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RECEPTOR BINDING AND BIOLOGICAL ACTIVITY OF [Ser^{B24}]-INSULIN, AN ABNORMAL MUTANT INSULIN

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[Ser^{B24}]-insulin, the second structurally abnormal mutant insulin, and [Ser^{B25}]-insulin were semisynthesized and were studied for receptor binding and biological activity. Receptor binding and biological activity determined by its ability to increase 2-deoxy-glucose uptake in rat adipocytes were 0.7-3% of native insulin for [Ser^{B24}]-insulin and 3-8% for [Ser^{B25}]-insulin. Negative cooperative effect of these analogues was also markedly decreased. Immunoreactivity of [Ser^{B24}]-insulin was decreased whereas that of [Ser^{B25}]-insulin was normal. Markedly decreased receptor binding of [Ser^{B24}]-insulin appeared to be due to substitution of hydrophobic amino acid, Phe, with a polar amino acid, Ser, at B24.

The first structurally abnormal mutant insulin found in the diabetic patient was first suggested to be [Leu^{B24}]-insulin by Tager et al on the basis of its antagonistic effect(1). On the contrary, we suggested [Leu^{B25}]-insulin was the mutant insulin because of its biological effect and immunoreactivity(2). Finally it was proved to be [Leu^{B25}]-insulin by HPLC(3) and by analysis of patient's insulin gene(4). This insulin, [Leu^{B25}]insulin, has only 2-5% of normal insulin in receptor binding and biological activity (2). The second case of a diabetic patients with an abnormal insulin was recently demonstrated to be [Ser^{B24}]-insulin by gene technology (5). Since B-24 is also the site for receptor binding (6), the abnormal insulin appeared to demonstrate decreased receptor binding ability, which may contribute to the pathogenesis of diabetes in the patient. We have reported that B-24 is a unique site since substitution of B24 L-Phe to a stereoisomer D-Phe led to a supernormal insulin with increased affinity for insulin receptors (7) and that substitution with Leu

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led to 40% of normal in biological activity(2). Thus, substitution with these hydrophobic amino acids at B24 maintained a relatively high biological acivity. Because serine is a polar amino acid, it is of interest to see the effect of substitution with serine at B24 on the receptor binding. We have semisynthesized [Ser^{B24}]-insulin by enzyme assisted coupling method (8) and characterized receptor binding and biological activity of the synthesized structurally abnormal mutant insulin.

Materials and methods

Materials:Porcine insulin (lot lFJ91, 26.2 units/mg) was kindly supplied by Eli Lilly and Co. $Na^{125}I$ 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-^{14}C]glucose and L-[^{14}C]glucose from New England Nuclear Corp.
Semisynthesis of human [Ser^{B24}]- and [Ser^{B25}]-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 (8). The semi-synthetic insulins thus obtained were found to be homogeneous on high-performance liquid chromatography.
Insulin binding studies:Human cultured lymphocytes (IM 9) 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. The methods of insulin binding studies, as well as dissociation studies, were previously described (2,7).
Iodination of insulins:Porcine I^{125}-labelled insulin, ^{125}I-labelled analogues were prepared at a specific radioactivity of 100-150 μCi/μg by the method of Freychet et al (9).
Glucose uptake studies:The method used to study glucose uptake in isolated rat adipocytes was as previously described (10).
Insulin binding to anti-insulin antibodies:Porcine [^{125}I]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 (11).

Results

Fig. 1.a. shows the ability of [Ser^{B24}]- and [Ser^{B25}]-insulin to compete with ^{125}I-labelled insulin for insulin receptor binding. Human [Ser^{B24}]-insulin and human [Ser^{B25}]-insulin demonstrated 0.7-3% and 3-8% respectively of the receptor binding potency of human insulin. We labelled the insulin analogues and examined the ability of the labelled analogues to bind to cells. Fig. 1.b. shows binding isotherms of human ^{125}I-labelled [Ser^{B24}]- and [Ser^{B25}]-insulin. However, because the both analogues showed extremely low binding, it was impossible to characterize the binding curves by Scatchard analysis. The degradation of labelled [Ser^{B24}]- and [Ser^{B25}]-insulin during incubation

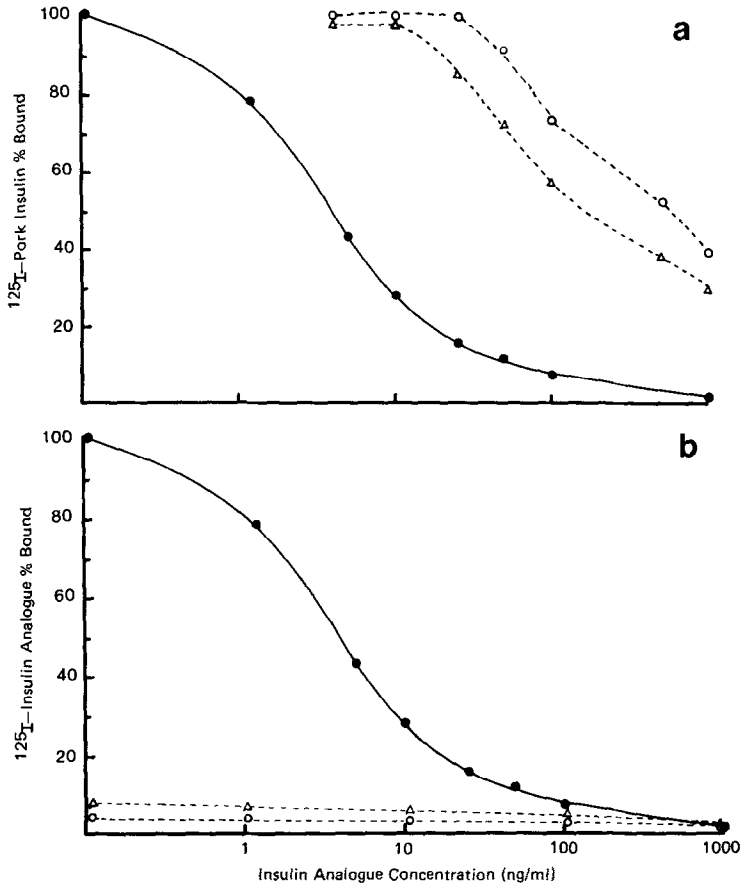

Figure 1.a. Ability of insulin analogues to displace [^{125}I]-pork insulin from cultured human lymphocytes (IM9). Cells were incubated with [^{125}I]pork insulin, and unlabelled human insulin (●), [Ser^{B24}]insulin (○) or [Ser^{B25}]insulin (△) at the indicated concentrations for 120 min at 15 C. Non-specific binding was subtracted from each data. Data represent one of three separate experiments. Figure 1.b. Ability of labelled insulin analogues to bind to cultured lymphocytes. Cells were incubated with [^{125}I] labelled human insulin (●), [Ser^{B24}]- (○) or [Ser^{B25}]-insulin (△), and various concentrations of unlabelled homologous insulin analogues.

with adipocytes ($2x10^5$ cells/ml) for 30 min at 37 C, assessed by precipitation method using 7.5% trichloroacetic acid, was 17.2 ± 2.1 % and 7.4 ± 1.8 % respectively compared to 29.1 ± 2.1% of normal insulin, when trace amounts of labelled insulin analogues were present in the buffer. However, when the large doses of unlabelled insulin (20 µg/ml) were also present in the buffer, the degradation of labelled native insulin was significantly decreased to 9.1 ± 0.5%, but the degradation of the analogues was unchanged(17.0 ± 1.0% for [Ser^{B-24}]-insulin and 7.1 ± 1.2% for [Ser^{B25}]-insulin).

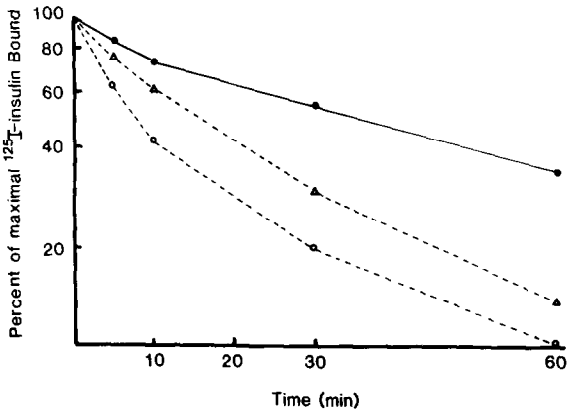

Figure 2. Dissociation of the bound labelled analogues. Cultured lymphocytes were allowed to associate with [^{125}I]human insulin (●), [^{125}I]-[Ser^{B24}]insulin (○) or [^{125}I]-[Ser^{B25}]insulin (△) for 90 min at 24 C and the cells were centrifuged at 4 C and the buffer was removed and replaced 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 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.

Decreased affinity of these analogues may be due to decreased association rate or increased dissociation rate. Since these analogues showed marked decreased binding ability, it is difficult to perform association study to determine the association rate of these analogues. As for dissociation studies, increased dissociation rates of these analogues were found (Fig. 2). Dissociation rates of human [Ser^{B24}]- , [Ser^{B25}]-insulins and normal human insulin were estimated by calculating the time required for a 50% decrease in binding during dilution, and they were 7, 16 and 39 min for [Ser^{B24}]- and [Ser^{B25}]-insulin and normal human insulin respectively. Thus, the decreased affinity of these analogues may be partly due to increased dissociation rate.

Next, we examined negative cooperative effect of these analogues by determining the dissociation rate of pre-bound labelled insulin in the presence of unlabelled these analogues in the buffer. Figure 3 shows the negative cooperative effect of analogues at a concentration of 100 ng/ml, which produces almost maximal effects with normal human insulin. The negative cooperative effect of [Ser^{B24}]- and [Ser^{B25}]-insulin was markedly decreased

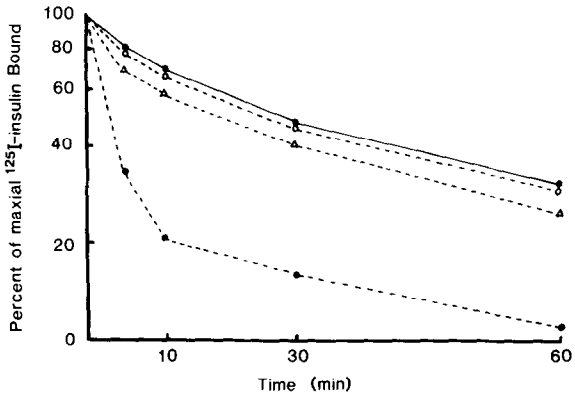

Figure 3. Negative cooperative effects of the analogues. [^{125}I] labelled porcine insulin was transferred into dissociation media where 100 ng/ml of human insulin (●), or 100 ng/ml of [Ser^{B24}]- (○) or 100 ng/ml of [Ser^{B25}]-insulin (△) were present.—●—;no insulin added in the dissociation media.

since time required for half maximal binding was 3 min for normal, 32 min for [Ser^{B24}]insulin and 14 min for [Ser^{B25}]insulin.. When concentration of these analogues was increased, the negative cooperative effect also increased, but they failed to reach the maximal level of that normal human insulin. Once the effect reached the maximal level, it started decreasing as the insulin concentrations increased (Fig. 4).

The biological activities of these analogues were determined by measuring their ability to stimulate glucose transport in isolated rat adipocytes. As

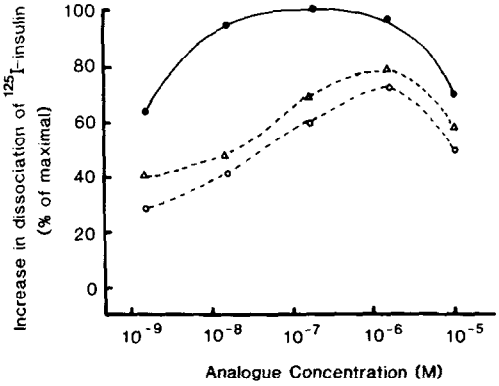

Figure 4. Negative co-operative effects of the analogues at various concentrations. The dissociation was allowed to proceed for 30 min as described in Fig.3. and then the amount of radioactivity bound was measured. The data are plotted as the radioactivity dissociated in the presence of the indicated 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; ○ ,[Ser^{B24}]insulin; △ ,[Ser^{B25}]insulin.

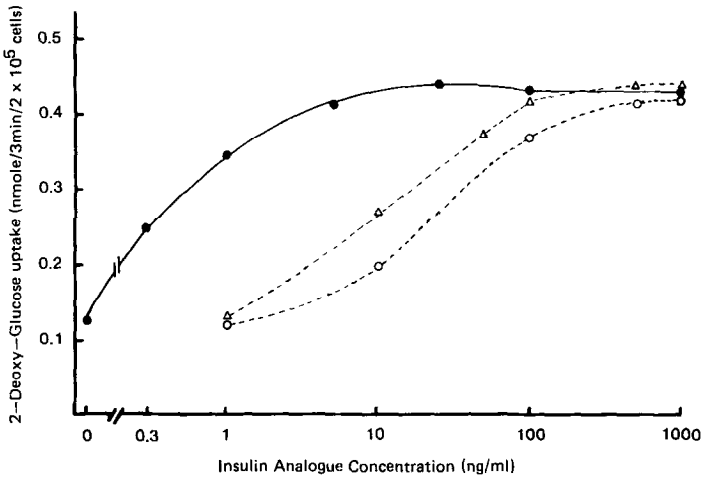

Figure 5. Ability of insulin analogues to stimulate 2-deoxyglucose uptake in isolated adipocytes. Cells were preincubated with human insulin(●), [Ser^{B24}]insulin(○), or [Ser^{B25}]insulin(△) at the indicated concentration for 60 min at 24 C and then 2-Deoxy-D[1-^{14}C]glucose uptake was measured. The values were means for three separate experiments.

shown in Fig. 5, the biological activities of the Ser^{B24} and Ser^{B25} analogues were 0.7-3% and 4-6% of that of human insulin. However, these analogues were able to demonstrate a maximal activity of normal insulin at the high concentration.

Guinea-pig anti-(porcine insulin) antiserum was used for testing the immunoreactivity of these analogues. Porcine, human and [Ser^{B25}]-insulin behaved similarly in binding to anti-(porcine insulin) antibody, whereas [Ser^{B24}]insulin reacted less with antibody (Fig. 6). Two other different batches of guinea-pig anti-porcine sera also demonstrated similar results.

Discussion

The second structurally abnormal mutant insulin, [Ser^{B24}]insulin, was found to demonstrate markedly decreased ability in receptor binding and biological activity. These decreased ability of [Ser^{B24}]insulin (0.7-3%) was similar to or less than the ability of the first structurally abnormal insulin (2-5%) as we previously reported (2). Thus, it is interesting to note that the both abnormal insulins found in the patients showed extremely low activity, which may be relevant to the cause of diabetes in these patients. As shown in [Leu^{B25}]insulin, increased dessociation rates seem in part to contribute to

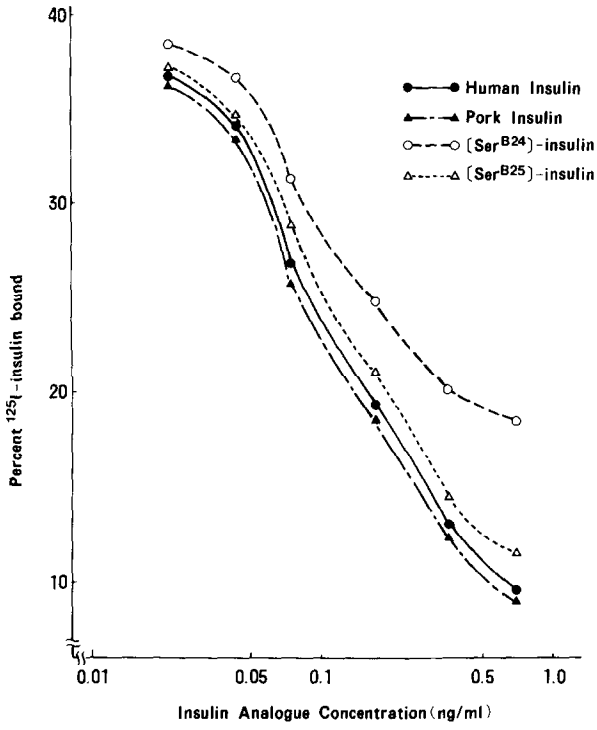

Figure 6. Immunoreactivity of insulin analogues. The methods are shown in Methods.

the decreased affinity in these analogues. Negative cooperativity, i.e. the other feature of insulin receptor binding, was also markedly decreased in these abnormal insulins. Thus, B24 as well as B25 plays a crucial role in negative cooperativity as well as in receptor binding.

The biological activities of these analogues are proportional to receptor binding activity and the analogues have full activity at the high concentrations.

The immunoreactivity of [Ser^{B24}]insulin was decreased compared with that of [Ser^{B25}]-, human and porcine insulin. The true value of the circulating abnormal insulin in the patient appeared to be higher than the value measured by radioimmunoassay using anti-(porcine insulin) antiserum. Since leucine substitution at B24 also demonstrated decreased immunoreactivity, determinant sites for immunoreactivity include B24 but not residue B25 as we suggested previously (2).

Decreased degradation of the analogues is probably due to decreased receptor mediated degradation since the presense of large amount of native insulin did not decrease degradation of the labelled analogues. It is interesting to point out that [Ser^{B24}]insulin is more degraded compared to [Ser^{B25}]insulin whereas the receptor binding ability of [Ser^{B25}]insulin is greater than that of [Ser^{B24}]insulin. The cause for this phenomenon is not clear at present.

B24 is a unique site since substitution with aliphatic amino acid (Leu) showed mild depression in receptor binding (2) and substitution with D-Phe showed increased receptor binding(7). On the contrary, substitution with aliphatic amino acid (Leu and Ala) or D-Phe at B25 all resulted in decreased receptor binding. These results suggest that B25 rather than B24 may be a key amino acid residue for receptor binding. However, as reported in this paper, substitution with a polar amino acid , serine, at B24 markedly decreased receptor binding, indicating hydrophobicity of B24 Phe may play an important role for receptor binding and negative cooperativity. B25 Phe, surrounded by polar amino acids, appears to forms the center of hydrophobic nucleus of insulin-receptor complex and hydrophobicity of B23 and B24 amino acid residues may also play some role in receptor binding.

In summary, [Ser^{B24}]insulin, the second structurally abnormal mutant insulin, demonstrates markedly decreased ability in receptor binding, bilogical activity and negative cooperativity. Furthermore, immunoreactivity of this insulin was also decreased compared to that of native insulin and [Ser^{B25}]insulin. Hydrophobicity of B24 amino acid residues may play an imporatnt role in receptor binding.

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