Characterization of [Leu^{B-24}]- and [Leu^{B-25}]-insulin analogues

Receptor binding and biological activity

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Human [Leu^{B-24}]- and [Leu^{B-25}]-insulins were semi-synthesized from porcine insulin by an enzyme-assisted coupling method. The receptor-binding ability of [Leu^{B-24}]- and [Leu^{B-25}]-insulins was 30–48% and 2–5% respectively of that of human insulin. There was no significant difference in degradation between human insulin and these analogues on incubation with isolated adipocytes. The decreased affinity of these analogues was due to an increased dissociation rate rather than a change in the association rate of their binding to human cultured lymphocytes. The negative co-operative effect of [Leu^{B-24}]- and [Leu^{B-25}]-insulin was decreased to 50 and 1% respectively of that of human insulin at a concentration of 100 ng/ml. The ability of [Leu^{B-24}]- and [Leu^{B-25}]-insulin to stimulate 2-deoxyglucose uptake in isolated rat adipocytes was 35 and 4% respectively of that of human insulin. These analogues did not have an antagonistic effect on the biological activity of human insulin. The immunoreactivity of [Leu^{B-25}]-insulin was similar to that of porcine or human insulin, whereas [Leu^{B-24}]-insulin demonstrated decreased binding to anti-(porcine insulin) antibodies. These findings suggest that B-chain phenylalanine-25 residue is more crucial for receptor binding and negative co-operativity, whereas the B-chain phenylalanine-24 residue may play a more important role in binding to anti-insulin antibody.

The first step of insulin action is binding to its receptors on cell membranes. The receptor-binding region of the insulin molecule includes C-terminal amino acid residues, among which residues 24 and 25 of the B-chain are most important (Pullen *et al.*, 1976). The hydrophobic residues Phe^{B-24} and Phe^{B-25} seem to be buried in the receptor–insulin complex and their modification may affect their affinity for insulin receptors. The first case of a diabetic patient associated with a structurally abnormal insulin was fairly recently reported (Tager *et al.*, 1979). The serum of the patient contained both a normal and an abnormal insulin, which was shown to have a substitution of leucine for phenylalanine at the position 24 or 25 of the B-chain of the insulin molecule. Subsequently the abnormal mutant insulin was specifically separated from normal insulin and was demonstrated to have antagonistic properties towards the effects of native insulin on glucose metabolism in rat adipocytes (Olefsky *et al.*, 1980). Tager *et al.* (1979) found that porcine [Leu^{B-24}]-insulin, but not porcine [Leu^{B-25}]-insulin, demon-

strated an antagonistic effect and that the Leu^{B-24} and Leu^{B-25} analogues had only 9 and 1% respectively of the activity of native insulin in rat adipocytes (Tager *et al.*, 1980). They concluded that the mutant insulin found in the patient was [Leu^{B-24}]-insulin. In contrast with the findings of Tager and his associates (Tager *et al.*, 1979, 1980), Keefer *et al.* (1981) reported that neither human [Leu^{B-24}]- nor human [Leu^{B-25}]-insulin was an antagonist of the biological effects of native insulin.

We have reported that human [Leu^{B-24}]- and [Leu^{B-25}]-insulin demonstrated 20–30% and 1–2% respectively of the activity of native insulin in rat adipocytes (Inoue *et al.*, 1981a,b). To clarify the characteristics of these analogues, we examined the receptor-binding kinetics of these insulins and their biological activities under conditions where both native insulin and the analogues were present in the incubation media to test whether the analogues have an antagonistic effect. Furthermore, we examined the immunoreactivity of these analogues with anti-insulin antibodies, since it was reported that

guinea-pig anti-(porcine insulin) sera could not distinguish the mutant insulin from native insulins (Tager *et al.*, 1979).

We report here that these analogues did not show any antagonistic effects towards native insulin and that the Leu^{B-24} analogue demonstrated a decreased immunoreactivity with anti-(porcine insulin) antibody, whereas immunoreactivity of Leu^{B-25} analogue was similar to that of native insulin. Therefore the patient's mutant insulin would appear to be [Leu^{B-25}]insulin, rather than [Leu^{B-24}]insulin, on the basis of our studies of the biological activities and immunoreactivity of these analogues.

Materials and methods

Materials

Porcine insulin (lot 1FJ91, 26.2 units/mg) was kindly supplied by Eli Lilly and Co. Na¹²⁵I was purchased from New England Nuclear Corp., and culture medium RPMI 1640, and foetal-calf serum from Gibco. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co., collagenase from Worthington Biochemical Corp., 2-deoxy-[1-¹⁴C]glucose and L-[¹⁴C]glucose from New England Nuclear Corp.

Semi-synthesis of human [Leu^{B-24}]- and [Leu^{B-25}]-insulins

The method for the semi-synthesis of human insulin, by using trypsin-catalysed coupling of insulin (B-23–B-30)-desoctapeptide with a synthetic octapeptide corresponding to human insulin, was as previously reported (Inouye *et al.*, 1979). This technique was successfully applied to prepare human [Leu^{B-24}]- and [Leu^{B-25}]-insulin analogues. The resulting material was purified on a Sephadex LH-20 column, followed by Sephadex G-50 and QAE (quaternary aminoethyl)-Sephadex A-25 column chromatography. The semi-synthetic insulins thus obtained were found to be homogeneous on high-performance liquid chromatography.

Insulin-binding studies

Human cultured lymphocytes (RPMI 1788) were found to have insulin receptors on the plasma membranes (Kobayashi & Meek, 1977). These cells were maintained in the medium RPMI 1640 with 10% foetal-calf serum and were used for binding studies when they were in the stationary phase. Studies of ¹²⁵I-labelled insulin binding to lymphocytes were conducted in plastic tubes containing cells (10⁶/ml), labelled insulin (0.2 ng/ml) and various concentrations of unlabelled insulin in a buffer containing NaCl (135 mM), KCl (2.2 mM), CaCl₂ (2.5 mM), MgSO₄ (1.25 mM), Na₂HPO₄ (2.5 mM), KH₂PO₄ (2.5 mM), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] 10 mM and bovine serum albumin (1.0%), pH 7.6. After

90 min incubation at 15°C, separation of cells from medium was done in a Beckman microcentrifuge. Non-specific binding was determined by incubating cells and labelled insulin with 200 µg of unlabelled insulin/ml. All the data were corrected for non-specific binding (Kobayashi *et al.*, 1980a,b).

Dissociation studies

Cultured human lymphocytes were allowed to associate with ¹²⁵I-labelled insulin for 90 min at 24°C and the cells were centrifuged at 4°C and the buffer was removed and replaced with equal amount of iced insulin-free media. The cell pellet was disrupted and the cells were distributed in 100 µl portions into tubes containing 10 ml of buffer at 24°C, which resulted in a 100-times dilution of the cell suspension. After the indicated time for dissociation, the cells in these tubes were centrifuged at 200 g for 5 min and the buffer was removed and the radioactivity remaining in the cell pellet was determined. The same studies were carried out in the presence of 200 µg of unlabelled porcine insulin/ml and the radioactivity of the cell pellet was determined and was subtracted from the data.

Iodination of insulins

Human ¹²⁵I-labelled insulin, ¹²⁵I-labelled [Leu^{B-24}]-insulin and human ¹²⁵I-labelled [Leu^{B-25}]-insulin were prepared at a specific radioactivity of 100–150 µCi/µg by the method of Freychet *et al.* (1971).

Preparation of isolated adipocytes

Male Sprague–Dawley rats (160–200 g) were stunned by a blow to the head and decapitated, and epididymal fat-pads were removed. Isolated fat-cells were prepared by shaking at 37°C for 60 min in Krebs–Ringer bicarbonate buffer, containing collagenase (3 mg/ml) and bovine serum albumin (40 mg/ml), as described by Rodbell (1964). Cells were filtered through 250 µm-pore-size nylon mesh, centrifuged at 30 g for 4 min, and washed twice in buffer. Adipocyte counts were performed by the modified method III of Hirsch & Gallian (1968), in which the cells were fixed in 2% OsO₄ in 0.05 M-collidine buffer for 24 h at 37°C and then taken up in a known volume of 0.154 M-NaCl for counting with a Coulter counter (model Industrial D).

Glucose-uptake studies

The method used to study glucose uptake was as previously described (Kobayashi *et al.*, 1981). In brief, isolated adipocytes (2 × 10⁵ cells/ml) were incubated with 2-deoxy-D-[1-¹⁴C]glucose (specific radioactivity 2 mCi/mol) at a concentration of 0.125 mM in Krebs–Ringer bicarbonate, pH 7.4, containing bovine serum albumin (10 mg/ml) at 24°C. The assay was terminated at the end of 3 min

by transferring 200 μ l portions from the assay mixture to plastic microtubes containing 100 μ l of silicone oil with a relative density between those of the buffer and the adipocytes. The tubes were centrifuged for 30 s in a Beckman Microfuge, and the assay was considered terminated when centrifugation began. When the stimulatory effect of insulin on uptake was measured, the cells were preincubated with insulin for 60 min at 24°C. The amount of sugar trapped in the extracellular water space of the cell layers was determined with L-[¹⁴C]glucose. The extracellular water space was measured in each experiment and all glucose-uptake data are corrected for this factor.

Insulin binding to anti-insulin antibodies

Porcine [¹²⁵I]insulin and insulin analogues at various concentrations were incubated with sera for 48 h at 4°C and bound and unbound insulin were separated with poly(ethylene glycol) as described previously (Kobayashi *et al.*, 1981).

Results

Receptor-binding ability

Fig. 1 shows the ability of insulin analogues to compete with [¹²⁵I]-labelled insulin binding to the cells. Human [Leu^{B-24}]insulin and human [Leu^{B-25}]insulin demonstrated 30 and 2% respectively of the receptor-binding potency of human insulin. To determine the direct binding ability of these analogues, we labelled the insulin analogues and examined the ability of the labelled analogues to bind to cells. Fig. 2 shows binding isotherms of human [¹²⁵I]-labelled [Leu^{B-24}]- and [Leu^{B-25}]-insulins when labelled analogues and homologous unlabelled analogues were incubated with cultured human lymphocytes. The binding potencies of labelled [Leu^{B-24}]- and [Leu^{B-25}]-insulins were 48 and 5% respectively of that of normal human insulin. Insulin concentrations for 50% of maximal binding were 4.6 ng/ml for normal human insulin, 8.5 ng/ml for human [Leu^{B-24}]insulin and 10.7 ng/ml for human [Leu^{B-25}]insulin, indicating decreased affinity in these analogues. To examine the affinity of these analogues more clearly, Scatchard analysis was performed on the data of Fig. 2(a). The slopes of the curves of analogues were decreased compared with that of normal human insulin appreciate real differences in the curvature of Scatchard plots, the data of Fig. 2(b) were plotted with the initial bound/free ratio normalized to 100%. As shown in Fig. 2(c), the line of [Leu^{B-25}]insulin was less curved, but not straight, compared with those of normal and [Leu^{B-24}]insulin. The average affinity shown by the data of Fig. 2(a) was calculated by the method of De Meyts & Roth (1975), and Fig. 2(d) shows a decreased K_e in both [Leu^{B-24}]insulin

Fig. 1. Ability of human insulin and analogues to compete with [¹²⁵I]-labelled porcine insulin for binding to cultured human lymphocytes (RPMI 1788)

[¹²⁵I]-labelled porcine insulin and indicated concentrations of unlabelled human insulin (●), [Leu^{B-24}]- (○) and [Leu^{B-25}]-insulin (▲) were incubated with cultured human lymphocytes (RPMI 1788) at 15°C for 90 min. The maximal percentage bound was defined as 100. Results are means for three separate experiments.

($1.48 \times 10^9 \text{ M}^{-1}$) and [Leu^{B-25}]insulin ($0.18 \times 10^9 \text{ M}^{-1}$) compared with that of normal human insulin ($2.62 \times 10^9 \text{ M}^{-1}$).

The degradation of the Leu^{B-24} and the Leu^{B-25} analogues, which was assessed by trichloroacetic acid precipitation, was $10.1 \pm 1.0\%$ (mean $\pm$ S.E.M.) and $8.4 \pm 1.2\%$ respectively during incubation with adipocytes, and these values were not significantly different from that of human insulin ($10.4 \pm 1.2\%$).

Decreased affinity (K_a) may be due to decreased association rate (k_a) or increased dissociation rate (k_d). Fig. 3 shows that the association rate of the insulin analogues and the time to reach 50% maximal insulin binding is essentially comparable among the three insulins (7 min for normal human insulin, 8 min for human [Leu^{B-24}]insulin and 7 min for human [Leu^{B-25}]insulin). Thus the association rates are not essentially different among analogues. Fig. 4 shows dissociation of the bound analogues from the cultured human lymphocytes. Dissociation rates (k_d) of human [Leu^{B-24}]- and [Leu^{B-25}]-insulins and normal human insulin were estimated by calculating the time required for a 50% decrease in binding during dilution and they were 26, 13 and 4 min for human normal, [Leu^{B-24}]- and [Leu^{B-25}]-insulins respectively. Thus the decreased affinity of these analogues is due to increased dissociation rate rather than decreased association rate.

Negative-co-operative effects of analogues

We examined the ability of these analogues to accelerate the dissociation of bound insulin from receptor sites, i.e. a negative co-operative effect (De Meyts *et al.*, 1973). Fig. 5 shows the negative-co-operative effect of analogues at a concentration

Fig. 2. Ability of ^{125}I -labelled insulin and analogues to bind to cultured human lymphocytes

Cells were incubated with 0.2 ng of labelled human insulin (●), labelled [Leu^{B-24}]-insulin (○) or [Leu^{B-25}]-insulin (▲), and unlabelled homologous analogues at the indicated concentrations; the subsequent binding

of 100 ng/ml, which produces almost maximal effects with normal human insulin. The negative-co-operative effect of [Leu^{B-24}]- and [Leu^{B-25}]-insulin was markedly decreased to 50 and 1% respectively of that of human insulin, and no co-operative effect was demonstrated by insulin desoctapeptide, which actually slightly slowed down the dissociation rate. However, when greater amounts of [Leu^{B-24}]- and [Leu^{B-25}]-insulins were added in the media, the negative-co-operative effect of these analogues increased, but they never reached the maximal level of that of normal human insulin. Once the negative-co-operative effect of the insulins reached the maximal level, it started decreasing as the insulin concentrations increased (Fig. 6).

Biological activity of the analogues

The biological activities of these analogues were determined by measuring their ability to stimulate glucose transport in isolated rat adipocytes. As shown in Fig. 7, the biological activities of the Leu^{B-24} and Leu^{B-25} analogues were 35 and 4% of that of human insulin. To test whether these analogues act as antagonists, both these analogues and human insulin were simultaneously incubated with adipocytes and then glucose uptake was measured in these cells. As shown in Table 1, neither the Leu^{B-24} nor the Leu^{B-25} analogue inhibited glucose uptake stimulated by human insulin. Furthermore, in the range of physiological concentration, human insulin demonstrated a full activity even in the presence of Leu^{B-24} or Leu^{B-25} analogues.

Immunoreactivity of analogues

Guinea-pig anti-(porcine insulin) antiserum was used for testing the immunoreactivity of these analogues. Porcine, human and [Leu^{B-25}]-insulin behaved similarly in binding to anti-(porcine insulin) antibody, whereas human [Leu^{B-24}]-insulin reacted

procedures are described in the Materials and methods section. Results are means from three separate experiments. (a) Binding isotherms of labelled analogues. (b) Scatchard plot of the data of (a). (c) Modified Scatchard plot of (b). The data of (b) were plotted with the initial B/F (bound/free ratio) normalized to 100%. (d) Average affinity profile of the data of (b). Average affinity (K) equals $[(B/F)/(R_0 - B)]$ (R_0 is the total receptor concentration) and is plotted as a function of fractional receptor occupancy (Y) (De Meyts & Roth, 1975). The highest observable K is designated K_e (empty-site affinity).

Fig. 3. Time course of labelled-analogue binding to cultured human lymphocytes

Labelled human insulin (●), [Leu^{B-24}]- (○) and [Leu^{B-25}]-insulin (▲) were incubated with lymphocytes at 24°C and the percentage binding to the cells was determined at the indicated time. Non-specific binding was subtracted from the binding data.

Fig. 5. Ability of analogues to accelerate dissociation of bound ¹²⁵I-labelled porcine insulin

Dissociation studies were performed in a similar manner to those described in Fig. 4 except that the dilution media contained no insulin (●), human insulin (100 ng/ml) (□), insulin desoctapeptide (5 μg/ml) (■), [Leu^{B-24}]insulin (100 ng/ml) (△) and [Leu^{B-25}]insulin (100 ng/ml) (▲).

Fig. 4. Dissociation of bound labelled analogues from cultured human lymphocytes in insulin-free media

Cells were allowed to associate with ¹²⁵I-labelled human insulin (●), [Leu^{B-24}]- (○) and [Leu^{B-25}]-insulin (▲) for 90 min at 24°C and then the bound labelled analogues were allowed to dissociate from the cells into insulin-free buffer at 24°C as described in the Materials and methods section. The percentage bound before dissociation started was defined as 100. Results are means for three separate experiments.

Fig. 6. Negative-co-operative effects of the analogues at various concentrations

The dissociation was allowed to proceed for 30 min as described in Fig. 5 and then the amount of radioactivity bound was measured. The data are plotted as the radioactivity dissociated in the presence of the indicated concentrations of analogues, minus the dissociation in the absence of the analogues (as a percentage of the maximal effect) as a function of the analogue concentration. ●, Human insulin; ○, [Leu^{B-24}]insulin; ▲, [Leu^{B-25}]insulin; △, insulin desoctapeptide.

less with the antibody (Fig. 8). Two other different batches of guinea-pig anti-porcine sera also demonstrated similar results (results not shown).

Fig. 7. Ability of insulin analogues to stimulate 2-deoxyglucose uptake in isolated rat adipocytes. Cells were preincubated with human insulin (●), [Leu^{B-24}]insulin (○) and [Leu^{B-25}]insulin (▲) at the indicated concentration for 60 min at 24°C and then 2-deoxy-D-[1-¹⁴C]glucose uptake was measured as described in the Materials and methods section. The values were means for three separate experiments.

Table 1. Ability of human [Leu^{B-24}]- and [Leu^{B-25}]-insulin to increase glucose uptake in rat adipocytes

The values of 2-deoxyglucose uptake are means $\pm$ S.E.M. for five separate experiments. There was no statistical difference between the values of human insulin only and those of the total of human insulin and insulin analogues in each concentration group.

Insulin or analogue	Concn. (ng/ml)	2-Deoxyglucose uptake (nmol/3 min per 2×10^5 cells)
Human insulin	0.3	0.298 ± 0.018
+ [Leu ^{B-24}]insulin	0.3	0.308 ± 0.010
+ [Leu ^{B-25}]insulin	0.3	0.296 ± 0.014
Human insulin	1.0	0.436 ± 0.030
+ [Leu ^{B-24}]insulin	1.0	0.440 ± 0.033
+ [Leu ^{B-25}]insulin	1.0	0.442 ± 0.028
Human insulin	5.0	0.482 ± 0.021
+ [Leu ^{B-24}]insulin	5.0	0.480 ± 0.034
+ [Leu ^{B-25}]insulin	5.0	0.478 ± 0.028

Discussion

The C-terminal region of the insulin molecule is important for receptor binding (Pullen *et al.*, 1976), and modification of this portion leads to decreased biological activity, as shown in the present study. The B-chain residue-24 and -25 positions of insulin are occupied by phenylalanine, which forms a hydrophobic patch that seems to be important for receptor binding. Thus substitution of these amino acid residues by alanine leads to decreased receptor binding as we reported previously (Inouye *et al.*, 1981b). Leucine substitution also causes decreased receptor binding, and substitution of B-chain residue-25 with either alanine or leucine resulted in

Fig. 8. Immunoreactivity of insulin analogues. ¹²⁵I-labelled porcine insulin and indicated concentrations of unlabelled human insulin (●), porcine insulin (■), [Leu^{B-24}]insulin (○) and [Leu^{B-25}]insulin (▲) were incubated with guinea-pig anti-(porcine insulin) sera for 48 h at 4°C. The bound and unbound insulins were separated by the poly(ethylene glycol) method described in the Materials and methods section.

further deterioration in binding ability, as shown in the present and previous studies (Inouye *et al.*, 1981a,b). Tager *et al.* (1980) reported that the Leu^{B-24} and Leu^{B-25} analogues had about 10 and 1% respectively of the activity of porcine insulin in receptor binding. Keefer *et al.* (1981) also reported similar values: 8–12% and 0.9–1.1% for the Leu^{B-24} and Leu^{B-25} analogues in receptor-binding activity. These values are lower than those for our analogues and these differences may be due partially to the use of non-homogeneously ¹²⁵I-labelled preparations.

The receptor-binding affinities of Leu^{B-24} and Leu^{B-25} analogues (K_d) were decreased to 56 and 7% respectively of that of normal insulin when these values were calculated by the method of De Meyts & Roth (1975). Kinetic studies suggest that increased dissociation rates seem to contribute to the decreased affinity in these analogues.

In accord with the results of Keefer *et al.* (1981), the substitution of the B-chain residue-25 leads to a more striking impairment in the negative-co-operative effect, suggesting a crucial role of the B-chain phenylalanine-25 in negative co-operativity as well

as in receptor binding. However, the Leu^{B-25} analogue demonstrated a very low, but still significant, negative-co-operative effect at high concentrations. Thus other amino acid residues of the insulin molecule, such as B-chain residue-26 or others, may also contribute to the negative-co-operative effect, since the insulin desoctapeptide does not show any negative-co-operative effect, as shown in Fig. 7. All these data confirm that the C-terminal region of the insulin molecule is important for negative co-operativity (De Meyts *et al.*, 1978). Decreased negative co-operativity at high insulin concentration was accounted for by dimerization of insulin molecules (De Meyts *et al.*, 1978). Studies of circular dichroism and dimerization showed decreased dimerization in [Leu^{B-24}]insulin and almost normal findings for [Leu^{B-25}]insulin (Inouye *et al.*, 1981b). Therefore, decreased negative co-operativity at high [Leu^{B-24}]insulin concentration seems not to be related to dimerization.

The biological activities of these analogues are proportional to receptor-binding activity. Both the Leu^{B-24} and Leu^{B-25} analogues have full activity when these insulins were present in sufficient amount. Tager *et al.* (1980) reported that an antagonistic effect was demonstrated with the Leu^{B-24} analogue, i.e. the Leu^{B-24} analogue inhibited the activity of native insulin. We could not confirm this antagonistic activity and found only that the activity of normal insulin and of the analogue were not strictly additive, but the activity of normal human insulin was not actually inhibited in the presence of the analogue. Keefer *et al.* (1981) also found no antagonistic effect in these analogues and only showed a cumulative effect of both analogues in stimulating lipogenesis in isolated adipocytes (Keefer *et al.*, 1981). The reasons for the discrepancies among these studies are not known and may be explained by minor structural differences between these analogues or their degradation during incubation with adipocytes. In our assay, no difference was observed in the degradation of the analogues during incubation.

The immunoreactivities of these analogues are different and the Leu^{B-24} analogue bound less to anti-insulin antibodies. In contrast, the B-chain residue-25 substitution did not cause any change in immunoreactivity compared with porcine or human insulin. Thus the determinant for immunoreactivity in the C-terminal region may include B-chain residue-30 and -24, but not residue-25 (Kobayashi *et al.*, 1981).

The structurally abnormal mutant insulin found in the patient was reported to have less than 10% of normal insulin activity (Olefsky *et al.*, 1980) and could not be distinguished from human insulin by immunoassay (Tager *et al.*, 1979). Therefore, from the present results, the Leu^{B-25} analogue rather than

the Leu^{B-24} analogue seems, on the basis of biological and immunological properties, to be the insulin found in the patient. Gene technology to produce the mutant insulin from the patient's DNA seems to be necessary to clarify whether B-chain residue-24 or -25 is the site of substitution with leucine in this patient.

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