Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 329-336

SUPERNORMAL INSULIN: [D-Phe^{B24}]-INSULIN WITH INCREASED AFFINITY FOR INSULIN RECEPTORS.

Masashi Kobayashi, Seiji Ohgaku, Makoto Iwasaki, Hiroshi Maegawa, Yukio Shigeta and Ken Inouye*.

The Third Department of Medicine, Shiga University of Medical Science, Ohtsu and Shionogi Research laboratories*, Osaka, Japan.

Received June 7, 1982

[D-Phe^{B24}]- and [D-Phe^{B25}]-human insulin were semisynthesized from porcine insulin by enzyme assisted coupling method. Receptor binding ability of [D-Phe^{B24}]- and [D-Phe^{B25}]-insulin was 180% and 4%, respectively, of that of human insulin. Increased affinity of [D-Phe^{B24}]-insulin was ascribed to markedly decreased dissociation rate in binding to human cultured lymphocytes. Negative cooperative effect of [D-Phe^{B24}]insulin was also increased to twice of that of human insulin. Biological activity of these analogues was assessed by 2-deoxy-glucose uptake studies in isolated adipocytes and the ability of [D-Phe^{B24}]- and [D-Phe^{B25}]-insulin was 140% and 4%, respectively, of that of human insulin. These findings suggest that B25 L-Phe is more crucial for receptor binding and that [D-Phe^{B24}]-insulin is the first semisynthetic insulin to show increased affinity for insulin receptors.

The first step of insulin action is binding to insulin receptors on plasma membranes. Thus, affinity of insulin for receptors, in most cases, determines biological activity of the insulin. For example, chicken insulin with increased affinity for receptors demonstrate increased biological activities(1, 2). Receptor binding region includes carboxyterminal amino acid residues, among of which B24 and B25 are most important and are not varied among insulins of various animals(3). Thus, modification of this region resulted in decreased affinity for receptors as we found that substitution of B24 L-Phe to Leu or Ala led to 30% of potency of native insulin in receptor binding and biological activity(4). These amino acid residues also important for negative cooperativity which is a unique phenomenon for insulin receptors(5). Therefore, insulin analogues with substitution at B24 and B25 with Leu resulted in decreased negative cooperativity(6).

Thus far, no sythetic insulin with increased affinity for receptors has been found. Since modification of B24 or B25 of insulin molecule seems to influence

0006-291X/82/130329-08$01.00/0

receptor affinity, it seemed to be of special interest to examine the effect of substitution of Phe-B24 or Phe-B25 by D-Phe on the action of insulin.

We now report that substitution of B24 L-Phe with stereoisomer D-Phe led to increased affinity for insulin receptors and biological activity. This is the first synthetic insulin with increased affinity for insulin receptors by modification of B-chain carboxyterminal region.

MATERIALS AND METHODS

Semisynthesis of analogues: The method of semisynthesis of human insulin using trypsin catalyzed coupling of desoctapeptide-(B23-B30)-insulin with a synthetic octapeptide corresponding of human insulin was previously reported(7). This technique was successfully applied to prepare (D-Phe^{B24})- and (D-Phe^{B25})-human insulin analogues, although the yields of enzymatic peptide bond synthesis were much lower than usual because of the presenece of the D-amino acid residues in the vicinity of the bond to be formed. The resulting material was purified by Sephadex LH-20 column, Sephadex G-50 and QAE-Sephadex A-25 column chromatography. The semisynthtic insulins thus obtained were found to be homogeneous in high performance liquid chromatography.

Insulin binding studies: Human cultured lymphocytes (RPMI 1788) were known to have abundant insulin receptors on their cell membranes (8). Studies of labelled insulin binding to lymphocytes were conducted in plastic tubes containing cells, labelled insulin and varying concentration of unlabelled insulin in the buffer containing NaCl 135 mM, KCl 2.2 mM. $CaCl_2$ 2.5 mM, $MgSO_4$ 1.25 mM, $NaHPO_4$ 2.5 mM, KH_2PO_4 2.5 mM, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 mM and bovine serum albumin 1.0%, pH 7.6. After 90 min incubation at 15°C, separation of cells from media was done by Beckman microcentrifuge. Non-specific binding was determined by incubating cells with 200 µg/ml of insulin and labelled insulin. All the data were corrected for non-specific binding.

For dissociation studies, cultured human lymphocytes were allowed to associate with 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 aliquots into tubes containing 10 ml of buffer at 24° C, which resulted in 100 times dilution of the cell suspension. After indicated time for dissociation, the cells in these tubes were centrifuged at 200g for 5 min and the buffer was removed and the radioactivity remaining in the cell pellet was determined. The same sutdies were carried out in the presence of 200 µg/ml of unlabelled insulin and the radioactivity of the cell pellet was determined and was subtracted from the each data.

Glucose Uptake Studies: The method of glucose uptake studies was previously described (9). In brief, isolated adipocytes were preincubated with insulin analogues for 60 min at 24° C and then incubated with 2-deoxy-[1-^{14}C]-D-glucose (specific activity 2 mCi/mM) at a concentration of 0.1 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 µl aliquots from the assay mixture to plastic microtubes containing 100 µl silicone oil with specific gravity between those of the buffer and adipocytes. The tubes were centrifuged for 30 s in a Beckman microfuge, and the assay was considered terminated when centrifugation begun. The amount of sugar trapped in the extracellular water space was measured in each experiment and all data of glucose uptake were corrected for this factor.

Iodination of insulin: [^{125}I]-human insulin, -[D-Phe^{B24}]-human insulin and -[D-Phe^{B25}]-human insulin were prepared at a specific activity of 100-150 µCi/µg according to the method of Freychet et al (10).

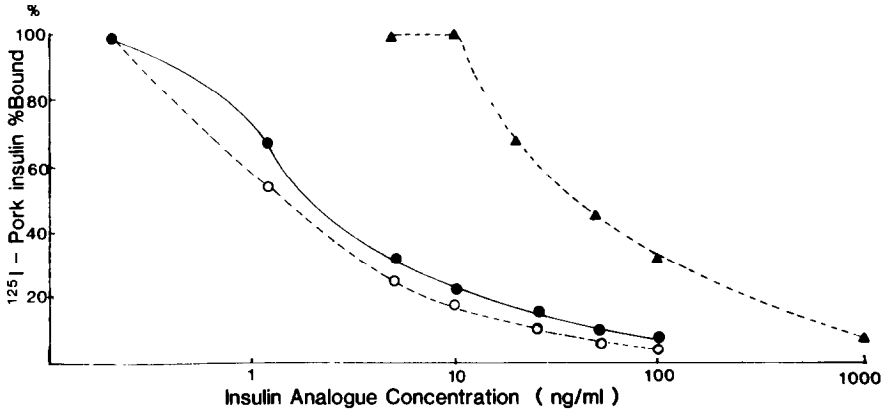
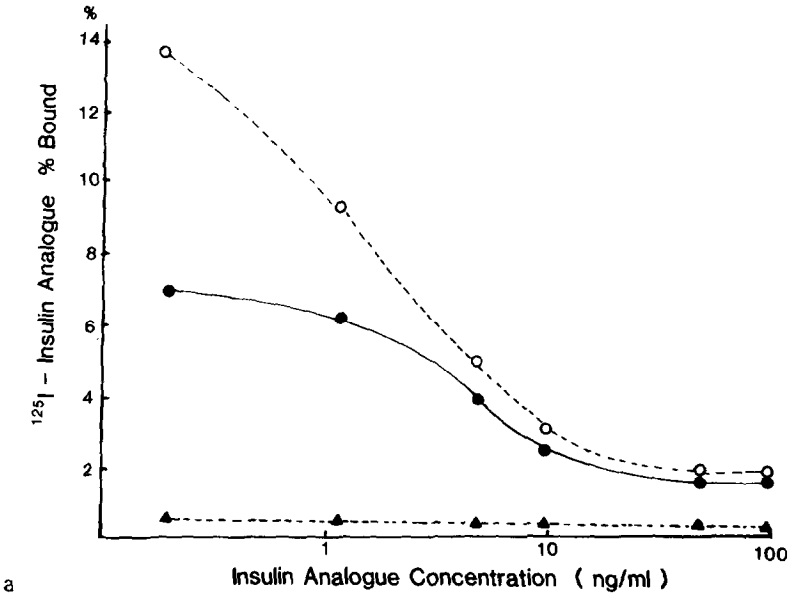


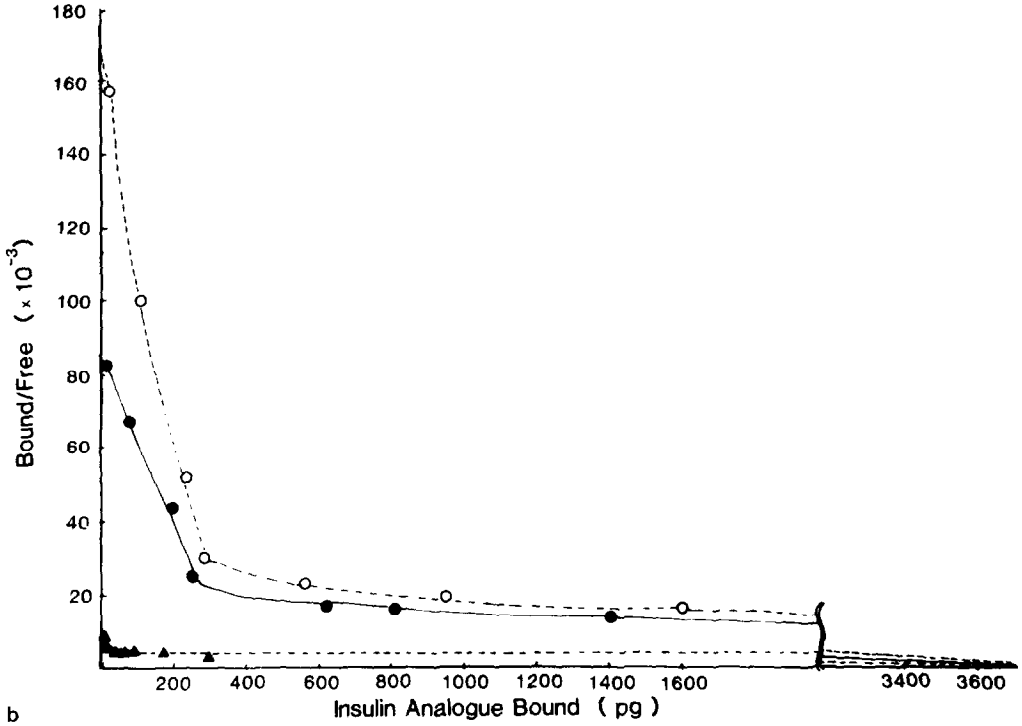
Figure 1.. Ability of insulin analogues to displace [^{125}I]-human insulin from cultured human lymphocytes (RPMI 1788). Cells were incubated with [^{125}I]-pork insulin, and unlabelled human insulin (●), [D-Phe^{B24}]-human insulin (○) or [D-Phe^{B25}]-human insulin (▲) at the indicated concentrations for 120 min at 15 C. Non-specific binding was subtracted from each data. Data represent mean of three separate experiments.

RESULTS

First, we examined the ability of these analogues to compete with [^{125}I]-pork insulin for insulin receptors on cultured human lymphocytes (RPMI 1788). As shown in Figure 1, the ability of [D-Phe^{B24}]-and [D-Phe^{B25}]-human insulin to bind to receptors was estimated to be 180% and 4%, respectively, of that of human insulin. Next, we labelled these analogues with [^{125}I] in order to directly investigate affinity of these analogues for receptors and kinetics of receptor binding. Labelled analogues demonstrated similar results of Fig. 1 and the Scatchard analysis revealed increased affinity at low insulin receptor occupancy in [^{125}I][D-Phe^{B24}]-human insulin binding (Fig. 2). To further characterize this increased affinity of [D-Phe^{B24}]-human insulin, we performed kinetic studies on receptor binding to cultured human lymphocytes, in which human insulin and the [D-Phe^{B-24}]-analogue showed essentially the same association rate and the time required to reach 50% of maximal binding was 6 and 7 min ,respectively, at 24°C. The dissociation rate of the bound labelled analogue was estimated by dilution method. The results are shown in Figure 3, in which a marked decrease in the rate was observed with [D-Phe^{B24}]-insulin. The time required for [D-Phe^{B24}]-insulin and normal human insulin to reach a 50% decrease in bound radioactivity during dilution was 13 min and 61 min respectively. These results clearly indicate that



a



b

Figure 2. a. Ability of labelled insulin analogues to bind to cultured lymphocytes (RPMI 1788). Cells were incubated with [^{125}I] labelled human insulin (●), [D-Phe^{B24}]- (○) or [D-Phe^{B25}]-human insulin (▲), and various concentrations of homologous insulin analogues. Figure 2.b. Scatchard analysis of Fig. 2.a.

the increased affinity of [D-Phe^{B24}]-human insulin is due to a remarkable decrease in the dissociation rate rather than a certain change in the association rate. Another unique effect of insulin, i.e. negative cooperative effect was determined

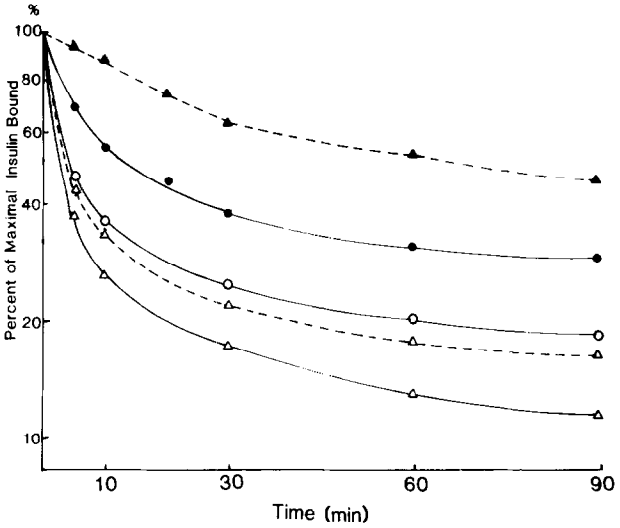


Figure 3. Dissociation of the bound labelled analogue and negative cooperative effect of the analogue. Cultured human lymphocytes were allowed to associate with [^{125}I]-human insulin (●) or [^{125}I]-[D-Phe^{B24}]-human insulin (▲) for 90 min at 24°C and the cells were centrifuged at 4°C and the buffer 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 aliquots into tubes containing 10 ml of buffer at 24°C, which resulted in 100 times dilution of the cell suspension. After indicated time for dissociation, the cells in these tubes were centrifuged 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/ml of unlabelled porcine insulin in the association phase and the radioactivity of the cell pellet was determined. This value was subtracted from each data. To determine negative cooperative effect of analogues, cells with bound [^{125}I] labelled human insulin (solid line) or labelled analogue (broken line) were transferred into dissociation media where 100 ng/ml of human insulin (○), or 100 ng/ml of [D-Phe^{B24}]-human insulin (△) was present.

by estimating the ability of the analogue to accelerate dissociation of bound insulin from the cells. The time required for [^{125}I]-human insulin to dissociate from the cells to the level of 50% of the maximal binding in the presence of [D-Phe^{B24}]-human insulin (100 ng/ml) was approximately half of that in the presence of human insulin (100 ng/ml). Furthermore, homologous insulin to elicit negative cooperative effect was also greater in [D-Phe^{B24}]-human insulin (Fig. 3).

Biological effect of these analogues was examined by glucose uptake studies in isolated rat adipocytes. Since these cells possess spare receptors for insulin action and only 10% of receptor occupancy is necessary for stimulating activity of glucose uptake, difference of insulin potency appears only at low insulin concentrations, i.e. less than 5 ng/ml. As shown in Figure 4, significantly increased activity of [D-Phe^{B24}]-human insulin was demonstrated at the concentration of 0.3 ng/ml and 1.0 ng/ml, but the maximal effect of the analogue was

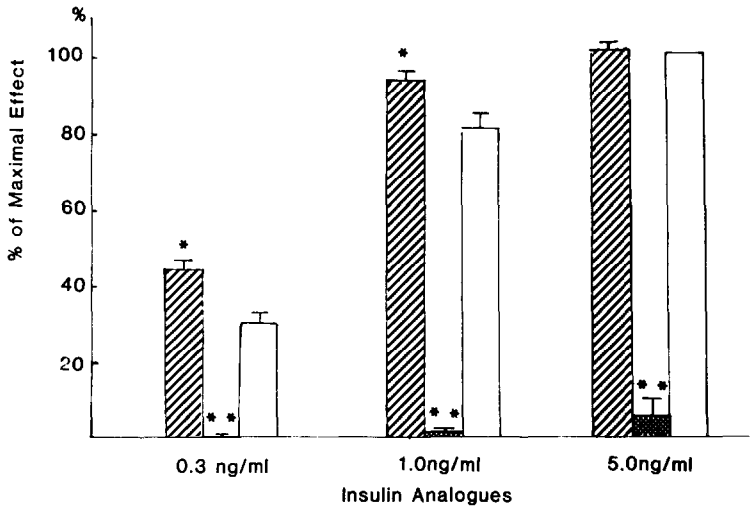


Figure 4. Ability of insulin analogues to stimulate 2-deoxy-glucose uptake in isolated rat adipocytes. The method of glucose uptake studies is described in Methods. ☐ :human insulin, ▨ :[D-Phe^{B24}]-human insulin, ▩ :[D-Phe^{B25}]-human insulin. *p<0.05, **p<0.01 vs human insulin.

similar to that of human insulin. The biological potency of [D-Phe^{B24}]-human insulin was estimated to be 140% of that of normal. In contrast, [D-Phe^{B25}]-human insulin demonstrated only 4% of control. Degradation of these analogues during incubation with adipocytes was assessed by precipitation with 10% trichloroacetic acid. After 90 min incubation with cells at 24°C, percent degradation of labelled [D-Phe^{B24}]-, [D-Phe^{B25}]- and human insulin was 10.2 ± 0.9%, 4.8 ± 0.3% and 8.7 ± 0.4% (mean±SEM), respectively, indicating that [D-Phe^{B24}]-human insulin demonstrated significantly increased degradation compared to human insulin ($p < 0.05$).

DISCUSSION

The present study clearly indicates that [D-Phe^{B24}]-human insulin is the first semisynthetic insulin to demonstrated increased affinity for insulin receptors and biological activity. B-chain 24 and 25 amino acid residues have been demonstrated to play an important role in receptor binding as we previously reported that Leu or Ala substitution resulted in decreased affinity (4, 11). Furthermore, it has been shown that B25 was more crucial than B24 for receptor binding (4,6,11). Substitution of B25 with stereoisomer led to a marked decreased affinity whereas B24 substituion elevated affinity for receptors as shown in this report and these

findings support our previous results. Thus, B25 may be a key amino acid residue for receptor binding whereas B24 may be a site to influence affinity in binding to receptors rather than a decisive center for receptor binding region. Production of synthetic insulin with increased affinity for receptors by modification of carboxyterminal region has not been reported. Among native insulins, chicken or turkey insulin have been shown to demonstrate increased affinity for insulin receptors. However, these insulins possess similar amino acid residues (Phe) at B24 and B25. Since substitution of B24 Phe with aliphatic amino acids (Leu or Ala) led to decreased affinity for insulin receptors and substitution with D-Phe at B24 led to increased affinity, hydrophobic amino acid at this region appeared to be necessary for receptor binding. Kinetic studies of [D-Phe^{B24}]-human insulin binding suggested that the markedly decreased dissociation rate appeared to account for increased affinity. Thus, change of direction of phenylalanine residue with stereoisomer at B24 made insulin molecule more firmly bound to receptors.

Degradation of [D-Phe^{B24}]-human insulin was increased and this was probably ascribed to increased receptor mediated degradation, although more detailed study is necessary to prove this speculation.

Negative cooperative effect of [D-Phe^{B24}]-human insulin was also increased to twice of that of native insulin and was well correlated with increased affinity for receptors in a general agreement of theory of DeMeyts (5).

Although biological activity of [D-Phe^{B24}]-human insulin was also increased, there was a discrepancy between receptor binding (180% of normal) and biological activity (140% of normal). The relative potency of these analogues may be apparent than real and this discrepancy may be due to differences in experimental conditions of binding assay (at 15°C) and sugar uptake studies (at 24°C).

Further studies to perform the analogue receptor binding and biological assays under the same condition and to investigate conformational structure of carboxy-terminal region of this analogue are under way.

In summary, with modification of receptor binding region of insulin molecule, this is the first analogue to show increased affinity for insulin receptors and

increased biological activity. This analogue appears to be useful for further study of insulin molecular structure-action relationship.

REFERENCES

1. Simon, J., Freychet, P., and Rosselin, G. (1974) Endocrinology 95,1439-1449.
2. Simon, J., Freychet, P., Rosselin, G. and DeMeyts, P. (1977) Endocrinology 100, 115-121.
3. Pullen, R.A., Lindsay, D.G., Wood, S.P., Tickle, I.J., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Glieman, J. and Gammeltoft, J. (1976) Nature 259, 369-373.
4. Inouye, K., Watanabe, K., Tochino, Y., Kobayashi, M., and Shigeta, Y. (1981) Biopolymers 20, 1845-1858.
5. DeMeyts, P., VanObberghen, E., Roth, J., Wollmer, A. and Brandenburg, D. (1978) Nature 273, 504-509.
6. Keefer, L.M., Piron, M., DeMeyts, P., Gattmer, H., Diaconescu, C., Saunders, D. and Brandenburg, D. (1981) Biochem. Biophys. Res. Commun. 100, 1229-1236.
7. Inouye, K., Watanabe, K., Morihara, K., Tochino, Y., Kanaya, T., Emura, J., and Sakakibara, S. J.Am. Chem. Soc. 101, 751-752.
8. Kobayashi, M. and Meek, J.C. (1977) Diabetologia 13, 251-255.
9. Kobayashi, M., Mondon, C., and Oyama, J. (1980) Am. J. Physiol. 238, E330-335.
10. Freychet, P., Roth, J., and Neville, D.M.Jr. (1971) Biochem, Biopys. Res. Commun. 43, 400-408.
11. Inouye, K., Watanabe, K., Morihara, K., Tochino, Y., Kanaya, T., Kobayashi, M., and Shigeta, Y. (1981) Experientia 37, 811-813.