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RECEPTOR BINDING AND NEGATIVE COOPERATIVITY OF A MUTANT INSULIN, [Leu^{A3}]-insulin

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[Leu^{A3}]-insulin, the third mutant insulin, was semisynthesized and was studied for receptor binding and negative cooperative effects. Receptor binding and biological effects of the mutant insulin were 0.3-0.5% of normal, the lowest among three mutant insulins. However, negative cooperative effects of the mutant insulin were almost normal at higher concentration($>10^{-6}$M). Monoclonal anti-insulin antibody binding studies revealed that carboxyterminal region of B chain was relatively unchanged. These results suggest that N-terminal region of A-chain extending to A3 is important for receptor binding and confirm that A3 does not play an important role for negative cooperativity.

The third family of the patients with a mutant insulin has recently been found in Japan (1). In contrast to the previous mutant insulins with abnormalities in carboxyterminal region of B chain (B24 and B25), the new mutant insulin exhibited substitution of Val with Leu at A3, i.e. [Leu^{A3}]-insulin (1). However, A3 Val was not included for putative receptor binding region, although N-terminal amino acid residues were reported to participate in receptor binding (2). Previous mutant insulins, [Leu^{B25}]- and [Ser^{B24}]-insulin, showed decreased negative cooperative effects since B24 and B25 were important for the effects (3,4). But, A3 Val was not included for negative cooperative region (5). Thus, it is of interest to examine receptor binding ability and negative cooperative effect of the new mutant insulin.

In this communication, [Leu^{A3}]-insulin showed marked decreased receptor binding and relatively intact negative cooperative effects.

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Materials and Methods

Materials:Porcine insulin (lot 1FJ91, 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 RPMI1640, 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 [Leu^{A3}]- and [Ala^{A3}]-insulins:Porcine insulin was acylated with Boc-N_3 and the resulting $N^{\alpha A1}$, $N^{\varepsilon B29}$-(Boc)$_2$-insulin was treated with trypsin to give $N^{\alpha A1}$-Boc-Desoctapeptide insulin (DOI). This compound was acylated and then treated with trifluoroacetic acid (TFA)-anisole to give $N^{\alpha B1}$-Fmoc-DOI. Subsequently, Edman degradation of the compound to remove Gly^{A1}, Ile^{A2} and Val^{A3} to give $N^{\alpha B1}$- Fmoc-des (Gly^{A1}, Ile^{A2} Val^{A3})-DOI. The tripeptide derivatives corresponding to [Leu^{A3}]- or [Ala^{A3}]-insulin were synthesized by conventional solution method. Boc-Gly-Ile-Leu-NHNH$_2$ was reacted with $N^{\alpha B1}$-Fmoc-des (A1, A2, A3)-DOI. The resulting $N^{\alpha A1}$-Boc, $N^{\alpha B1}$-Fmoc-[Leu^{A3}]-DOI was reacted with octapeptides by trypsin-catalyzed coupling method (6). The product of [Leu^{A3}]-insulin was homogenous by HPLC.

Insulin binding studies:Human cultured lymphocytes(IM9) 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 (7).

Iodination of insulins:Porcine ^{125}I-labelled insulin, ^{125}I-labelled analogues were prepared at a specific radioactivity of 100-150 μCi/μg by the method of Freychet et al (8).

Glucose uptake studies:The method used to study glucose uptake in isolated rat adipocytes was as previously described (9).

Insulin binding to anti-insulin antibodies:Porcine [^{125}I]insulin and insulin anlogues at various concentrations were incubated with sera or monoclonal antibody for 48 h at 4 C and bound and unbound insulin were separated with poly(ethylene glycol) as described previously (10). Anti-bovine insulin monoclonal antibody (OXI-005) recognizing carboxyterminal area of B-chain was purchased from Novo company (11).

Results

Fig. 1. shows the ability of three mutant insulins, [Leu^{A3}]-, [Ser^{B24}]- and [Leu^{B25}]-insulin to compete with ^{125}I-labelled insulin for insulin receptor

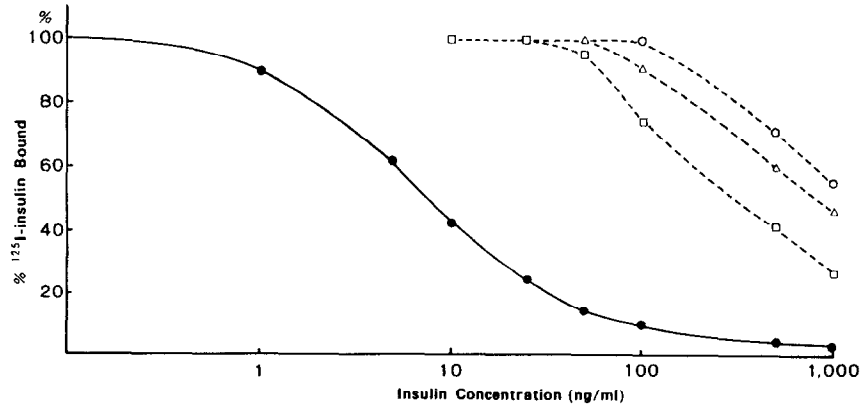
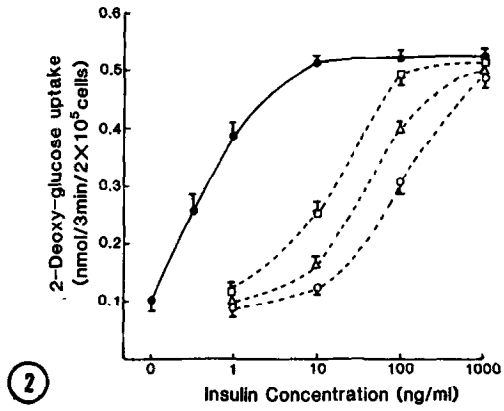


Figure 1. Ability of three mutant insulins to bind to insulin receptors. IM-9 cells were incubated with ^{125}I-porcine insulin and various concentrations of unlabelled human (●), [Leu^{A3}]- (○), [Ser^{B24}]- (△), and [Leu^{B25}]-insulin (□). Maximal binding of ^{125}I-insulin was defined as 100 %.



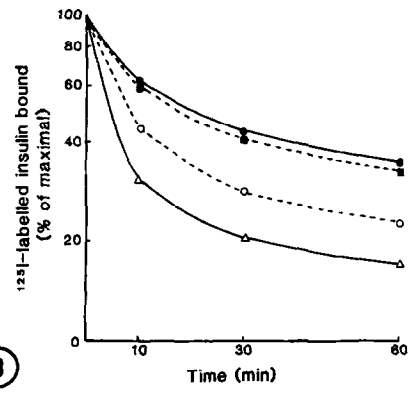


Figure 2. Ability of three mutant insulins to stimulate 2-deoxy-glucose uptake in rat adipocytes. Cells were preincubated with human (●), [Leu^{A3}]- (○), [Ser^{B24}]- (△), and [Leu^{B25}]-insulin (□) and then 2-deoxy-glucose uptake was determined. Differences of the values between human and other mutant insulins were statistically significant ($p<0.01$) at the concentration from 1 to 100 ng/ml except for 100 ng/ml of [Leu^{B25}]-insulin. The values are mean ± SEM of four separate experiments.

Figure 3. Negative cooperative effects of [Leu^{A3}]-insulin. ^{125}I labelled porcine insulin was transferred into dissociation media where 100 ng/ml of human insulin (△), or 100 ng/ml of [Leu^{A3}]- (○) or [Leu^{B25}]-insulin (■) were present. —●— ;no insulin added in the dissociation media.

binding. Human [Leu^{A3}]-, [Ser^{B24}]- and [Leu^{B25}]-insulin demonstrated 0.3-0.5%, 0.8-1.0% and 3-5% respectively of the receptor binding potency of human insulin. Thus, [Leu^{A3}]-insulin has the lowest receptor binding ability among three mutant insulins. We labelled the insulin analogues and examined the ability of the labelled analogues to bind to cells and the results were similar to that of Fig.1. as we previously reported for [Leu^{B25}]- and [Ser^{B24}]-insulin (3,4).

Biological activity of the mutant insulin assessed by glucose uptake in adipocytes was similar to the receptor binding ability(Fig. 2).

We examined negative cooperative effects of the mutant insulin by determining the dissociation rate of the prebound labelled insulin in the presence of unlabelled mutant insulin in the buffer. Fig. 3 shows the negative cooperative effect of analogues at a concentration of 100 ng/ml, which produces almost maximal effects of negative cooperativity with normal insulin. The negative cooperative effects of [Leu^{A3}]-insulin was relatively maintained compared with [Leu^{B25}]-insulin. When the cooperative effects were examined through the wide range of insulin concentrations, as shown in Fig. 4, [Leu^{A3}]-insulin was more

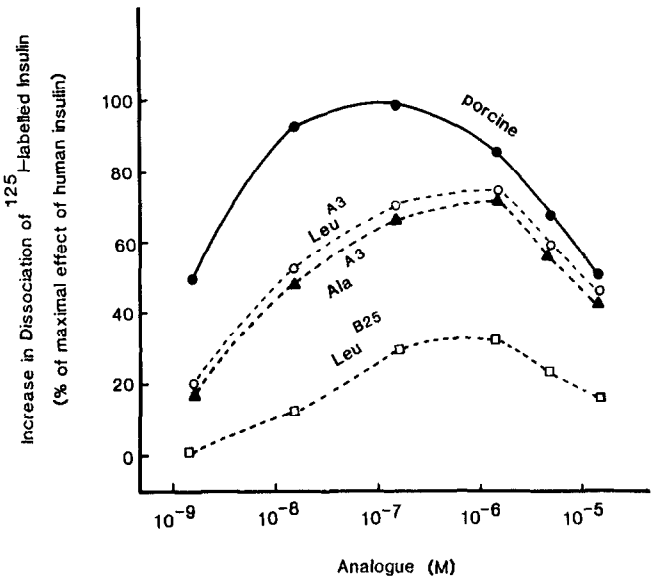


Figure 4. Negative cooperative effects of the analogues at various concentrations. The dissociation was allowed to proceed for 30 min as described in Fig. 3. and then the amount of radioactivity bound was measured. The data are plotted as the radioactivity dissociated in the presence of the indicated concentrations of anlogues, minus the dissociation in the absence of the anlogues (as a percentage of the maximal effect) as a function of the analuge concentration. ●, Porcine insulin;○, [Leu^{A3}]-insulin;▲, [Ala^{A3}]-insulin;□, [Leu^{B25}]-insulin.

potent than [Leu^{B25}]-insulin in negative cooperative effects in contrast to the results of receptor binding ability of these mutant insulins. Higher concentrations ($> 10^{-6}$M) of [Leu^{A3}]-insulin reached to the extent of normal insulin in the negative cooperative effect.

Immunoreactivity of the mutant insulin was tested by guinea pig anti-(porcine insulin) antiserum and anti-bovine insulin monoclonal antibody which mainly reacts with carboxyterminal region of B-chain. As shown in Fig. 5.a, [Leu^{A3}]- and [Ala^{A3}]-insulin reacted less with the polyclonal antibody compared with human insulin. When monoclonal antibody recognizing carboxyterminal of B-chain was used, [Leu^{A3}]-insulin almost similarly bound to the antibody compared to human insulin (Fig. 5.b). In contrast, [Leu^{B25}]- and [Ser^{B24}]-insulin were not able to react with the monoclonal antibody. Thus, the result of monoclonal antibody binding study suggests that carboxyterminal area of B-chain of [Leu^{A3}]-insulin is not greatly affected by substitution of Val with Leu at A3.

Degradation of [Leu^{A3}]-insulin with insulin degrading enzyme was tested with supernatant of IM-9 cells and was equal to that of normal insulin (Fig. 6).

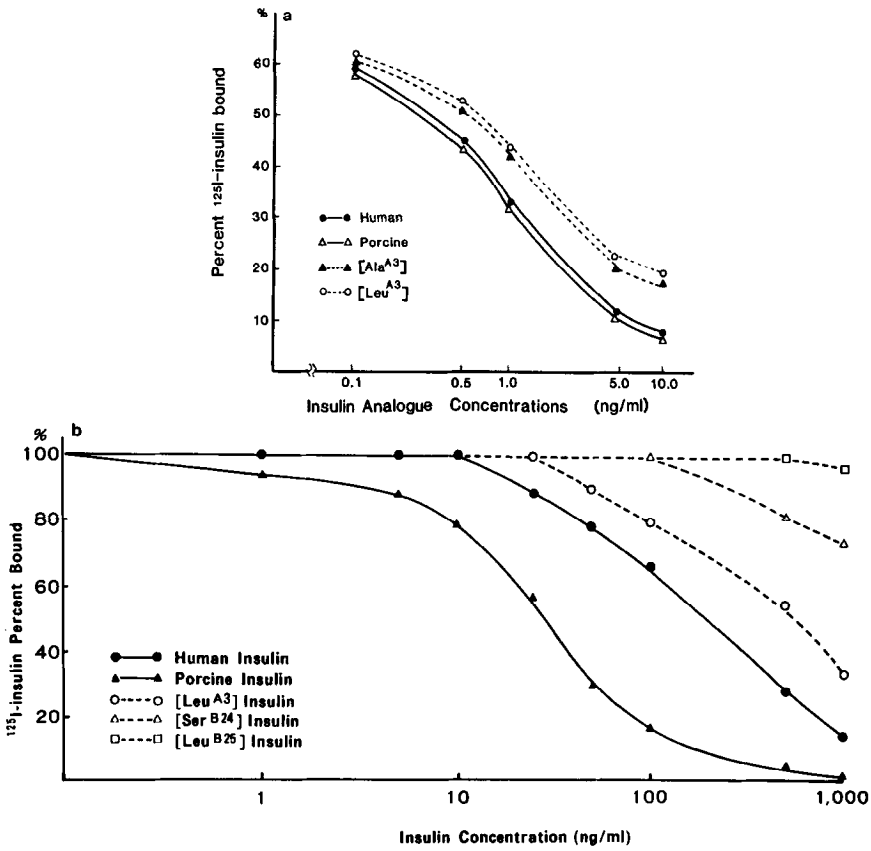


Figure 5.(a) Immunoreactivity of insulin analogues. Binding to polyclonal antibody. (b) Monoclonal antibody binding to three mutant insulins.

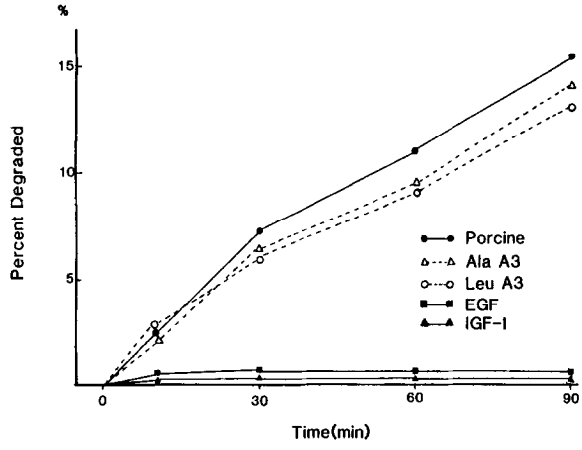


Figure 6. Degradation of mutant insulins by supernatant of IM-9 cells. IM-9 cells (10^6 cells/ml) were incubated in phosphate saline (pH 7.8, 20 mM sodium phosphate) for 4 h at 37°C. The cells were then centrifuged and the supernatant (100µl) was incubated at 37°C with labelled analogues in HEPES buffer (total volume 1 ml). Percent degradation was assessed by 7.5% trichloroacetic acid.

However, when a tracer amount of labelled insulin was incubated with adipocytes for 30 min at 37°C, degradation of [Leu^{A3}]-insulin and human insulin was 8.1% ± 2.2% and 28.4 ± 1.8%, respectively. The large amounts of unlabelled insulin (20μg/ml) decreased the degradation to 7.4 ± 1.1% and 8.9 ± 1.4%, respectively. Thus, receptor mediated degradation appeared to be decreased in the mutant insulin.

Discussion

We previously suggested that B25 may be a key amino acid residue to form a hydrophobic core with a binding region of receptor surrounded by hydrophilic amino acids (3,4,7). However, Val at A3 also plays an important role because of more decreased binding ability of [Leu^{A3}]- or [Ala^{A3}]-insulin than the previous mutant insulins, indicating that N-terminal regions of A-chain, at least A1 to A3 and A5 may be as important as B24 and B25. Although the amino acid residues of A1 and A5 were included for the putative receptor binding region (2), our results indicate that A3 also plays some role in receptor binding. Kitagawa and his coworkers suggested that the side chain of Ile of A2 played a critical role for high biological activities since Ile A2 had a close contact with Tyr A19 by van der Waals bond between the residues (12). Therefore, substitution of Val with Leu or Ala at A3 may disturb the proper disposition of the A19 residue, one of the putative receptor binding residues, and the maintenance of the A2-A8 helical structure. Tyr A19 also is in close proximity to carboxyterminal area of B chain, especially Phe B25. Thus, N-terminal area of A chain may indirectly affect the regional structure of carboxyterminal area of B chain. However, the monoclonal antibody binding study suggested that the region largely remained unchanged.

Although [Leu^{A3}]-insulin exhibited much lower binding activity compared to [Leu^{B25}]-insulin, the negative cooperative effect of [Leu^{A3}]-insulin was higher than that of [Leu^{B25}]-insulin. This confirmed that B25 was more important than A3 for negative cooperative effects. Thus, the results of negative cooperative effects and the monoclonal antibody binding study suggest the relatively

unchanged regional structure of carboxyterminal area of B-chain of the new mutant insulin.

Degradation of the new mutant insulin by insulinase was equal to that of normal insulin and the receptor mediated degradation was significantly decreased. Similar findings were obtained in two other mutant insulins (13). Thus, these regions including A3, B24 and B25 did not appear to be the recognition site of insulinase. We previously reported that decreased receptor binding and degradation will prolong the disappearance rate of the mutant insulin from the circulation (14). The new mutant insulin may show more delayed metabolism in vivo compared to [Leu^{B25}]-insulin because of much decreased receptor binding ability of [Leu^{A3}]-insulin.

In summary, studies on the new mutant insulin indicate an importance of N-terminal region of A-chain extending to A3 as well as carboxyterminal region of B-chain for receptor binding and confirm that A3 does not play an important role for negative cooperativity and degradation by insulinase.

Acknowledgments

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