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BINDING OF MUTANT INSULINS TO A MUTATED INSULIN RECEPTOR

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SUMMARY We studied the binding of mutant insulins to both the normal human insulin receptor and an insulin receptor in which the sequence 240-250 of the receptor α subunit was mutated to provide an additional net positive charge. One mutant insulin (AspB10), which has an additional negative charge, bound to both types of receptors with a higher affinity than native insulin. Moreover, this mutant insulin was more effective in activating the tyrosine kinase activity of both types of receptors. This study suggests, therefore, that charge interactions between insulin and its receptor may play a role in insulin receptor binding and action.

Insulin initially interacts with its specific receptor located in the plasma membrane (1). The insulin receptor is a disulfide-linked tetrameric glycoprotein composed of two extracellular 130-kDa α subunits containing the insulin-binding site(s) and two transmembrane 95-kDa ß subunits containing tyrosine kinase activity in their intracellular domains (1-4). When insulin binds to the α subunit, tyrosine kinase activity in the ß subunit is activated, and insulin action ensues.

We have previously reported that amino acid residues 240-250 in the cysteine-rich region of the human insulin receptor α subunit may constitute a site in which insulin binds (5). We then constructed a human insulin receptor mutant in which 3 residues in this sequence were altered to add a net positive charge, and expressed this mutant in rat hepatoma cells (HTC) (6). When compared with cells transfected with normal insulin receptors, cells transfected with mutant receptors had an increase in insulin-binding affinity and a decrease in the dissociation of bound ^{125}I-insulin (6). These findings further suggested that amino acid sequence 240-250 of the human insulin receptor α subunit may contain one site that interacts with insulin, and that mutations in this site can influence insulin receptor binding.

Several recombinant mutant insulin analogues have been prepared and shown to have altered biological potencies (7). In particular an analogue, in which the B10 histidine residue is replaced by aspartic acid thus adding a net negative charge to the insulin molecule, has been shown to have a biological potency greater than native insulin itself. In the present study we have investigated the interaction of this and other mutated insulins with our mutated insulin receptor.

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METHODS AND MATERIALS

HTC rat hepatoma cells expressing transfected normal human insulin receptors (HTC-IR), and rat hepatoma cells expressing transfected mutated receptors whereby amino acid sequence 240-250 of the human insulin receptor α subunit was altered from TCPPPYYHFQD to TCPRRYYDFQD (HTC-IR mutant) were obtained and grown as previously described (6). Recombinant insulin analogues were obtained from Dr. Jens Brange of the Novo Research Institute.

Insulin Binding to Intact Cells HTC-IR cells and HTC-IR mutant cells were grown to confluence on 1 cm^2 plastic multiwell plates. Cells were then incubated for 3 h at 15 C in bicarbonate-free Dulbecco's modified Eagle's H-16 medium supplemented with 15 mM HEPES (pH 7.4) and 10% fetal bovine serum with 6 pM ^{125}I-insulin and various concentrations of unlabeled insulin. After incubation, cells were washed with 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), and specific binding of insulin was determined as described previously (8). Data obtained were analyzed by Scatchard plots (9).

Membrane Preparation Confluent cells from 5 flasks (150 cm^2) were harvested and centrifuged. The cell pellet was resuspended in 50 mM Tris buffer, pH 7.9, containing 10 mM benzamidine, 30 μg/mL aprotinin, 0.2 mg/mL bacitracin, 10 mM PMSF, sonicated three times for 20 s and centrifuged for 10 min at 2,500 rpm. The supernatants of each centrifugation were pooled and centrifuged at 11,000 rpm for 10 min. The pellet was resuspended in 1-2 ml of Tris buffer, aliquoted in 100 μL fractions, and microfuged for 30 min. The supernatant was removed, and the pellet was stored at -70C.

Insulin Binding to Membranes Insulin binding and competition were carried out essentially as described previously (10). Membranes (5 μg of protein) were incubated 18 h at 4 C with 40,000 cpm of [^{125}I-Tyr^{A14}] insulin in Tris-HCl buffer (pH 7.4) containing 0.1% bovine serum albumin and 1 mM bacitracin in a final volume of 200 μL. Membrane bound radioactivity was precipitated by the addition of 50 μL of 0.4% bovine g-globulin and 250 μL of 20% poly(ethylene glycol).

Activation of Insulin Receptor Kinase Normal and mutant insulin receptors from transfected cells were partially purified by wheat germ agglutinin chromatography as described (11). Receptor protein was quantitated by radioimmunoassay, and the concentration of receptor was adjusted to 50 ng/mL. Insulin-dependent tyrosine kinase activity was determined by preincubating 2 ng of receptor with various concentrations of insulin for 16 h at 4 C in buffer containing 50 nM HEPES (pH 7.4), 0.1% triton X-100, 0.25% bovine serum albumin, 1 mM PMSF. Next, 15 μL of 2 mM $MnCl_2$, 10 mM $MgCl_2$ and 10 mM [γ-^{32}P]ATP were added for 30 min. The synthetic substrate poly(Glu-Tyr) was then added at a final concentration of 2.5 mg/mL, and the incubation was continued for 30 min at 20 C. Finally, aliquots were spotted on 4-cm^2 discs of Whatman 3MM paper, and the dried disc were washed in four changes of 10% trichloroacetic acid containing 10 mM sodium phosphate. Radioactivity was determined by scintillation counting. The background controls were determined from duplicate reactions lacking poly(Glu-Tyr).

RESULTS

Binding of Insulin and Mutant Insulins to Intact Cells and Membranes We first studied the binding of insulin and the insulin analogues AspB10 to both HTC-IR cells and HTC-IR mutant cells. As previously reported, HTC-IR mutant cells bound native insulin with an affinity that was over twice that of HTC-IR cells (Figure 1, Table I). In both cell lines, the mutant insulin was more effective than native insulin in competing with ^{125}I-insulin for binding to the receptor. Several other mutant insulins were similarly tested in both cell lines, and found to be either equal to or less effective than native insulin (Table I).

We next studied the binding of insulin and insulin AspB10 to membranes prepared from HTC-IR and HTC-IR mutant cells. As with intact cells, the insulin analogue was more effective than native insulin in binding to membranes from both cell types (Figure 2).

Tyrosine Kinase Assay The ability of the mutant insulin to activate insulin receptor tyrosine kinase activity was then studied. In both HTC-IR cells and HTC-IR mutant cells,

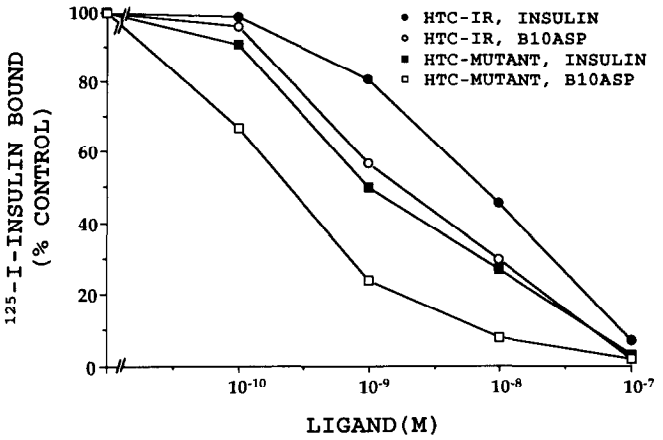

Figure 1 Binding of insulin analogue AspB10 (AspB10) and native insulin (insulin) to normal human insulin receptors (HTC-IR) and mutant insulin receptors (HTC-Mutant) expressed in intact HTC cells. Shown are competition inhibition curves for binding of ^{125}I-insulin to HTC cells transfected with normal insulin (IR), and mutant (IR-mutant) receptors. A representative experiment is shown.

AspB10 was more potent than native insulin in activating this function (Figure 3). In HTC-IR cells, the ED_{50} of insulin was 4.5 ± 2.9 nM (mean ± SD, n=3) and the ED_{50} of AspB10 insulin was 2.0 ± 0.2 nM. In HTC-IR mutant, cells the ED_{50} of insulin was 2.4 ± 0.6 nM and the ED_{50} of AspB10 insulin was 0.8 ± 0.3nM.

DISCUSSION

Previously we reported that when the insulin receptor was mutated at sequence 240-250 of the α subunit to produce a net positive charge in the molecule, both insulin binding and activation of tyrosine kinase were enhanced 2-3 fold (6). We postulated therefore that sequence 240-250 comprised an insulin-binding site within the insulin receptor α subunit, and that both charge and hydrophobicity were required for optimal interaction between insulin and its receptor.

TABLE I

BINDING OF INSULIN AND ANALOGUES TO HTC-IR AND HTC-IR MUTANT CELLS

	One half maximal of Inhibition of ^{125}I-insulin Binding (nM)	
Analogue	HTC-IR	HTC-IR Mutant
Insulin	6.9±1.8	2.0±0.8
AspB10	2.1±0.6	0.8±0.4
GluB22	6.6±2.7	2.6±1.6
AspB28	10.6±2.6	3.3±1.7
AspB9	19.1±4.1	10.3±3.3

Each value is the mean ± SD for 4 separate experiments.

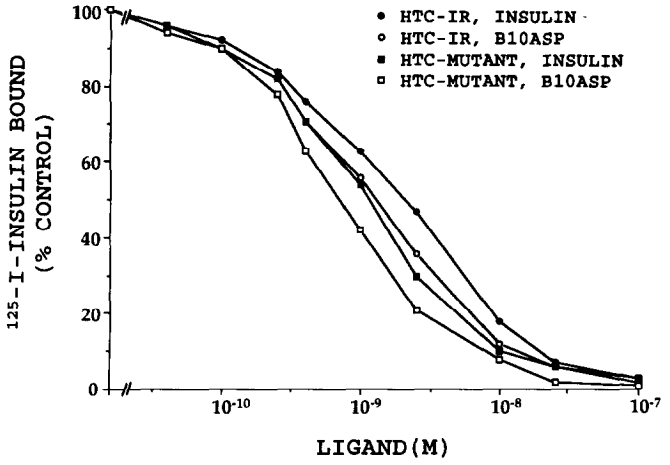

Figure 2 Binding of Asp B10 insulin (B10Asp) and native insulin (insulin) to membranes prepared from HTC cells expressing normal insulin receptors (HTC-IR) and HTC cells expressing mutant receptors (HTC-mutant). A representative experiment is shown.

Subsequently, using an antibody generated against this sequence, we presented additional data suggesting that this sequence formed part of the insulin-binding domain of the insulin receptor, and that the binding of insulin to its receptor a subunit induces a conformational change of the receptor in this region (5).

Insulin analogues with enhanced potencies have been synthesized and studied. An insulin analogue in which B10 His is replaced by aspartic acid has been found to have increased affinity of binding and increased biological activity (7,12). In contrast, replacement of B10 His with asparagine results in reduced biological activity (13). One of the possible explanations for the increased activity of AspB10 insulin analogue is that this negatively charged AspB10 residue

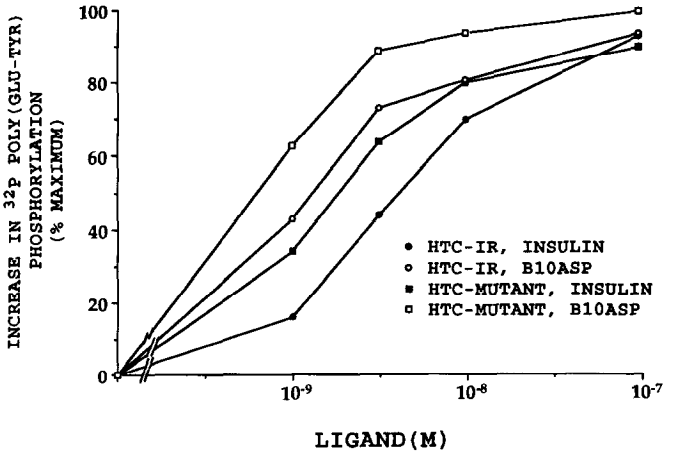

Figure 3 Tyrosine kinase activity of the insulin receptor toward the synthetic substrate poly(Glu-Tyr) in HTC cells transfected with normal insulin receptors (HTC-IR) or mutant receptors (HTC- mutant). Receptors solubilized and purified by wheat germ affinity chromatography were assayed for tyrosine kinase activity as described in methods. A representative experiment is shown.

interacts directly with a positively charged site on the receptor. More recently it was reported (14) that the analogue, des-(B26-B30)-[B10Asp,TyrB25-NH_2] insulin was 11-13 fold more potent than intact insulin with respect to receptor binding affinity and stimulation of lipogenesis. These studies suggested, therefore, that residues B10 and B25 could be components of two distinct receptor-binding domains.

In the present study we find that the negatively charged mutant insulin analogue AspB10 had increased binding affinity to both the normal and the mutated insulin receptor. Moreover, increased binding activity was reflected in increased tyrosine kinase activity. These findings, therefore, support the concept that sequence 240-250 comprises an insulin-binding site within the insulin receptor, and that ionic interactions in this region may play a role in binding affinity. However, the presence of an additional negative charge in the insulin molecule, per se, is itself insufficient for enhancing binding affinity since other mutant insulins having additional negative charges at sites other than B10 (Table I) did not exhibit an enhancement of binding. Further studies utilizing additional mutant insulin receptors and mutant insulins should produce more information on the mechanism of insulin-insulin receptor interaction.

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