Immunoreactivity and Biologic Activity of Semisynthetic [Leu^{B-30}]-Insulin

Potential Value in the Treatment of Insulin Antibody-Mediated Insulin Resistance

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SUMMARY

Insulin analogues with different amino acids, including threonine, alanine, L-leucine, D-leucine, L-leucine amide, phenylalanine, tri-alanine, or desalanine, at the B-30 position were semisynthesized from pork insulin by the new enzymatic method. The order of ability of the insulin analogues to bind to anti-insulin sera was [Ala^{B-30}] > desalanine > [Thr^{B-30}] > [Ala-Ala-Ala^{B-30}] > [D-Leu^{B-30}], [Leu-NH$_2$$^{B-30}$], [Phe^{B-30}] > desoctapeptide ≥ [Leu^{B-30}]. The ability of insulin analogues with different amino acids at B-30 to bind to receptors, as well as their biologic potency tested with glucose uptake in isolated rat adipocytes, was comparable among the analogues. These results suggest that [Leu^{B-30}]-insulin demonstrated the least immunoreactivity and has full activity in receptor binding and biologic effect, and that it may be useful for treatment of anti-insulin antibody-mediated insulin resistance.

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Highly purified pork insulin is now being used clinically, but 70% of the patients treated develop anti-insulin antibodies.[1] For those patients with marked insulin antibody-mediated insulin resistance, an insulin derivative with the least immunoreactivity and full biologic activity is needed for treatment of acute metabolic decompensation.

The B-chain carboxy-terminal amino acid residue, B-30, is present on the outer surface of the insulin molecule[2] and is substituted with various amino acids in different animal species,[3] suggesting that it may be a potential immunoreactive determinant. Therefore, a simple alteration of the amino acid residue at B-30 may produce an insulin less reactive to the patients' anti-insulin sera.

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We report here that [Leu^{B-30}]-insulin may be such an insulin of potential value in treatment of insulin resistance mediated by insulin antibody, since it has the least immunoreactivity to anti-insulin antibodies and yet has full biologic activity.

MATERIALS AND METHODS

Materials. Porcine insulin (Lot. 1FJ91, 26.2 U/mg) was kindly supplied by Eli Lilly and Company. Na[^{125}I], [^{3}H]-2-deoxy-glucose, and [^{3}H]-L-glucose were purchased from New England Nuclear and collagenase (type 2) from Worthington Biochemicals.

Insulin analogues. Desoctapeptide-(B23-B30)- (DOI) or desalanine-(B-30)-pork insulin (DAI) was obtained by digestion of pork insulin with TPCK-treated trypsin[4] or with *Achromobacter lyticus* protease I,[5] respectively, as described previously. The coupling of DAI with Leu-OBu^t or other amino acid-OBu^t was performed enzymatically using *Achromobacter lyticus* protease I.[5] The products were isolated by a preparative apparatus of reverse-phase liquid chromatography. The tertiary butyl ester of the coupled materials was deprotected with trifluoroacetic acid in the presence of anisole. Insulin analogues thus obtained were identified by high performance liquid chromatography, polyacrylamide gel electrophoresis, and amino acid analysis.[6]

Insulin binding to anti-insulin antibodies. [^{125}I]-pork insulin and insulin derivatives at various concentrations were incubated with sera for 48 h at 4°C. Bound and unbound insulin were separated by the polyethylene glycol (PGE) method.[7] After 48 h of incubation, PGE was added to the incubation tubes to a final concentration of 12.5%. Finally, the bound and unbound insulins were separated by centrifugation.

Anti-insulin sera were obtained from three patients previously treated with lente insulin (mixture of beef and pork insulins) and from three guinea pigs immunized with pork insulin.

Insulin binding to placenta membranes. Placenta membranes were prepared by the method of Posner.[8] [^{125}I]-pork

insulin and insulin analogues were incubated with the membranes (membrane protein 0.8 mg/ml) for 90 min at 15°C. Nonspecific binding was measured by incubating the membranes with labeled insulin and 200 μg/ml of pork insulin. All the binding data are shown as specific binding, calculated by subtracting the nonspecific binding from the total binding. Separation of bound and unbound [^{125}I]-insulin was done by microfuge centrifugation.

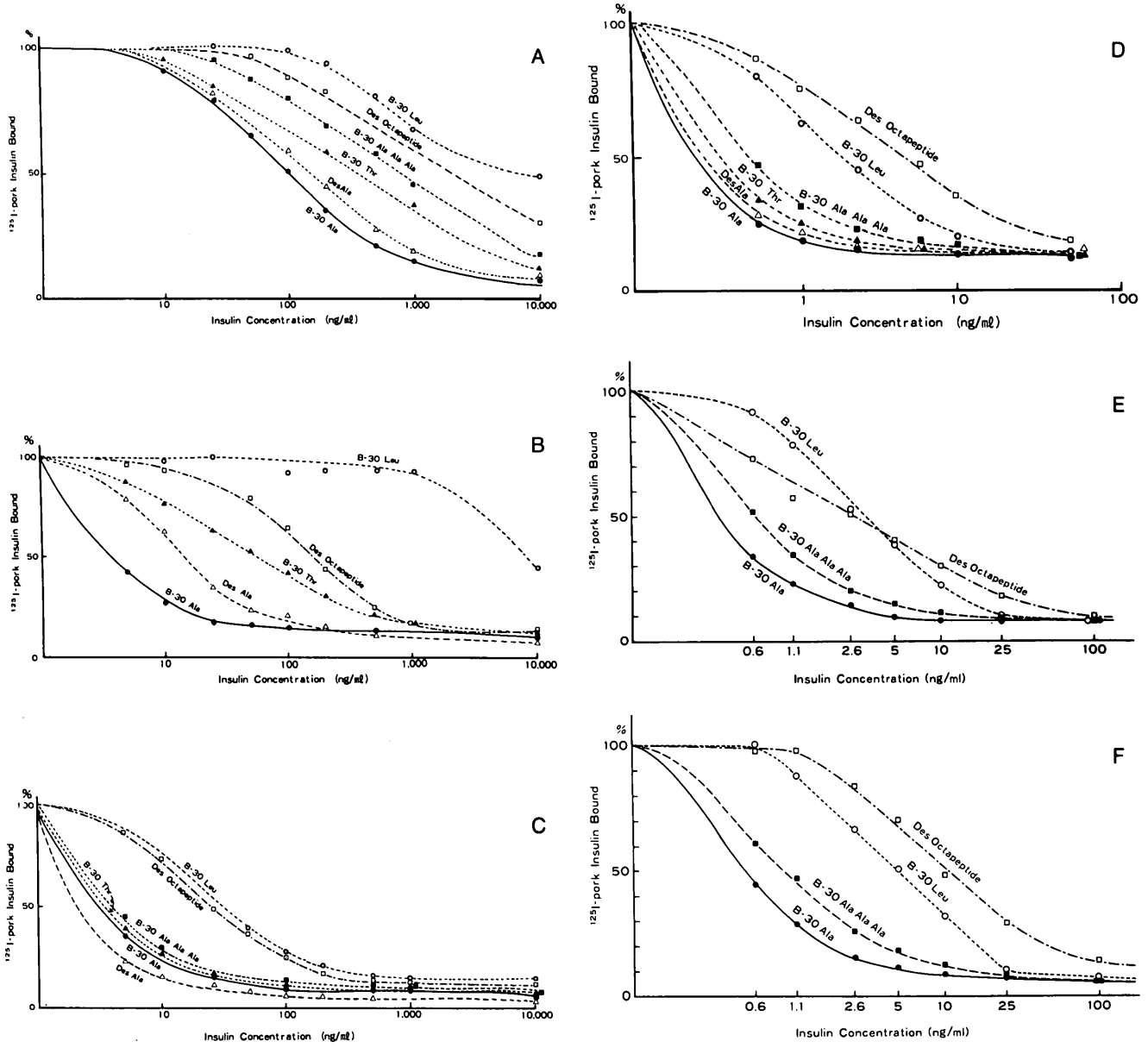
Glucose uptake studies. The details of this method have been previously described.[9] Isolated rat adipocytes were preincubated with insulin for 60 min at 24°C. Next, the cells were incubated with [^{3}H]-2-deoxy-glucose at a concentration of 0.1 mM in Krebs-Ringer buffer. The assay was terminated at the end of 3 min by transferring 200-μl portions from the assay mixture to microtubes containing 100 μl of silicone oil. The tubes were centrifuged for 30 s in a Beckman microfuge, and the assay was considered terminated when centrifugation was begun. The extracellular water space was calculated from the data of incubation of [^{3}H]-L-glucose with the cells and all the data of glucose uptake were corrected for this factor.

RESULTS

Insulin binding to anti-insulin serum. We tested the reactivity of insulin analogues with three patients' anti-insulin sera and three guinea pig anti-insulin sera. Figure 1 A-C shows the reactivity of insulin analogues with patients' sera. [Ala^{B-30}]-insulin (pork insulin) reacted the most with the sera and [Leu^{B-30}]-insulin the least. Interestingly, human insulin ([Thr^{B-30}]-insulin) reacted less with the sera than pork insulin. The reactivities of [Phe^{B-30}]-, [Leu-NH$_2$$^{B-30}$]-, and [D-Leu^{B-30}]-insulins were between those of [Thr^{B-30}]-insulin and DOI (data not shown).

Anti-insulin antibodies from three guinea pigs immunized

FIGURE 1. Ability of various insulin analogues to displace ^{125}I-pork insulin from anti-insulin sera. Anti-insulin sera from patients treated with lente insulin (mixture of beef and pork insulin) (A-C) and from guinea pigs immunized with pork insulin (D-F) were incubated with ^{125}I-pork insulin and the various insulin analogues at indicated concentrations.



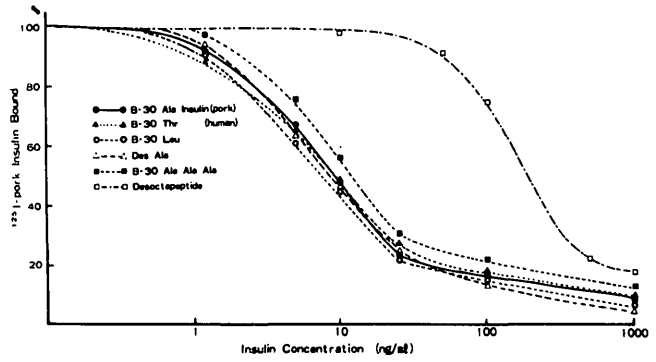


FIGURE 2. Ability of various insulin analogues to bind to human placenta membranes. Human placenta membranes were incubated with ^{125}I-pork insulin (0.2 ng/ml) and various concentrations of insulin analogues. Percent binding of ^{125}I-pork insulin in the absence of insulin analogues was defined as 100%.

with pork insulin reacted with these insulin analogues in the same manner as those in the human sera, although DOI cross-reacted less with two of guinea pig sera than [Leu^{B-30}]-insulin (Figure 1 D-F).

Insulin binding to human placenta membranes. To examine whether alteration of the amino acid residue at the carboxy terminal (B-30) affects insulin binding to insulin receptors, we studied the ability of insulin analogues in binding to human placenta membranes. As shown in Figure 2, alteration of the amino acid to Ala (pork insulin), Thr (human insulin), Leu, or desalanine at the B-30 position does not produce any difference in ability to bind to insulin receptors on human placenta membranes. Furthermore, insulins with Phe, Leu-NH_2, or D-Leu at B-30 are also capable of binding to insulin receptors to the same degree as pork insulin (data not shown). Trialanine in the carboxy-terminal position showed a slight decrease in binding ability. The ability of DOI to bind to receptors is 0.5–1.0% of that of pork insulin. Thus, the alteration of B-30 amino acid residues does not affect insulin receptor binding.

Biologic effect of insulin analogues. All the insulin analogues, including insulin with Thr, Ala, or Leu at B-30, have the full activity to increase glucose uptake in isolated rat adipocytes (Figure 3). Other insulins with D-Leu, Leu-NH_2, Phe, or desalanine at B-30 also show the same activity (data not shown). However, the activity of the insulin analogue with trialanine at B-30 was decreased at the analogue concentrations of 0.3 and 1.0 ng/ml ($P < 0.05$), and the activity

FIGURE 3. Ability of various insulin analogues to increase 2-deoxy-glucose uptake in isolated rat adipocytes. Data represent mean ± SE of three separate experiments for each analysis.

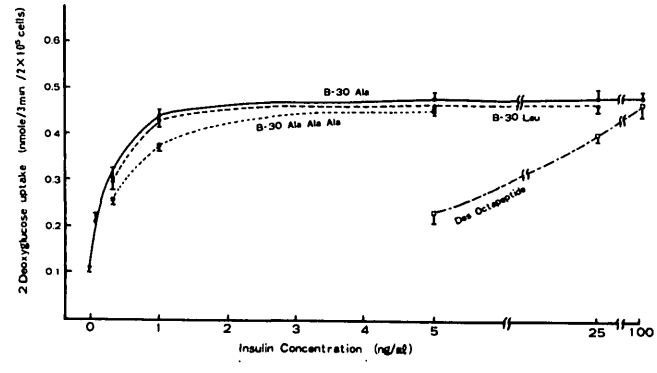


TABLE 1
Ability of insulin analogues to decrease plasma glucose in rats (mean ± SE)*

Insulin analogues	Plasma glucose levels (mg/dl)	
	Before injection	30 Min after injection
Pork insulin (N = 4)	121 ± 3	57 ± 6
[Leu^{B-30}]-insulin (N = 4)	124 ± 8	59 ± 8†
DOI (N = 3)	123 ± 7	112 ± 9‡

* Insulin analogues (8.4 μg/kg) (=0.2 U/kg for pork insulin) were injected to rats via tail vein at 9 a.m.
† Not statistically significant versus pork insulin.
‡ $P < 0.01$ versus pork insulin.

of DOI was only 1–2% of that of pork insulin. These results show the close relationship between insulin binding and its biologic activity.

Next, we tested the in vivo effect of pork insulin, [Leu^{B-30}]-insulin, and DOI in rats. The activity of [Leu^{B-30}]-insulin to decrease plasma glucose levels was comparable with that of pork insulin, whereas DOI showed marked decreased activity (Table 1). Therefore, [Leu^{B-30}]-insulin has full activity with respect to receptor binding and biologic effect, whereas its ability to react with anti-insulin antibodies is only 10% of that of pork insulin.

DISCUSSION

From these studies, it is clear that the alteration of amino acid residues at B-30 of the insulin molecule affects neither insulin ability to bind to insulin receptors nor biologic activity. The important receptor binding regions of the insulin molecule are located at amino acid residues A-1, A-5, A-19, A-21, B-12, B-16, B-22, B-23, B-24, B-25, and B-26; the B-chain carboxy-terminal region (B-30) does not participate in insulin receptor binding.[10] Therefore, insulins with various amino acids at B-30, or desalanine insulin, show full activity in both receptor binding and biologic effect.

In contrast to receptor binding, these insulin analogues behave differently in antibody binding. Anti-insulin sera from patients treated with a mixture of pork and beef insulins and three sera from three separate guinea pigs immunized with pork insulin react with [Ala^{B-30}]-, [Thr^{B-30}]-, desalanine-, [Leu^{B-30}]-insulin, and DOI in decreasing order. Therefore, [Leu^{B-30}]-insulin reacts much less with insulin antibodies and yet has full biologic activity.

Since the B-30 position is located on the outer surface of the insulin molecule, the region may be important for immunoreactivity. Kumar reported that desalanine insulin and human and pork insulins react differently with the patients' insulin antibodies.[11] Interestingly, we also found that human insulin binds less to anti-insulin sera than pork insulin, suggesting that these antibodies can recognize the difference in amino acid residues at B-30.

Fish insulins have been useful in some patients with extreme insulin resistance, since they are structurally much different from beef or pork insulin. However, because of shortage of resources, commercial production of fish insulin is difficult at present. On the other hand, [Leu^{B-30}]-insulin can be easily semisynthesized from pork insulin by the enzymatic method, which seems to be one of the best for

semisynthesis of human insulin. This analogue may be useful for patients with extreme insulin resistance due to anti-insulin antibodies.

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