasma analog curves) after intravenous and subcutaneous routes of delivery suggest that at least a portion of the extended time action after subcutaneous injection is due to retention of the analog at the subcutaneous site. This could be accounted for by binding of the acylated analog to tissue albumin as suggested by Kurtzhals et al. (30) and Markussen et al. (31).

Hashimoto et al. (24) synthesized [N^{ε}-palmitoyl Lys(B_{29})] bovine insulin to create a more lipophilic insulin for enhancement of transdermal and oral absorption of the drug. Although they observed an extension of time action of the acylated analog in comparison with unmodified insulin after intravenous administration in rats, they did not address the reason for the change. In addition, the effects of acylation demonstrated in their studies were much more modest than and inconsistent with those observed in our study and could not have predicted the results reported here.

The specific binding of [N^{ε}-palmitoyl Lys(B_{29})] human insulin to albumin and the prolongation of its time action observed in studies are consistent with results recently reported by Kurtzhals et al. (30) and Markussen et al. (31) with other fatty acid acylated insulin analogs. Although [N^{ε}-palmitoyl Lys(B_{29})] human insulin appeared to be at least as potent as intravenous human insulin and less potent (based on the glucose nadir reached) than subcutaneously administered human insulin, the lead analog reported by Markussen et al. (31), [N^{ε}-myristoyl Lys(B_{29})] des(B_{30}) human insulin, appeared to have decreased potency when given intravenously and equivalent potency when administered subcutaneously. This apparent discrepancy may represent actual differences in the properties of the two analogs or may be due to differences in the dosages of analogs used as well as the species (dog vs. pig) and metabolic states (insulin-withdrawn diabetic vs. normal) of the animals studied.

In conclusion, our studies support the concept that modification of insulin to promote binding to an existing serum protein can be used to create a soluble, extended-acting insulin. In addition, the time action, pattern, and decreased variability of response to [N^{ε}-palmitoyl Lys(B_{29})] human insulin support the development and further testing of this soluble insulin analog as a basal insulin to increase the safety of intensive insulin therapy.

ACKNOWLEDGMENTS

We would like to thank Ron Chance for expert advice and Ed Legan, Judy Heisserman, and Starr Johnson for excellent technical assistance.

REFERENCES

1. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977–986, 1993
2. Galloway JA, Chance RE: Improving insulin therapy: achievements and challenges. *Horm Metab Res* 26:591–598, 1994
3. Freeman SL, O'Brien PC, Rizza RA: Use of human ultralente as the basal insulin component in treatment of patients with IDDM. *Diabetes Res Clin Pract* 12:187–192, 1991
4. Lauritzen T, Pramming S, Gale EAM, Deckert T, Binder C: Absorption of isophane (NPH) insulin and its clinical implications. *Br Med J* 285:159–162, 1982

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/555018/46-4-637.pdf by guest on 04 March 2024

5. Binder C. Absorption of injected insulin. *Acta Pharmacol Toxicol* 27 (Suppl. 2):1–84, 1969
6. Jørgensen S, Vaag A, Langkjaer L, Hougaard P, Markussen J: NovoSol Basal: pharmacokinetics of a novel soluble long acting insulin analogue. *Br Med J* 299:415–419, 1989
7. Galloway JA, Hooper S, Spradlin CT, Howey DC, Frank BH, Bowsher RR, Anderson JH: Biosynthetic human proinsulin: review of chemistry, in vitro and in vivo receptor binding, animal and human pharmacology studies, and clinical trial experience. *Diabetes Care* 15:666–692, 1992
8. Seipke G, Geisen K, Neubauer HP, Pittius C, Roßkamp R, Schwabe D: New insulin preparations with prolonged action profiles: A21 modified arginine insulins. *Diabetologia* 35 (Suppl. 1):A4, 1992
9. Dreyer M, Pein M, Schmidt Chr, Heidtmann B, Schlünzen M, Roßkamp D: Comparison of the pharmacokinetics/dynamics of Gly(A21)-Arg(B31, B32)-human-insulin (HOE71GT) with NPH-insulin following subcutaneous injection by using euglycaemic clamp technique. *Diabetologia* 37 (Suppl. 1):A78, 1994
10. Hoffmann JA, Chance RE, Cusick T, Rabe CA, Tinsley FC, Williams VK, Myers SR: Synthesis and bioactivity of des(64,65)-human proinsulin (D) a soluble, intermediate-acting insulin agonist. *Diabetes* 42 (Suppl. 1):54A, 1993
11. Heinemann L, Heise T, Klepper A, Ampudia J, Bender R, Starke AAR: Time-action profiles of the intermediate-acting insulin analogue des(64,65)-human proinsulin. *Diabetes Metab* 21:415–419, 1995
12. Cascieri MA, Saperstein R, Hayes NS, Green BG, Chicchi GG, Applebaum J, Bayne ML: Serum half-life and biological activity of mutants of human insulin-like growth factor I which do not bind to serum binding proteins. *Endocrinology* 123:373–381, 1988
13. Bartalena L: Thyroid hormone transport proteins. *Clin Lab Med* 13:583–598, 1993
14. Bartalena L: Recent achievements in studies on thyroid-hormone-binding proteins. *Endocr Rev* 11:47–64, 1990
15. Baumann G, Ambum KD, Buchanan TA: The effect of circulating growth hormone binding protein in metabolic clearance, distribution, and degradation of human growth hormone. *J Clin Endocrinol Metab* 64:657–660, 1987
16. Van Haeften TW, Bolli GB, Dimitriadis GD, Gottesman IS, Horwitz DL, Gerich JE: Effect of insulin antibodies and their kinetic characteristics on plasma free insulin dynamics in patients with diabetes mellitus. *Metabolism* 35:649–656, 1986
17. Gray RS, Cowan P, DiMario U, Elton RA, Clarke BF, Duncan LJP: Influence of insulin antibodies on pharmacokinetics and bioavailability of recombinant human and highly purified beef insulins in insulin dependent diabetics. *Br Med J* 290:1687–1691, 1985
18. Peters T: Serum albumin. *Adv Protein Chem* 37:161–245, 1985
19. Doweiko JP, Nompleggi DJ: Role of albumin in human physiology and pathophysiology. *J Parenter Enteral Nutr* 15:207–211, 1991
20. Carter D, Ho JX: Structure of serum albumin. *Adv Protein Chem* 45:153–203, 1994
21. Spector AA: Fatty acid binding to plasma albumin. *J Lipid Res* 16:165–179, 1975
22. Lindsay DG, Shall S: The acetylation of insulin. *Biochem J* 121:737–745, 1971
23. Lindsay DG, Shall S: Acetoacetylation of insulin. *Biochem J* 115:587–595, 1969
24. Hashimoto M, Takada K, Kiso Y, Muranishi S: Synthesis of palmitoyl derivatives of insulin and their biological activities. *Pharm Res* 6:171–176, 1989
25. Markowitz J, Archibald J, Downie HG: Experimental surgery of the pancreas. In *Experimental Surgery Including Surgical Physiology.* 5th Ed. Baltimore, Williams & Wilkins, 1964, p. 236–252
26. Frank BH, Beckage MJ, Willey KA: High-performance liquid chromatographic preparation of single-site carrier-free pancreatic polypeptide hormone radiotracers. *J Chromatogr* 266:239–248, 1983
27. Shoelson SE, Polonsky KS, Zeidler A, Rubenstein AH, Tager HS: Human insulin B24 (phe → ser): secretion and metabolic clearance of the abnormal insulin in man and in a dog model. *J Clin Invest* 73:1351–1358, 1984
28. Howey DC, Bowsher RR, Brunelle RL, Woodworth JR: [Lys(B_{28}), Pro(B_{29})]-human insulin: a rapidly absorbed analogue of human insulin. *Diabetes* 43:396–402, 1994
29. Turnheim K, Waldhäusl WK: Essentials of insulin pharmacokinetics. *Wien Klin Wochenschr* 100:65–72, 1988
30. Kurtzhals P, Havelund S, Jonassen I, Kiehr B, Larsen UD, Ribel U, Markussen J: Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect *in vivo*. *Biochem J* 312:725–731, 1995
31. Markussen J, Havelund S, Kurtzhals P, Andersen AS, Halstrøm J, Hasselager E, Larsen UD, Ribel U, Schäffer L, Vad K, Jonassen I: Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs. *Diabetologia* 39:281–288, 1996

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/46/4/637/550518/46-4-637.pdf by guest on 04 March 2024