

New Concept for Long-Acting Insulin

Spontaneous Conversion of an Inactive Modified Insulin to the Active Hormone in Circulation: 9-Fluorenylmethoxycarbonyl Derivative of Insulin

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Insulin is a short-lived species in the circulatory system. After binding to its receptor sites and transmission of its biological signals, bound insulin undergoes receptor-mediated endocytosis and consequent degradation. An inactive insulin derivative that is not recognized by the receptor has a longer circulation life, but obviously is biologically impotent. (Fmoc)₂ insulin is an insulin derivative purified through high-performance liquid chromatography in which two 9-fluorenylmethoxycarbonyl (Fmoc) moieties are covalently linked to the α -amino group of phenylalanine B1 and the ϵ -amino group of lysine B29. It has 1–2% of the biological potency and receptor binding capacity of the native hormone. After incubation, (Fmoc)₂ insulin undergoes a time-dependent spontaneous conversion to fully active insulin in aqueous solution at 37° C and a pH range of 7–8.5. At pH 7.4, the conversion proceeds slowly ($t_{1/2} = 12 \pm 1$ days) and biological activity is generated gradually. A single subcutaneous administration of (Fmoc)₂ insulin to streptozocin-treated diabetic rats normalized their blood glucose levels and maintained the animals in an anabolic state over 2–3 days. A broad shallow peak of immunoreactive insulin was found to persist in circulation over this period. To confirm further that the long-acting effect of (Fmoc)₂ insulin proceeds via slow release in the blood circulation itself, we administered native insulin, NPH insulin, or the (Fmoc)₂ derivative intraperitoneally. The rats recovered from hypoglycemia at $t_{1/2} = 8.0 \pm 0.3$ and 10 ± 0.4 h after administration of native and NPH insulin, respectively. In contrast, (Fmoc)₂ insulin was active for a significantly longer time, with an extended onset of $t_{1/2} = 26 \pm 1$ h, and a glucose-lowering effect even 40 h after administration. (Fmoc)₂ insulin was also found to be more resistant to proteolysis. Finally, we found that (Fmoc)₂ insulin does not induce antigenic effects.

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Boc, t-butyloxycarbonyl; ELISA, enzyme-linked immunosorbent assay; Fmoc, 9-fluorenylmethoxycarbonyl; (Fmoc)₂ insulin, an insulin derivative in which two Fmoc moieties are covalently attached to phenylalanine B1 and lysine B29; HPLC, high-performance liquid chromatography; STZ, streptozocin; (t-Boc)₂O, di-tert-butylidicarbonate; TFA, trifluoroacetic acid; (TNB)₂ insulin, an insulin derivative in which two trinitrobenzenesulfonate moieties are attached to insulin.

In summary, we present here a new concept for prolonging the half-life of insulin in the circulatory system, in which receptor-mediated endocytosis and degradation is delayed and accompanied by a time-dependent generation of basal insulin. *Diabetes* 48:1437–1442, 1999

Insulin is the mainstay for treatment of virtually all type 1 and many type 2 diabetic patients. For long-term treatment, insulin is usually administered by subcutaneous injections. A longer duration of action is achieved by using insulin preparations made less soluble by complexing them with zinc and protamine so that the insulin is released gradually on dissolution (1–3). Once it reaches the circulatory system, insulin has a very short life, with an in vivo half-life of ~5–6 min (1). The concept of an insulin that is longer acting in circulation is appealing and has important pharmaceutical implications.

Insulin is degraded primarily in the liver and to a lesser extent in the kidneys and muscle tissue (4). After insulin binding to the cell-surface receptor, insulin-receptor complexes are internalized into small vesicles (endosomes), where degradation is initiated (4,5). Obviously, insulin from other species, chemically modified insulin (6,7), or proinsulin (8) that has a low receptor binding affinity lasts longer in the circulatory system, but these types of insulin have little biological activity.

We have used a new strategy to design a long-acting form of insulin. Insulin is converted into an inactive species that has a reduced binding capacity to the receptor and therefore avoids endocytosis and degradation. At physiological pH and temperature, however, it is capable of reverting to the native hormone and has the desired glucose-lowering action in hyperglycemic rats. Such a prototype derivative of insulin is described here in detail.

RESEARCH DESIGN AND METHODS

Materials. Human (Zn²⁺-free) insulin was donated by Novo Nordisk (Bagsvaerd, Denmark) or by Biotechnology General (Rehovot, Israel), and was used without further purification. D-[U-¹⁴C]Glucose (4–7 mCi/mol) was obtained from Du Pont-NEN (Boston, MA). Collagenase, type I, (134 U/mg) was purchased from Worthington (Freehold, NJ). 9-Fluorenylmethoxycarbonyl-N-hydroxysuccinimide (Fmoc-OSu) and di-tert-butylidicarbonate (t-Boc)₂O were obtained from Novabiochem (Laufelfingen, Switzerland). All other materials used in this study were of analytic grade.

Biological methods. Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion (9). Lipogenesis (incorporation of [U-¹⁴C]

glucose into lipids) was performed according to the method of Moody et al. (10). Displacement of ^{125}I -labeled insulin from rat adipocytes was carried out at 7°C , essentially as described in Shechter et al. (11). Diabetes was induced by a single intravenous injection of a freshly prepared solution of streptozocin (STZ; 55 mg/kg body wt) according to the method of Meyerovitch et al. (12). Rats were maintained at 24°C under conditions of controlled lighting and were fed ad libitum. Blood samples for the analysis of blood glucose were taken from the tail veins and measured with a glucose analyzer (Beckman Instruments, Fullerton, CA) by the glucose oxidase method. Groups consisted of five rats. Data are presented as means \pm SE.

Enzymatic degradation studies. Native insulin and an insulin derivative in which two Fmoc moieties are covalently attached to phenylalanine B1 and lysine B29 [(Fmoc) $_2$ insulin] (1 mg) were thoroughly dissolved in 1 ml HEPES buffer (50 mmol/l; pH 7.4) containing 5% DMSO and 4-nitrophenol (0.2 mg; used as an inert internal marker). Trypsin and chymotrypsin dissolved in 0.1 mmol/l HCl (10 μl , 1 mg/ml) were added and the solutions were incubated at 37°C . At the indicated time points, aliquots were withdrawn (25 μl), acidified with a 0.5 ml mixture of solutions A and B (60:40) (which is the initial gradient ratio used for high-performance liquid chromatography [HPLC] separations), and analyzed by HPLC. Each sample (containing 25 μg of protein at $t = 0$) was eluted with a gradient as described below for insulin derivatives at a flow rate of 1 ml/min. The area corresponding to the protein peak (in percentage, calculated by the integrator) is referred to as the internal peak of 4-nitrophenol. The protein peak area at $t = 0$ was designated as 100%.

Antigenicity. Groups of BALB/C mice, age 12 weeks, were initially treated with 100 μg of native insulin or 100 μg (Fmoc) $_2$ insulin emulsified in complete Freund's adjuvant oil (Difco, Detroit, MI) and then boosted at 2 weeks using incomplete Freund's adjuvant (Difco) (13,14). At 6 weeks, the mice were bled individually and their sera were diluted up to 1:1,000 and tested for antibodies to native insulin or (Fmoc) $_2$ insulin in an enzyme-linked immunosorbent assay (ELISA) (15). Briefly, 10 μg of the two antigens were applied to assay plates that bind the antigens and further incubated with the test sera. Antibodies binding to the adherent antigens were detected with alkaline phosphatase anti-mouse IgG + IgM conjugate. A significant quantity of antibody was defined as having absorbance at 405 nm >0.10 , which is 5 SD over the mean ELISA reading obtained in the sera of six normal mice.

Quantitation of immunoreactive insulin. Immunoreactive insulin was analyzed by a standard radioimmunoassay (RIA) using guinea pig anti-porcine insulin antibodies (Linco, St. Charles, MO) with human insulin standard. Iodinated ^{125}I -labeled porcine insulin was purchased from Diagnostic Products (Los Angeles, CA). Bound and free hormones were separated with goat anti-guinea pig IgG serum and 4% polyethylene glycol. The minimal detectable level for human insulin was 44 pmol/l (0.26 ng/ml).

TABLE 1
Chemical and biological features of (Fmoc) $_2$ insulin

Characteristic	(Fmoc) $_2$ insulin
Position of modification	Phe ^{B1} , Lys ^{B29}
Fmoc (mol)/insulin (mol)	1.9
Retention time (min)	20.9
Mass spectra (m/z)	
Calculated	6,252
Found	6,254
Solubility in aqueous buffer (pH 7.4; mg/ml)	1 \pm 0.1
Relative receptor binding potency (%)	2.0
Relative biological activity (%)	1.4
Biological potency following incubation at pH 8.5 and 37°C (%)	
9 h	10
20 h	20
45 h	98

Position of modification determined according to Sanger's procedure using dinitrofluorobenzene (16). Fmoc/insulin determined spectroscopically (experimental part). Retention time established with analytical HPLC procedure (experimental part); insulin elutes under the same experimental conditions with retention time = 12 ± 0.2 min. Mass spectra were determined using the electrospray ionization technique. Receptor binding capacity was determined by a binding assay and displacement of ^{125}I -insulin from rat adipocytes (11,27). Biological potency was determined by a lipogenic assay with rat adipocytes.

Chemical procedures. Mass spectra were determined with a VG AutoSpec Q (EBE-qQ) mass spectrometer (VG Fisons, Altrincham, U.K.) using the electrospray ionization technique. Reverse-phase HPLC was performed with a Spectra-Physics SP8800 liquid chromatography system (Spectro-Physics, San Jose, CA) equipped with an applied Biosystem 757 variable wavelength absorbance detector. The column effluents were monitored by ultraviolet absorbance at 220 nm. Chromatograms were recorded on a Chrom-Jet integrator (Thermo-Separation, Riviera Beach, FL). HPLC prepacked columns used included LiChroCART 250 (10 mm) containing LiChrosorb RP-18 (7 μm) (Merck, Rahway, NJ) and LiChrospher 100 RP-8 (5 μm), 250 \times 4 mm (Merck). HPLC purification was achieved with a linear gradient formed using a combination of 0.1% trifluoroacetic acid (TFA) in H_2O (solution A) and 0.1% TFA in acetonitrile: H_2O (75:25) (solution B). For analytical HPLC procedures, a linear gradient of 40–100% of solution B was run for 40 min at a flow rate of 1 ml/min. Amino acid composition was analyzed after acid hydrolysis in 6N HCl at 110°C for 24 h. A Dionex automatic amino acid analyzer (HP 1090, Palo Alto, CA) was used. Covalently bound Fmoc was quantitated by treating the derivative with piperidine and determining absorbance at 301 nm ($\epsilon_{301} = 7800$). Sites of Fmoc insertions were determined according to the method of Sanger (16).

Fmoc^(Phe^{B1}, Lys^{B29}) insulin was synthesized as described in detail in our patent application (17). In short, (t-Boc) $_2\text{O}$ (3.75 mg, 17.2 μmol) was added gradually to a solution of insulin (100 mg, 17.2 μmol) in DMSO:triethylamine, (20:1 vol/vol; 3 ml). The reaction mixture was stirred for 20 h at room temperature, after which ethyl acetate and ether were added. The precipitate formed was then isolated by centrifugation, washed twice with ethyl acetate and ether, and purified further by an HPLC procedure to yield 35 mg of pure Boc^{GlyA1} insulin. The product was then reacted with 15 equivalents of Fmoc-OSu (30 mg) in dimethylformamide (1.5 ml) containing 10 equivalents of *N*-ethyl-diisopropylamine for 20 h at room temperature and then precipitated with ethyl acetate and ether. The product was then

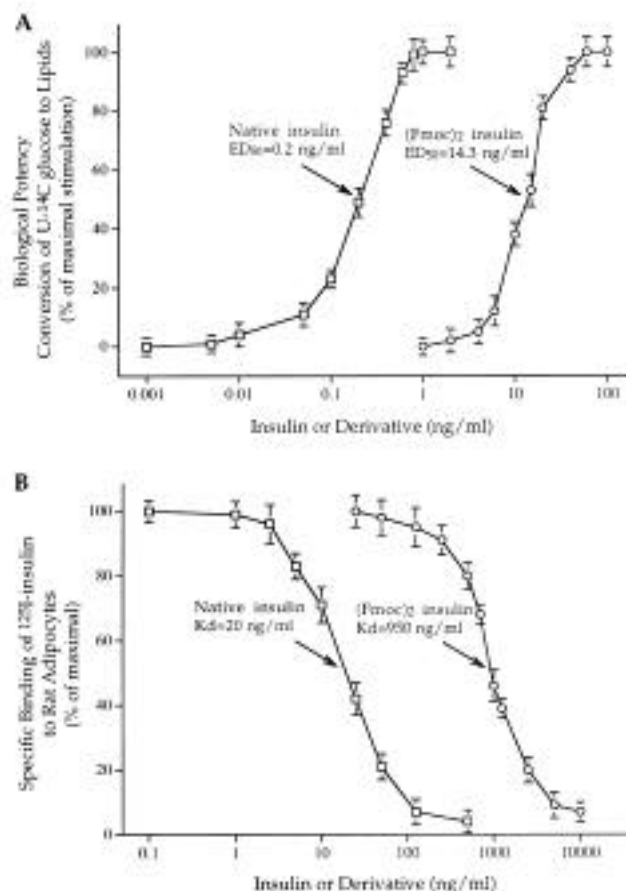


FIG. 1. Dosage-dependent stimulation of lipogenesis and displacement of ^{125}I -insulin from rat adipocytes with (Fmoc) $_2$ insulin. **A:** Lipogenesis was carried out for 1 h at 37°C in plastic vials containing 0.5 ml of fat cell suspensions (1.5×10^5 cells) and 0.2 mmol/l [^{14}C]glucose in the presence or absence of the indicated concentrations of native or (Fmoc) $_2$ insulin. Results are expressed as the percentage of maximal stimulation. Insulin (17 nmol/l)-stimulated lipogenesis was four- to five-fold higher than basal. **B:** Displacement of ^{125}I -insulin was performed for 1 h at 7°C (experimental procedure). ED₅₀, effective dose₅₀.

washed with ethyl acetate and ether and treated with 2 ml of TFA containing 5% H₂O for 0.5 h at room temperature. Cold methyl tert-butyl ether was added, and the precipitate formed was purified by HPLC to obtain the final product in 20% overall yield (20 mg).

(TNB)₂ insulin, an insulin derivative in which two trinitrobenzene-sulfonate moieties are attached to insulin, was prepared by reacting human insulin (5 mg/ml in 0.1 mol/l NaHCO₃) with 3 mol/l excess of trinitrobenzene sulfonic acid for 4 h at room temperature. The reaction mixture was then dialyzed for 48 h and purified by HPLC. Mass spectra found *m/z* 6232 (calculated 6229).

RESULTS

General features of (Fmoc)₂ insulin. We selected Fmoc^(PheB1, LysB29) insulin (abbreviated to [Fmoc]₂ insulin) as the representative prototype of an insulin-derivative with low biological activity and poor receptor binding affinity that is capable of reverting to the native hormone under physiological conditions. Table 1 summarizes its basic chemical and biological characteristics. The correct composition of (Fmoc)₂ insulin was verified by amino acid analysis (not shown). (Fmoc)₂ insulin has ~1.4% of the biological potency (ED₅₀ = 14.3 ± 0.4 ng/ml) of insulin (ED₅₀ = 0.2 ± 0.015 ng/ml), as judged by a lipogenic assay (Fig. 1A). It has ~2% receptor binding affinity (*K_d* = 950 ± 50 ng/ml) relative to the native hormone (*K_d* = 20 ± 2 ng/ml), as determined by displacement of ¹²⁵I-insulin from intact adipose rat cells (Fig. 1B).

(Fmoc)₂ insulin fully reverted to the native, biologically active hormone on incubation with 0.1 mol/l NaHCO₃ (pH 8.5) for 2 days at 37° C (*t*_{1/2} = 24–27 h) (Table 1). This was confirmed by HPLC and lipogenic assays. At pH 7.4, the rate of conversion to insulin proceeded more slowly (*t*_{1/2} = 12 ± 1 days) and was virtually linear (Fig. 2).

(Fmoc)₂ insulin was less soluble than native insulin in aqueous medium. A solution of ~1 mg/ml was prepared by dissolving the derivative at pH 8.5–9.0 followed by adjustment of the pH to 7.0–7.4 (Table 1).

(TNB)₂ insulin, a derivative with trinitrobenzene moieties that possesses ~1% of the biological potency of the native hormone, was also prepared. Unlike (Fmoc)₂ insulin, (TNB)₂ insulin does not revert to insulin after incubation and was

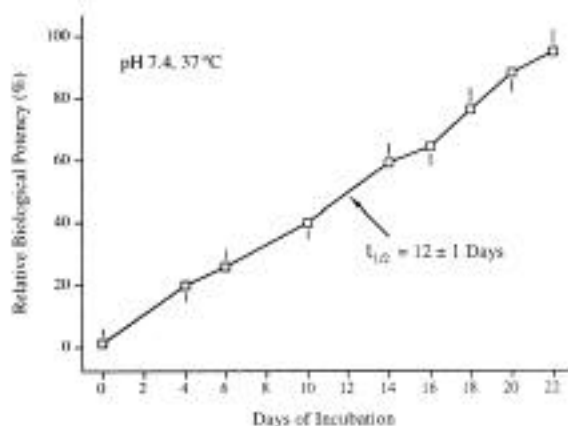


FIG. 2. Time course of reactivation of (Fmoc)₂ insulin. (Fmoc)₂ insulin (1 mg/ml in 50 mmol/l HEPES buffer containing 10% DMSO) was incubated at pH 7.4 and 37° C. Aliquots were withdrawn at the indicated time points and analyzed for biological activity in a lipogenic assay with rat adipocytes. Under our assay conditions, insulin activated lipogenesis four- to fivefold above basal with an ED₅₀ value = 0.2 ± 0.02 ng/ml. An insulin derivative exhibiting ED₅₀ = 20 ± 2 ng/ml in this assay was considered as having 1% of the native biological potency (26).

used as a nonreactive, negative control derivative for (Fmoc)₂ insulin.

A single subcutaneous administration of (Fmoc)₂ insulin to hyperglycemic rats induced prolonged normoglycemia and weight gain. The STZ-treated diabetic rat is a suitable model for analyzing a long-acting insulin. STZ-treated diabetic rats are hyperglycemic (>350 mg/dl glucose), hypoinsulinemic (5–10% of normal β-cell function), and underweight. Daily administration of insulin restores normal functions (18). Figure 3 compares the effect in STZ-treated diabetic rats of a single subcutaneous injection of native insulin, (Fmoc)₂ insulin, or (TNB)₂ insulin (*n* = 5 for each group, 3 mg/rat) on blood glucose (see Fig. 3 legend for further experimental details). Circulating glucose levels, immunoreactive insulin content, and daily weight gain were measured over 4 days. After a single administration of (Fmoc)₂ insulin, circulating glucose levels fell to normal values within 12 h. Normoglycemia was maintained for 2 days,

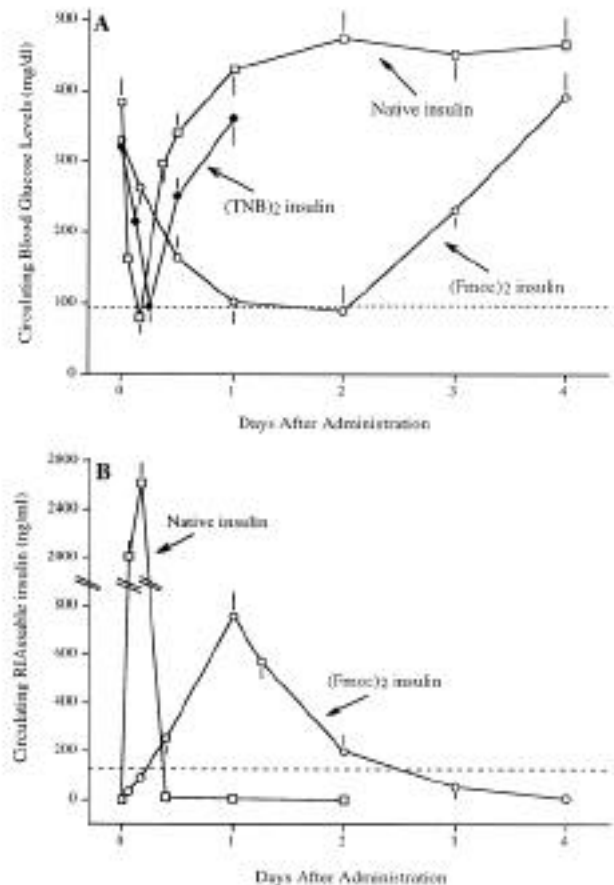


FIG. 3. Circulating glucose levels and appearance of immunoreactive insulin after a single subcutaneous administration of (Fmoc)₂ insulin to STZ-treated diabetic rats. **A:** STZ-treated diabetic rats received (Fmoc)₂ insulin, native insulin, or (TNB)₂ insulin (3 mg/rat, 0.75 ml). Blood glucose levels were determined at the indicated time points. Each point represents the arithmetic mean of blood glucose of five rats. The horizontal dashed line indicates the arithmetic mean of blood glucose of control healthy rats. **B:** Aliquots of serum from STZ-treated diabetic rats given insulin or (Fmoc)₂ insulin were withdrawn at the indicated time points for determining immunoreactive insulin. The horizontal dashed line represents the threshold level of immunoreactive insulin that is required to maintain normoglycemia in STZ-treated diabetic rats (determined with native insulin administered to a group of STZ-treated diabetic rats).

TABLE 2

Daily weight gain of STZ-treated diabetic rats after a single subcutaneous administration of (Fmoc)₂ insulin

Treatment	Daily weight gain (g · rat ⁻¹ · day ⁻¹)			
	Day 1	Day 2	Day 3	Day 4
Vehicle	0.7 ± 0.05	0.9 ± 0.1	1.2 ± 0.1	1.1 ± 0.1
Native insulin (3 mg/rat)	5.2 ± 0.5	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
(Fmoc) ₂ insulin (3 mg/rat)	9.4 ± 1.0	13.1 ± 1.7	10.7 ± 2.0	2.0 ± 0.2

Data are means ± SE and are expressed as daily weight gain in g/rat. STZ-treated diabetic rats (each group *n* = 5) received native insulin, (Fmoc)₂ insulin, or vehicle under the experimental conditions described in detail in Fig. 3. Rats were weighed at time of administration and then daily at 10:00 A.M.

and glucose levels were kept low for an additional day (Fig. 3A). With both native and (TNB)₂ insulin, normoglycemia was transient and hyperglycemia reoccurred within ~10 and ~15 h, respectively. Administration of 30 µg of native insulin to STZ-treated diabetic rats resembled the transient pattern of glucose-lowering action obtained by 3 mg of the 1% biologically active (TNB)₂ insulin (not shown).

Figure 3B shows levels of immunoreactive insulin in circulation after a single subcutaneous administration of insulin or (Fmoc)₂ insulin. With insulin, circulating levels reached a maximum high at 4 h (2.6 ± 0.2 µg/ml), followed by a sharp fall to undetectable levels (>0.3 ng/ml) 8 h after administration. After a single administration of (Fmoc)₂ insulin, however, a shallow and wide peak of immunoreactive insulin was obtained. It reached a maximum of 0.76 ± 0.03 µg/ml 1 day after administration, and then decreased gradually over the next 2 days, intercepting the RIA-insulin level for maintaining normoglycemia in STZ-treated diabetic rats (Fig. 3B, dashed line) only 2.6 ± 0.2 days after administration.

Anabolic effect of (Fmoc)₂ insulin in STZ-treated diabetic rats. Table 2 summarizes the daily weight gain of STZ-treated diabetic rats after a single administration of native insulin or (Fmoc)₂ insulin. Those receiving vehicle gained only an average of ~1 g · day⁻¹ · rat⁻¹ over the 4 days of this experiment. After a single administration of (Fmoc)₂ insulin, the weight gain averaged ~11 g · day⁻¹ · rat⁻¹ for the first 3 days. Native insulin had some anabolic effect in the first day only (Table 2), whereas (TNB)₂ insulin was indistinguishable from the vehicle (not shown).

(Fmoc)₂ insulin is also long acting after intraperitoneal administration to healthy rats. (Fmoc)₂ insulin is intrinsically less soluble than native insulin in aqueous medium (Table 1). Therefore, in theory, its prolonged action in vivo after subcutaneous administration could be attributed to a slower absorption rate, an ability to avoid receptor-mediated degradation in circulation, or both. To differentiate between the first two conditions, we bypassed the subcutaneous compartment by administering native insulin, long-acting insulin (NPH), or (Fmoc)₂ insulin intraperitoneally to healthy rats. Glucose levels were monitored and the time required to recover from hypoglycemia was determined. As expected, native insulin and NPH insulin were not significantly different from each other in this experimental system. Recovery from hypoglycemia proceeded at *t*_{1/2} = 8.0 ± 0.3 and 10 ±

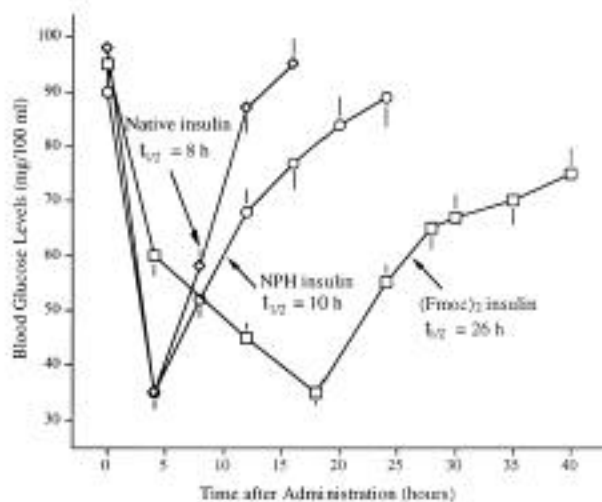


FIG. 4. Effect of a single intraperitoneal administration of (Fmoc)₂ insulin on the blood glucose levels of normal healthy rats. Groups of five normal healthy rats were given (Fmoc)₂ insulin, native insulin, or NPH insulin (3 mg/rat, 1.0 ml). (Fmoc)₂ insulin was dissolved in 50 mmol/l HEPES (pH 7.4) containing 10% DMSO. Blood glucose levels, determined at the indicated time points, are the arithmetic mean of four rats. Arrows indicate half-life of recovery from hypoglycemia for each treatment.

0.4 h, respectively (Fig. 4). (Fmoc)₂ insulin, however, was active for a significantly longer time with an extended onset at *t*_{1/2} = 26 ± 1 h and had a glucose-lowering effect even at 40 h after administration (Fig. 4). Thus (Fmoc)₂ insulin showed a prolonged glucose-lowering action after intraperitoneal administration as well.

Stability toward proteolysis. In addition to avoiding receptor-mediated endocytosis, the stability of hormones to proteolysis in circulation or at tissue surfaces (4) can be a significant contributing factor for prolongation of activity. We therefore evaluated the susceptibility of (Fmoc)₂ insulin to a mixture of trypsin and chymotrypsin at 37° C (experimental part). Native insulin was fully degraded within ~6 h at a *t*_{1/2} = 0.8 h. In contrast, (Fmoc)₂ insulin was highly resistant to proteolysis, exhibiting a *t*_{1/2} = 7.0 h (Fig. 5), thereby indicating the stability of (Fmoc)₂ insulin to proteolysis in comparison with the native hormone.

Antigenicity of (Fmoc)₂ insulin. To consider the clinical implications of (Fmoc)₂ insulin, we evaluated its potential antigenicity compared with that of native insulin. Figure 6 shows the level of antibodies elicited after treating mice with native or (Fmoc)₂ insulin. Measurements of antibody levels were performed in an ELISA and indicated that the native hormone and the derivative induced antibodies to insulin to the same extent. Moreover, a lower incidence of antibodies to (Fmoc)₂ insulin were found in the sera of (Fmoc)₂ insulin-treated mice (*P* < 0.01) (Fig. 6). Sera of untreated control mice had a negligible level of antibodies (OD₄₀₅ < 0.025; not shown). (Fmoc)₂ insulin was thus slightly less immunogenic than native insulin. In a separate set of experiments, we found that antibodies to insulin react with (Fmoc)₂ insulin (~4%; not shown) to a small extent.

DISCUSSION

In addition to rapid-acting insulin, long-acting preparations are also required for maintaining a basal level of the hormone over

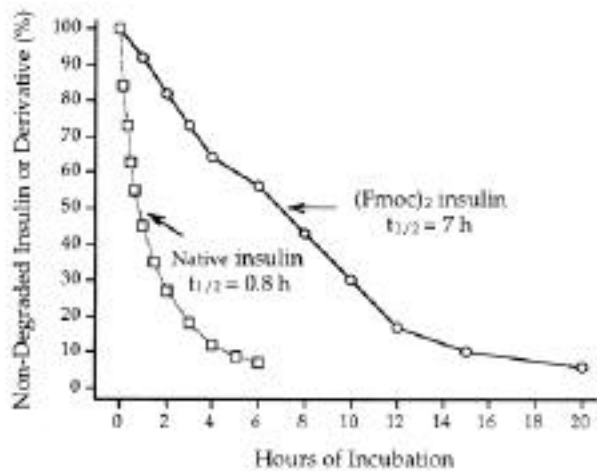


FIG. 5. Stability of (Fmoc)₂ insulin toward enzymatic degradation in comparison with native insulin. (Fmoc)₂ insulin and native insulin were incubated with a mixture of trypsin and chymotrypsin at 37° C. At the indicated time points, aliquots were subjected to analytical HPLC. The quantity of the hormone (peak area) at $t = 0$ was assigned to 100%.

prolonged periods (2,19,20). An ideal therapeutic long-acting insulin is a preparation that releases insulin in an active form into the circulation after administration with a “zero-order” kinetics over a prolonged period. Such a mission has not been fully achieved solely by decreasing the rate of absorption of injected insulin from the subcutaneous compartment; therefore, all existing insulin preparations do not have sufficiently prolonged action to provide a basal insulin supply throughout the day (1). Furthermore, reproducible administrations of such suspensions are difficult to achieve due to lack of homogeneity (2,21). We have reported here on a different approach that circumvents the existing technology.

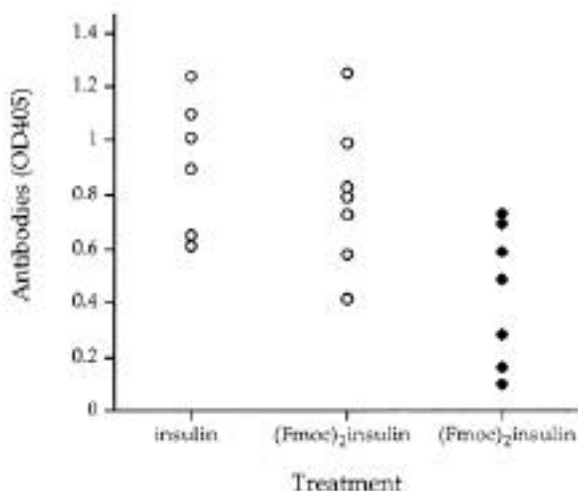


FIG. 6. Antigenic capacity of (Fmoc)₂ insulin in mice. Treatment with insulin and (Fmoc)₂ insulin-induced antibodies to insulin to the same extent. A lower incidence of antibodies to (Fmoc)₂ insulin was found in the sera of (Fmoc)₂ insulin-treated mice. Groups of seven BALB/C mice (age 12 weeks) were treated with 100 µg of native insulin or 100 µg of (Fmoc)₂ insulin emulsified in complete Freund's adjuvant oil, boosted at 2 weeks, and analyzed for antibodies to insulin or (Fmoc)₂ insulin in an ELISA assay at 6 weeks (experimental part). A significant amount of antibody was defined as an optical density 405 nm reading >0.10, which is 5 SD over the mean ELISA reading obtained in the sera of six normal mice. $P < 0.01$ by Kruskal-Wallis nonparametric test. ○, antibody to native insulin; ◆, antibody to (Fmoc)₂ insulin.

Fmoc moieties are covalently coupled to insulin to obtain a derivative that has a decreased receptor-binding capacity and thus avoids receptor-mediated degradation, thereby living longer in circulation. At physiological conditions, it is capable of reverting back to the native, biologically active hormone with the desired kinetics and glucose-lowering action. In the present study, we described the design, preparation, and use of (Fmoc)₂ insulin. This derivative is modified on the ε-amino group of lysine B29 and on the α-amino group of phenylalanine B1. It has 1–2% of the native receptor binding affinity and biological activity (Fig. 1, Table 1). After incubation at pH 7.4, it shows a slow and linear elevation in biological potency ($t_{1/2} = 12 \pm 1$ days) until it reaches a maximum that is almost equivalent to that of native insulin (Fig. 2). We have demonstrated that a single subcutaneous administration of (Fmoc)₂ insulin has a prolonged effect in STZ-treated diabetic rats (Fig. 3). Blood glucose levels were reduced to normal values over 2 days and continued to remain low on day 3 (Fig. 3). Neither native insulin nor (TNB)₂ insulin (which has a reduced receptor binding affinity, but does not revert to the native hormone) showed any prolonged effect under similar experimental conditions. Analysis of immunoreactive insulin levels in the serum of (Fmoc)₂ insulin-treated rats was found to correlate well with the capacity to lower blood glucose levels within this period. The STZ-treated diabetic rats given (Fmoc)₂ insulin gained $11 \text{ g} \cdot \text{rat}^{-1} \cdot \text{day}^{-1}$ over 3 days, a value close to the daily weight gain of healthy male Wistar rats of matched age and of STZ-treated diabetic rats receiving daily injections of insulin ($0.2 \text{ mg} \cdot \text{rat}^{-1} \cdot \text{day}^{-1}$; not shown). We have also confirmed that (Fmoc)₂ insulin has a prolonged action within the circulatory system itself by applying the derivative intraperitoneally (Fig. 4). In this experimental system, recovery from hypoglycemia proceeded with $t_{1/2} = 26 \pm 1$ h, as opposed to 8 ± 0.3 and 10 ± 0.4 h for native and NPH insulin, respectively. (Fmoc)₂ insulin was also found to be resistant to proteolysis as compared with the native hormone (Fig. 5). Finally, we evaluated the antigenicity of our derivative and found it to be less immunogenic than the native hormone (Fig. 6).

What is the general advantage of applying an inactive drug at the time of administration? Provided that the full structural identity and activity are subsequently restored, several advantages for administering a therapeutically inactive drug were a priori envisioned and or found in the course of this study. The advantage is that it enabled administration of a high dosage without the fear of toxicity, hypoglycemia, or desensitization. The prolonged action is maintained by applying a larger dosage that is gradually converted to the active state, while avoiding receptor-mediated endocytosis and subsequent degradation and being resistant to proteolysis.

Why was Fmoc selected as the conjugating moiety? Fmoc is widely used in organic and peptide synthesis for the reversible protection of amino groups (22,23). Its removal occurs under basic conditions. For example, in organic solvents it is cleaved-hydrolyzed within several minutes after the addition of piperidine. We envisioned that in aqueous buffers, at neutral and slightly alkaline pH, Fmoc proteins would be hydrolyzed at slow rates and in a homogeneous fashion. This was indeed the case, as is indicated by the results (Fig. 1, Table 1). (Fmoc)₂ insulin was hydrolyzed to the native hormone at a $t_{1/2} = \sim 1.0$ and 12 ± 1 days at pH 8.5 and 7.4, respectively (Fig. 1, Table 1) and was not hydrolyzed at all at pH 6.5 (not shown). With this pH profile of (Fmoc)₂ insulin

hydrolysis, we anticipated that this derivative would fulfill our expectations in vivo. Mammals maintain strict homeostasis and preserve a circulating pH of 7.4 (24); because the pH does not become elevated, there is no risk of accelerated conversion of (Fmoc)₂ insulin to the native hormone. Under the rare pathological conditions in which mild acidification does occur, circulating (Fmoc)₂ insulin would be hydrolyzed even more slowly.

In summary, we have presented here a novel concept for long-acting insulin—(Fmoc)₂ insulin—which is a derivative having ~2% biological and binding affinity. It undergoes homogenous and spontaneous activation at pH 7.4 and 37° C due to slow conversion of the derivative to the monomodified intermediates and to the native hormone. Monomodified insulin derivatives with bulky groups on phenylalanine B1 and lysine B29 were documented to preserve 30–70% of the biopotency of insulin (6). All three species therefore contribute to the glucose-lowering effect in vivo at any time point after administration. The excellent correlation in lowering glucose and circulating immunoreactive insulin levels found after (Fmoc)₂ insulin administration (Fig. 3) confirmed previous findings of close relationships between immunoreactivities and biological potencies of derivatized insulins (6). We discovered that (Fmoc)₂ insulin is not immunogenic. Finally, Fmoc amino acids were found to be nontoxic after chronic administration to rodents (25). Thus the Fmoc moieties also satisfy the criterion of having a low index of toxicity. We are currently investigating several aspects of this approach for the general application of this technology to drugs other than insulin.

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