Shortened Insulin Analogues: Marked Changes in Biological Activity Resulting from Replacement of TyrB26 and N-Methylation of Peptide Bonds in the C-Terminus of the B-Chain†

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ABSTRACT: The role of three highly conserved insulin residues PheB24, PheB25, and TyrB26 was studied to better understand the subtleties of the structure−function relationship between insulin and its receptor. Ten shortened insulin analogues with modifications in the β-strand of the B-chain were synthesized by trypsin-catalyzed coupling of *des*-octapeptide (B23−B30)-insulin with synthetic peptides. Insulin analogues with a single amino acid substitution in the position B26 and/or single N-methylation of the peptide bond at various positions were all shortened in the C-terminus of the B-chain by four amino acids. The effect of modifications was followed by two types of in vitro assays, i.e., by the binding to the receptor of rat adipose plasma membranes and by the stimulation of the glucose transport into the isolated rat adipocytes. From our results, we can deduce several conclusions: (i) the replacement of tyrosine in the position B26 by phenylalanine has no significant effect on the binding affinity and the stimulation of the glucose transport of shortened analogues, whereas the replacement of TyrB26 by histidine affects the potency highly positively; [HisB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide and [NMeHisB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide show binding affinity 529 and 5250%, respectively, of that of human insulin; (ii) N-methylation of the B24−B25 peptide bond exhibits a disruptive effect on the potency of analogues in both in vitro studies regardless the presence of amino acid in the position B26; (iii) N-methylation of the B23−B24 peptide bond markedly reduces the binding affinity and the glucose transport of respective analogue [NMePheB24]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide.

Recently, much attention has been paid to the insulin receptor molecule and its interaction with insulin. Although the structure of the insulin receptor or other receptors from tyrosine-kinase superfamily has not been solved completely, structures of individual domains of some of these receptors were determined (*1*−*3*).

The insulin receptor is a heterotetramer $\beta-\alpha-\alpha-\beta$, in which two heterodimers $\alpha-\beta$ are linked by two disulfide bridges (*4*). The parts of the insulin receptor responsible for interaction are located on α-subunit, mainly in N-terminal domains designated as L1−CR−L2 (*5*, *6*). Garrett et al. (*1*) solved the crystal structure of L1−CR−L2 domains of the α-subunit of the homologous type-1 insulin-like growth-factor (IGF-1) receptor. Luo et al. (*7*) tried to clarify the insulin−insulin receptor complex by using electron cryomicroscopy with insulin labeled by gold marker.

Although the interaction of insulin with its receptor has been outlined, particular residues involved in this contact have not yet been specified. Almost all studies (*5*, *7*−*15*) are consistent that main ligand-binding sites on the insulin receptor are located on the L1 and L2 domains. Alanine scanning mutagenesis of the insulin receptor ectodomain determined important residues for these interactions (*16*−*18*). These studies support previously published results that not only the N-terminus of the α-subunit but also the C-terminus of the α-subunit (residues 704−716) is essential for ligand binding (*19*). Two insulin monomers might bind to the receptor, but only one insulin molecule is necessary for high-affinity binding (*10*, *20*).

Studies on human insulin mutants, such as Chicago PheB25 → LeuB25, Los Angeles PheB24 → SerB24 (*21*−

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[1] Abbreviations: BSA, bovine serum albumin; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DOI, *des*-octapeptide (B23−B30)-insulin; DTI, *des*-tetrapeptide (B26−B30)-insulin; EC_{50}, the concentration required for 50% maximal effect; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HI, human insulin; IC_{50}, the concentration required for 50% inhibition; IGF-I, insulin-like growth factor I; MALDI-TOF, matrix assisted laser desorption and ionization-time-of-flight; NMP, 1-methyl-2-pyrrolidinone; PMSF, phenylmethylsulfonyl-fluoride; PyBroP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; RP-HPLC, reverse phase-high performance liquid chromatography; RT, room temperature; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TIS, triisopropylsilane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane;

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23), and new synthetic or semisynthetic insulin analogues confirmed the importance of invariant residues in the C-terminal part of the B-chain (*24*−*27*). Insulin analogues with modification in the C-terminal domain of the B-chain, particularly in PheB25, demonstrated that the replacement of residues in this region with branched-chain hydrophobic or hydrophilic amino acids causes a pronounced decrease of the biological activity.

Crystallographic analyses (*28*) have shown that the C-terminal part of the B-chain is vital for the dimerization of two insulin monomers (designated as molecules I and II). Residues B24−B25−B26 of the molecule I pack against the equivalent region of molecule II and form antiparallel β-sheet conformation (*28*, *29*). PheB24 and TyrB26 form hydrogen bonds with the same residues in the second molecule of insulin. In contrast to minor conformational movement of the side chains of PheB24 and TyrB26, the side chains of both PheB25 undergo significant changes. In molecule I, PheB25 is folded back against TyrA19. The carbonyl oxygen of TyrA19 subsequently creates a hydrogen bond with the amide hydrogen of PheB25. In molecule II, the side chain of PheB25 turns across the surface of the dimer interface toward molecule I (*29*). It appears that while PheB24 and TyrB26 play a fundamental role in the self-association of insulin (which is important for storage of the hormone in the pancreas), PheB25 is probably critical for ligand binding to the insulin receptor (*26*, *30*, *31*), and its side chain seems to be involved in a direct hormone−receptor contact (*19*, *20*, *32*).

Moreover, the C-terminal of the B-chain, especially PheB25, is involved in the phenomenon described as negative cooperativity (*33*). It means that the binding of one ligand molecule decreases the binding affinity of other insulin molecules to neighboring binding sites (*14*).

Whereas the deletion of residues B26−B30 causes no change in the biological activity of insulin, providing that the C-terminus of B-chain is amidated (*34*−*38*), analogues modified in the last four amino acids of the C-terminal part of B-chain can provide interesting results (*13*, *39*, *40*), e.g., analogues with the highest observed receptor affinity were those modified in the position B26 (*40*−*42*).

For the above-mentioned reasons, and also because of the simplicity of synthesis and due to unclear significance of C-terminal amino acids B27−B30, all our analogues described in this study were *des*-tetrapeptide (B27−B30)-insulin-B26-amides (DTI-amides). The amidation of the C-terminal carboxy group neutralizes the negative charge of the respective carboxylate (*35*). Tyrosine, originally in position B26, was replaced by a residue with bulky side chain for better understanding of structure−activity relationships during insulin−insulin receptor interaction. The peptide bond between residues B24−B25 or B25−B26 was modified by substitution of the amide hydrogen with a methyl group and the resulting peptides were compared with the same analogues without N-methylation. In addition, the analogue [NMePheB24]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide ([NMePheB24]DTI-amide) was prepared to evaluate the influence of N-methylated B23−B24 peptide bond.

EXPERIMENTAL PROCEDURES

Materials. Amino acids were purchased from Nova Biochem (Laufelfingen, Switzerland) and Bachem (Bubendorf, Switzerland). Trypsin (E. C. 3.4.21.4.) and human insulin were purchased from Sigma-Aldrich (St. Louis, USA), and porcine insulin was from Spofa a.s. (Prague, Czech Republic). All other reagents were purchased from Nova Biochem, Sigma-Aldrich, and Lachema (Neratovice, Czech Republic). All chemicals used were of analytical grade. Gel chromatography was performed on Sephadex G-50 fine (Pharmacia, Uppsala, Sweden), analytical reversed-phase chromatography on column Watrex (Nucleosil 120−5μm C18 250 × 4 mm; San Francisco, CA) column and the preparative reversed-phase chromatography on Vydac (218TP510 25 × 1 cm; Columbia, MD) column. For gradient RP-HPLC analysis, the high-pressure liquid chromatography Waters LC 625 system (Milford, MA) was used. Different gradients of acetonitrile (8−72%) in water containing 0.1% (v/v) of TFA were used for peptide and protein elution. The mass spectroscopy was performed using a ZAB-EQ spectrometer with BEQQ geometry (VG Analytical; Manchester, UK) for FAB analysis and a Bruker Reflex IV (Billerica, MA) TOF mass spectrometer for MALDI-TOF data. Amino acid analyses were performed on Durrum D-500 analyzer (Palo Alto, CA).

Preparation of Des-octapeptide-(B23−B30)-insulin (DOI). Zn-free porcine insulin (*43*) was dissolved in 0.05 M Tris/HCl pH 9.0−9.2, to which TPCK-trypsin in 0.1 M $CaCl_2$ was then added at a molar enzyme/substrate ratio of 1:50. This solution was left for 20 h at RT, and then the pH was adjusted to 5.4 by 1 M HCl. The solution was centrifuged at 2000*g* for 20 min, the supernatant was poured off, and the sediment was dissolved in 10% acetic acid. *Des*-octapeptide-(B23−B30)-insulin was isolated by gel chromatography.

Synthesis of Tetrapeptides. Tetrapeptides were synthesized by solid-phase peptide synthesis employing the Fmoc-protecting strategy (*44*) for α-amino groups of individual amino acids. Coupling of amino acids was carried out using HBTU/DIPEA coupling reagents (*45*) in NMP. Coupling of N-methylated amino acids was performed using PyBroP/DIPEA reagents (*46*) in NMP. The peptides were cleaved from the resin by TFA/TIS/H_2O (96:2:2 v/v).

General Procedure for the Enzymatic Semisynthesis of Insulin Analogues. The semisyntheses (*47*−*49*) of the shortened insulin analogues were performed according to the method of Svoboda et al. (*50*) with few modifications. The amino component (150 mM) and DOI (30 mM) were dissolved in a solution (the total volume of 200 μL) containing 55% aq. dimethylformamide, 10 mM $CaCl_2$, and 2.8 mg of TPCK-trypsin (enzyme/substrate molar ratio of 1:50). The pH value was adjusted by *N*-methylmorpholine to 6.9−7.0. The resulting mixture was incubated for 4−7 h at RT. The reaction, monitored by analytical RP-HPLC, was stopped by the addition of acetone (4 °C). The sediment was dissolved with 10% acetic acid, and the product was separated from trypsin, DOI, and side products by preparative reversed-phase HPLC using gradient of acetonitrile. The fractions containing the product were pooled, concentrated under reduced pressure, and lyophilized.

Isolation of Rat Adipose Tissue Plasma Membranes. Plasma membranes (*51*) were prepared from epididymal fat of adult male Wistar rats weighing 210−250 g. The fat (5−6 g) was homogenized using ultra-turrax with 20 mL of buffer consisting of 10 mM Tris/HCl, 250 mM sucrose, 1 mM

PMSF, and 1 mM benzamidine, pH 7.4. The mixture was centrifuged at 3000*g* for 15 min at 4 °C, and then the supernatant was centrifuged again at 17000*g* for 15 min at 4 °C. The resulting pellet was resuspended in 1 mL of 0.05 M Tris/HCl buffer, pH 7.6, and the protein concentration was determined according to Bradford (*52*).

Receptor Binding Studies. The modified assay of Zorad et al. (*51*) was used to determine relative receptor binding affinities to rat adipose plasma membranes. Mono component ^{125}I-iodotyrosyl^{A14}-porcine insulin was prepared using the lactoperoxidase method (*53*). Plasma membranes (50 μg of proteins) were incubated in 5-mL tubes with ^{125}I-insulin at a concentration of 2×10^{-10} M (~70 000 cpm) and various concentrations (range, $10^{-13}-10^{-6}$ M) of insulin or insulin analogues in the buffer composed of 100 mM Tris/HCl, 2 mM N-ethylmaleimide, 13.2 mM $CaCl_2$, and 0.1% (w/v) BSA, pH 7.6, in a total volume of 250 μL. The solution was incubated at 4 °C for 21 h, and then the reaction was terminated by the addition of ice-cold 120 mM NaCl followed by a quick filtration on a Brandel cell harvester (Biochemical Research and Development Laboratories, Gaithersburg, MD). Bound radioactivity was determined by γ-counting (Gamma Counter Minigamma 1275, LKB-Wallac, Sweden). The total binding (the binding in the absence of the competitor) was about 10% of the total radioactivity. The nonspecific binding (the binding in the presence of 10^{-5} M insulin) was determined to be less than 15% of the total binding.

Analysis of Binding Data. Competitive binding curves were plotted using Graph-Pad Prizm 3 software (San Diego, CA), comparing best fits for single binding site models. Half-maximal inhibition of binding of ^{125}I-insulin to receptor (IC_{50}) values were obtained from nonlinear regression analysis.

Preparation of Isolated Adipocytes. Male Wistar rats weighing 210−250 g were used in all experiments. Rats were decapitated and their epididymal fat was removed. Approximately 3 g of fat was cut, and the isolated fat cells were prepared by shaking at 37 °C for 60 min in Krebs-Ringer bicarbonate buffer containing collagenase (3 mg/mL) and 3.5% (w/v) BSA with 5 mM glucose under CO_2/O_2 atmosphere according to the method of Rodbell (*54*). Cells were then filtered through nylon mesh, centrifuged at 100*g* for 2 min, and washed three times in Krebs-Ringer bicarbonate buffer containing 16 mM Hepes, 2% (w/v) BSA. Adipocyte counts were performed according to modified method of Hirsch and Gallian (*55*), in which the percentual content of cells in suspense is determined.

Glucose Transport Studies. The assay measures the total uptake of the radiolabeled 2-deoxyglucose and is based on the principle that while 2-deoxyglucose is transported and phosphorylated by the same process as D-glucose, it cannot be further metabolized. We used modified methods of Cherqui (*56*) and Olefsky (*57*) for monitoring of the stimulation of glucose transport to the isolated adipocytes. Adipocytes (15% suspension of the cells) were incubated in Krebs-Ringer bicarbonate buffer, 16 mM Hepes, 2% (w/v) BSA, pH 7.6, in the absence and in the presence of various concentrations of insulin (range $10^{-12}-10^{-6}$ M) in total volume of 500 μL. Incubations were performed in polypropylene tubes in a shaking water bath at 37 °C for 30 min under CO_2/O_2 atmosphere. Glucose transport was then measured by a procedure starting with the addition of 10 μL of 2-deoxyglucose containing 50 μM 2-deoxy-1-^{3}H-glucose (Amersham, Little Chalfont, UK). After 5 min, the reaction was terminated by addition of 5 mL of ice-cold 120 mM NaCl and 1.5 mL of silicone oil. The tubes were centrifuged at 1000*g* for 3 min at 4 °C, and the adipocytes were collected with special absorbing material. The uptake of ^{3}H-2-deoxyglucose was determined by β-counting (Scintillation Beta Counter, Beckman LS6000, USA using scintillation Bray solution, Karlsruhe, Germany). The nonspecific transport was determined in the presence of 5 μM cytochalasin B, an inhibitor of glucose transport. Evaluation of glucose transport is termed as biological activity of insulin and its analogues throughout the paper.

Analysis of Glucose Transport Data. Sigmoidal dose−response curves for 2-deoxyglucose uptake by isolated rat adipocytes were plotted using Graph-Pad Prizm 3 software. EC_{50} values, i.e., the concentration giving half-maximum effect, were determined from nonlinear regression analysis. Data were expressed in % of maximal stimulation.

RESULTS

We prepared several new analogues of insulin for better understanding the detailed structure−function relationship of insulin upon the interaction with its receptor. The modifications were introduced into the β-strand in the B-chain, a part of insulin responsible for interaction with the receptor. All analogues were shortened in the C-terminus of the B-chain of insulin by four amino acids. As discussed previously, this part of insulin can be deleted without any influence on the biological activity of insulin (*26*, *34*). Although the C-terminal amidation is not a basic requirement for full biological activity (*42*, *58*), all analogues were prepared as carboxamides in the position B26 for suppression of possible negative effect of the C-terminal carboxylate of the B-chain (*34*, *35*).

We prepared 10 analogues of HI and determined their binding affinities and biological activities. The analogues were prepared by trypsin-catalyzed enzymatic semisynthesis from *des*-(B23−B30)-octapeptide-insulin and synthetic tetrapeptides. We decided to divide insulin analogues into three series (Tables 1 and 2). Each series differs from the two other in the amino acid in the position B26: tyrosine ("tyrosine B26 series"), phenylalanine ("phenylalanine B26 series"), or histidine ("histidine B26 series") were placed in the B26 position for their hydrophobic character (tyrosine occurs in this position in wild-type insulin). The second parameter in which the analogues differ is the methylation of peptide bonds in B24−B25 or B25−B26 positions.

Characterization of Insulin Analogues. Insulin analogues were purified by RP-HPLC and the yields were between 15 and 25%. The results of MALDI-TOF or FAB mass spectroscopy and results of amino acid analyses corresponded to the expected data within the error limits of the methods.

Receptor Binding Studies. The relative receptor binding potencies of semisynthetic analogues determined on the adipose plasma tissue are shown in Figure 1A−D and in Table 1. The first group (Figure 1A) represents "tyrosine B26 series", in which the shortened analogues contain tyrosine at position B26 as it is in the case of wild-type insulin. The data show a moderate improvement of receptor binding affinity of [NMeTyrB26]DTI-amide (180%) and a

Table 1: Values of IC_{50}[a] and Receptor Binding Affinities[b] of Insulin and Insulin Analogues

peptide	IC_{50} ± SEM [nM]	(*n*)	potency [%]
human insulin	1.626 ± 0.97	(9)	100
porcine insulin	0.510 ± 0.21	(10)	319
"*Tyrosine B26 series*"			
DTI-amide	0.396 ± 0.13	(7)	410
[NMePheB25]DTI-amide	110.8 ± 43.3	(4)	1.5
[NMeTyrB26]DTI-amide	0.900 ± 0.41	(7)	180
"*Phenylalanine B26 series*"			
[PheB26]DTI-amide	1.625 ± 0.69	(6)	100
[NMePheB25,PheB26]DTI-amide	81.37 ± 25.9	(6)	2.0
[NMePheB26]DTI-amide	2.02 ± 1.03	(4)	80
"*Histidine B26 series*"			
[HisB26]DTI-amide	0.307 ± 0.12	(7)	529
[NMePheB25,HisB26]DTI-amide	102.9 ± 50.3	(4)	1.6
[NMeHisB26]DTI-amide	0.031 ± 0.02	(5)	5250
[NMePheB24]DTI-amide	15.7 ± 5.9	(4)	10.4

[a] IC_{50} values represent concentration of insulin or insulin analogue causing half-maximal inhibition of binding of ^{125}I-insulin to insulin receptor. Each value represents the mean ± SEM of multiple determinations; the number of separate determinations is shown in parentheses. [b] Relative receptor binding affinity is defined as (IC_{50} of human insulin/IC_{50} of analogue) × 100. See Experimental Procedures for details.

Table 2: Values of EC_{50}[a] and Biological Activity Expressed by Ability to Stimulate of Glucose Transport[b] of Insulin and Insulin Analogues

peptide	EC_{50} ± SEM [nM]	(*n*)	potency [%]
human insulin	0.235 ± 0.331	(6)	100
porcine insulin	0.107 ± 0.115	(5)	220
"*Tyrosine B26 series*"			
DTI-amide	0.050 ± 0.075	(3)	470
[NMePheB25]DTI-amide	22.10 ± 11.95	(3)	1.1
[NMeTyrB26]DTI-amide	0.052 ± 0.019	(4)	450
"*Phenylalanine B26 series*"			
[PheB26]DTI-amide	0.160 ± 0.210	(3)	148
[NMePheB25,PheB26]DTI-amide	52.65 ± 25.24	(2)	0.45
[NMePheB26]DTI-amide	4.39 ± 1.37	(4)	5.35
"*Histidine B26 series*"			
[HisB26]DTI-amide	>0.01	(3)	>1000
[NMePheB25,HisB26]DTI-amide	10.46 ± 6.71	(2)	2.25
[NMeHisB26]DTI-amide	>0.01	(3)	>1000
[NMePheB24]DTI-amide	6.67 ± 5.04	(3)	3.52

[a] EC_{50} values represent effective concentration generating half-maximal response of insulin or insulin analogue. Each value represents the mean ± SEM of multiple determinations; the number of separate determinations is shown in parentheses. [b] Relative potency to stimulate incorporation of 2-deoxy-1-^{3}H-glucose into isolated adipocyte is defined as (EC_{50} of human insulin/EC_{50} of analogue) × 100. See Experimental Procedures for details.

high binding affinity (410%) of DTI-amide compared to human insulin. Despite the conservation of the crucially important phenyl group of PheB25, however, [NMePheB25]-DTI-amide exhibited major loss of binding affinity (1.5%).

The "phenylalanine B26 series" as the second group of analogues (Figure 1B) gives similar results as the "tyrosine B26 series". The replacement of the tyrosine in position B26 by phenylalanine nevertheless affected the binding affinity rather negatively. Although the [PheB26]DTI-amide analogue retains the full binding affinity (100%) of human insulin, it is less potent compared with DTI-amide. [NMePheB26]DTI-amide exhibits nearly full receptor binding affinity (80% of that of human insulin) but if compared with [NMeTyrB26]DTI-amide, it is less potent, as observed also in the case of the respective nonmethylated analogues. Further analogues, [NMePheB25,PheB26]DTI-amide and [NMePheB25]DTI-amide, show markedly reduced receptor binding affinity (2.0%) relative to the human insulin.

The third group, "histidine B26 series" (Figure 1C), presents the effects of imidazole side chain of histidine in the position B26 combined with N-methylation of various peptide bonds. Replacement of TyrB26 in insulin molecule by histidine has a very positive effect on the ability of the respective analogues to bind to the receptor. However, the [NMePheB25, HisB26]DTI-amide analogue exhibits a markedly high decrease in receptor binding affinity (1.6%), similarly as in the case of analogues with N-methylated peptide bond between B24−B25. The nonmethylated [HisB26]-DTI-amide analogue showed high affinity (529%) to the insulin receptor, and [NMeHisB26]DTI-amide surprisingly displayed one of the highest binding affinities of the analogues of human insulin for which the data are available, namely, 5250%.

The effect of N-methylation of the peptide bond between B23 and B24 on the binding potency is presented in Figure 1D. [NMePheB24]DTI-amide analogue decreased the receptor binding affinity 10-fold compared to wild-type insulin. Nevertheless, the effect of N-methylation of the B23−B24 peptide bond is less pronounced than N-methylation in the position B24−B25, which has a crucial effect on the respective analogues in all three series.

Glucose Transport Studies. The biological activity is expressed by the ability of analogues to stimulate the transport of 2-deoxyglucose into isolated rat adipocytes. Displacement curves and the data of the individual analogues are shown in Figure 2A−D and in Table 2. The results are similar to the data obtained from receptor affinity studies. In brief, the nonmethylated, shortened analogues displayed full or even higher biological activity, while the analogues with N-methylated peptide bond B24−B25 showed a marked decrease in their ability to stimulate glucose transport. In the "tyrosine B26 series" (Figure 2A), DTI-amide and [NMeTyrB26]DTI-amide analogues exhibited a more than 4-fold increase (470 and 450%, respectively) of biological activity relative to that of human insulin. [NMePheB25]DTI-amide retained only 1.1% activity of human insulin. In the "phenylalanine B26 series" (Figure 2B) the [PheB26]DTI-amide analogue had the same activity in comparison with human insulin, but lower in comparison with DTI-amide. Surprisingly, the [NMePheB26]DTI-amide analogue exhibited a very low (5.3%) biological activity relative to that of human insulin, even if its binding affinity was similar to human insulin. The biological activities of analogues [NMePheB25,PheB26]DTI-amide and [NMePheB25,HisB26]-DTI-amide were consistent with other results and showed very low ability (0.5 and 2.3%, respectively, to that of insulin) to stimulate the glucose transport into rat adipocytes. Analogues from "histidine B26 series" [HisB26]DTI-amide and [NMeHisB26]DTI-amide were able to increase the glucose transport more than 10-fold in comparison with human insulin.

DISCUSSION

Our results with new insulin analogues with selected amino acid substitutions at position B26, and with modifications

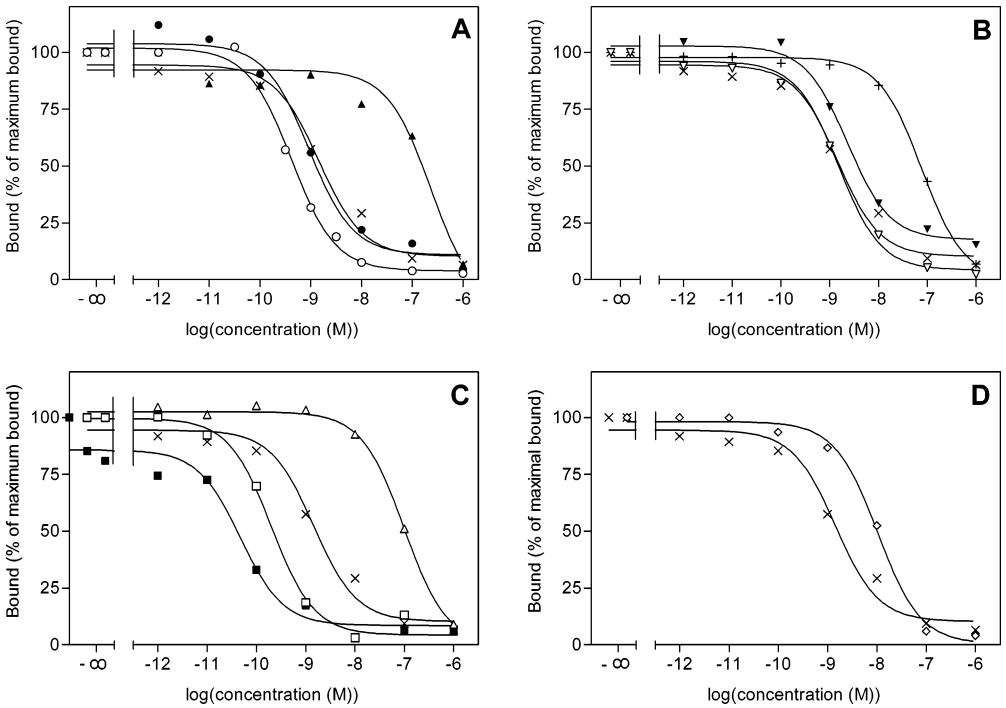

FIGURE 1: Inhibition of binding of ^{125}I-insulin to adipose tissue plasma membranes by insulin and insulin analogues. See Experimental Procedures for details. Quantitative information is provided in Table 1. The curves represent typical binding experiment. (A) Human insulin (×); *des*-tetrapeptide (B27−B30)-insulin-B26-amide (○); [NMePheB25]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (▲); [NMeTyrB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (●). (B) Human insulin (×); [PheB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (▽); [NMePheB25,PheB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (+);[NMePheB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (▼). (C) Human insulin (×); [HisB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (□); [NMePheB25,HisB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (△); [NMeHisB26]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (■). (D) Human insulin (×); [NMePheB24]-*des*-tetrapeptide (B27−B30)-insulin-B26-amide (◇).

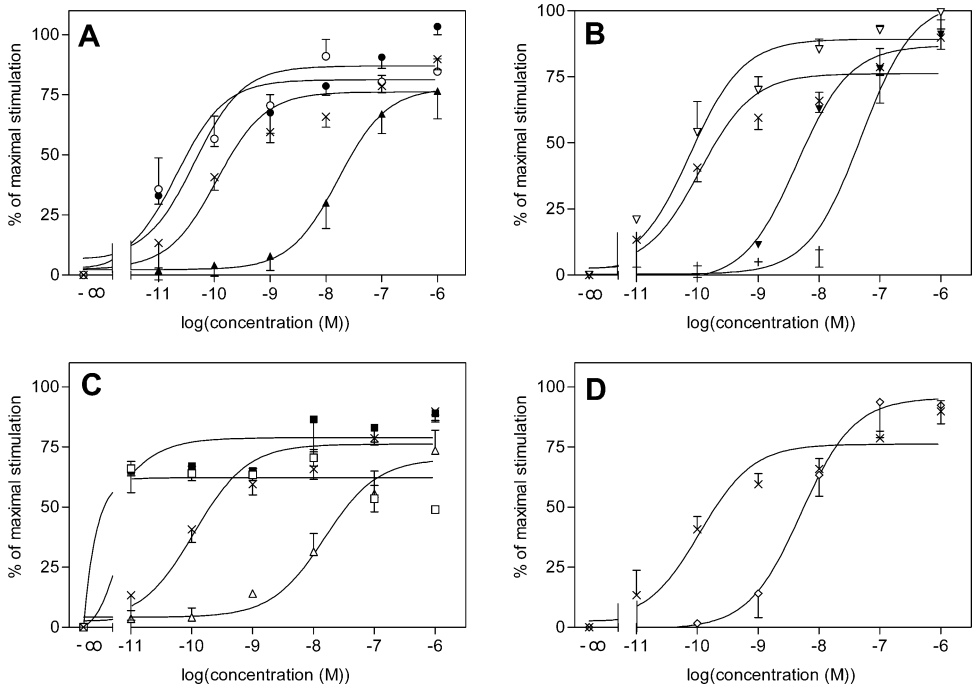

FIGURE 2: Stimulation of glucose transport into adipocytes by insulin and insulin analogues. See Experimental Procedures for details. Quantitative information is provided in Table 2. Values are mean ± SEM. (A) Human insulin (×); DTI-amide (○); [NMePheB25]DTI-amide (▲); [NMeTyrB26]DTI-amide (●). (B) Human insulin (×); [PheB26]DTI-amide (▽); [NMePheB25,PheB26]DTI-amide (+);[NMePheB26]-DTI-amide (▼). (C) Human insulin (×); [HisB26]DTI-amide (□); [NMePheB25,HisB26]DTI-amide (△); [NMeHisB26]DTI-amide (■). (D) Human insulin (×); [NMePheB24]DTI-amide (◇).

able to induce conformational changes of the peptide backbone, provide valuable proof of the importance of the C-terminus of the B-chain of insulin in the interaction with its receptor. We used enzyme catalyzed semisynthesis for the preparation of 10 analogues shortened in the C-terminus of the B-chain. We followed the well-known fact (*34*, *38*,

42) that the C-terminus of the B-chain can be shortened by no more than five residues without any loss of the receptor binding affinity. High-affinity binding of the shortened analogues, particularly, *des*-pentapeptide (B26−B30)-insulin, requires the elimination of the negative charge of the C-terminus of the B-chain by carboxamidation (*34*, *35*). The carboxamidation increases hydrophobicity (*42*), and the shortening of the C-terminus of the B-chain affects the conformation of this part of the insulin molecule. One of the structural consequences of the B-chain shortening is a better exposure of N-terminal residues of the A-chain due to the absence of the B27−B30 segment (*59*−*61*). The second consequence of the shortening could be the positive effect on the detachment of the β-strand from the central α-helix of insulin, which is the basic requirement for the accommodation of insulin in the "active conformation" (*62*−*65*).

In our experiments, the DTI-amide analogue shows an increased binding affinity (410%) and biological activity (470%) relative to those of human insulin. The DTI-amide analogue has been already prepared (*42*), and its binding affinity and biological activity were found 110 and 129%, respectively, higher if compared with porcine insulin. The discrepancy in obtained values may be because human insulin has a weaker IC_{50} value in rat adipocytes in comparison with porcine insulin. If compared with porcine insulin, our DTI-amide shows a 130% receptor binding affinity, which is in accordance with Lenz's results and other studies (*42*, *58*, *66*). We also synthesized *des*-tetrapeptide (B27−B30)-insulin without the C-terminal amidation of TyrB26 (data not shown) and compared it with the respective amidated analogue. *Des*-tetrapeptide (B27−B30)-insulin had a slightly reduced receptor binding affinity (90% of human insulin). It suggests that carboxamidation in the position B26 is no basic requirement for full binding affinity but that it enhances the binding affinity of shortened analogues (*42*, *66*) as well as of *des*-pentapeptide (B26−B30)-insulin (*24*, *35*).

Not only DTI-amide but also other nonmethylated analogues of all series show no reduction in binding affinity and biological activity. The [PheB26]DTI-amide analogue from "phenylalanine B26 series" retains full affinity (100%) and higher biological activity (148%) relative to those of human insulin, but both potencies are 4-fold lower than that of DTI-amide. This observation is in agreement with the properties of full-length [TyrB25,PheB26]-insulin analogue (*25*), which retains 80% of binding affinity of native insulin. It suggests that the replacement of TyrB26 by Phe has only little effect on the hormone analogue function, but it also shows that the tyrosine hydroxyl group may play a positive role during the interaction with the insulin receptor. Interestingly, the replacement of the amino acid in the position B26 by histidine seems to positively affect the biopotency. The [HisB26]DTI-amide analogue increased the receptor binding affinity more than 5-fold and the biological activity more than 10-fold. The imidazole ring with its capability to form a hydrogen bond can certainly affect the receptor binding as well as the ability to stimulate the transport of glucose into the cells. The positive effect of the presence of histidine in the putative receptor binding site of insulin is confirmed by the [HisB25]-*des*-pentapeptide (B26−B30)-insulin analogue (*38*) the receptor binding affinity of which is 313% of that of natural insulin.

Peptide bond N-methylation has very different effects on the binding affinity and biological activity of the analogues. It can be stated that N-methylation of a specific peptide bond affects analogues from all series in the same way. In general, the substitution of the amide hydrogen of a peptide bond with the methyl group has many implications. First, N-methylation of a peptide bond results in the increase of local flexibility of the main chain (*40*, *67*, *68*). Even if the flexibility of the C-terminus of the B-chain is necessary for high-affinity binding, several studies suggest that the excessive flexibility of peptide bonds in this part of insulin may entail low receptor binding affinity (*68*−*70*). Indeed, N-methylation of a peptide bond in this part of insulin does not cause such perturbations as does the replacement of a peptide bond by methylene (*68*) or by ester bond (*69*, *70*), but consequences should be very similar. Second, N-methylation results in the elimination of the ability to form intermolecular or intramolecular hydrogen bonds, which are important for stability and right folding of the insulin monomer, for dimer forming, and for interaction of insulin with its receptor. Furthermore, the introduction of the methyl group into the peptide chain increases hydrophobicity and can destabilize the β-strand of the B-chain.

N-methylation of the peptide bond between the residues B23 and B24 in [NMePheB24]DTI-amide analogue decreases the receptor binding affinity to 10.4% and the biological activity to 3.5%. Although N-methylation should also have similar conformational consequences as the replacement of PheB24 by D-amino acids, our result is not in agreement with the increased binding potency of [D-PheB24]-insulin and [D-AlaB24]-insulin analogues (*71*, *72*). It suggests that the possible perturbation of the β-strand in the position B24 due to the substitution by D-amino acid does not affect binding ability, whereas the elimination of the capability of the hydrogen bonding of PheB24 due to N-methylation is very important for the interaction with the insulin receptor, and it seems that capability of the amide nitrogen of PheB24 to donate hydrogen is crucial for full receptor binding potency.

All our analogues with N-methylation between residues B24 and B25, regardless of the amino acid in position B26, show markedly reduced binding affinity and biological activity. The [NMePheB25]DTI-amide, [NMePheB25,PheB26]-DTI-amide, and [NMePheB25,HisB26]DTI-amide analogues retain no more than 2.3% potency of that of human insulin in both in vitro studies. The most important implication resulting from N-methylation between PheB24 and PheB25 is the elimination of the hydrogen bond between PheB25 and TyrA19. Correct contacts between the β-strand of the B-chain (*65*) and the rest of insulin molecule ensure correct alignment of the entire insulin molecule and subsequently a proper rearrangement associated with receptor binding. Although N-methylation of the peptide bond between B24 and B25 could increase the flexibility and subsequently the possibility of the detachment, the resulting effect is rather unfavorable. The same results were achieved with the replacement of this peptide bond by a methylene group (*68*) or by an ester bond (*69*, *70*). The destabilization of the β-strand of the B-chain along with the steric effect of the methyl group can negatively affect the interaction with the insulin receptor, but it seems that the most important requirement for correct insulin−insulin receptor interaction

is the preservation of the hydrogen bond between the amino group of PheB25 and the carbonyl oxygen of TyrA19.

In contrast to N-methylation of the peptide bond between PheB24 and PheB25, N-methylation of the peptide bond between PheB25 and an amino acid in the position B26 has no fatal consequences for binding ability and biological activity. N-methylated amino acid in the position B26 affects the receptor binding affinity and transport of glucose, but the results are in parallel with the results obtained with our nonmethylated analogues. The [NMePheB26]DTI-amide analogue retains nearly full receptor binding affinity but has a low biological activity. This discrepancy could be explained by the potential antagonism of this analogue. The decreased potencies of [NMePheB26]DTI-amide, particularly if compared with DTI-amide, are consistent with the fact that not only the presence of phenyl ring but also the presence of a group (as is hydroxyl of tyrosine B26) capable of hydrogen bonding or another contacts is necessary for high-affinity receptor binding. From this point of view, it seems logical that [NMeTyrB26]DTI-amide analogue is superior in both these aspects, and it supports the hypothesis mentioned above that shortened analogues are probably more accessible for the interaction with the receptor. The [NMeHisB26]DTI-amide is the analogue with the highest binding affinity of all insulin analogues described so far. N-methylation of the peptide bond B25−B26 probably shapes the imidazole ring of HisB26 to a very favorable conformation. Then, elimination the hydrogen bond of blocked nitrogen of α-amino group of histidine is largely compensated by the ability to form a new hydrogen bond in another place (nitrogen of imidazole). This effect may probably be the reason for the "superpotency" of this analogue. While the insertion of a methylene group in this position in the full-length analogue leads to extremely low potency (*68*), the substitution of Tyr by D-amino acid in shortened analogues leads to higher binding affinity, namely, to 200% in case of D-TyrB26 (*42*), and to 1250% in case of D-AlaB26 (*41*). The substitution of TyrB26 by sarcosine (*N*-methylglycine) in a shortened analogue was also very successful and enhanced the receptor binding affinity 11-fold (*40*). Moreover, all analogues N-methylated between positions B25 and B26 do not exhibit the necessity to form a hydrogen bond with the respective peptide bond nitrogen, the hydrogen bond which is naturally one of four H-bonds stabilizing the insulin dimer and which participates in insulin−insulin receptor interaction.

We hope that our new analogues shed more light on the importance of the C-terminus of the B-chain, mainly the B24−B26 fragment and its role in intramolecular contacts and in the interaction of insulin with its receptor.

CONCLUDING REMARKS

In the present study, we investigated the effect of the substitution of TyrB26 by Phe or His combined with the effect of N-methylation of three different peptide bonds at the C-terminus of the B-chain of insulin analogues shortened by four amino acids on the binding affinity and biological activity of resulting insulin analogues. Our results suggest that Tyr and especially His in the position B26 positively affect the binding affinity and the biological activity of respective analogues. This effect, which is not possible in the case of PheB26, is certainly due to the ability of hydrogen bonding of side chains of TyrB26 and HisB26. N-methylation of peptide bonds B23−B24 or B24−B25 markedly reduces both affinity and activity of analogues, and this effect is particularly significant for the B24−B25 position. On the other hand, the N-methylation of peptide bond B25−B26 has no negative effect on the receptor binding affinity, but it does have different effects on the biological activity. In fact, if the combination of B25−B26 N-methylation with PheB26 results in a very weak analogue, the combination of B25−B26 N-methylation with HisB26 leads to a "superpotent" insulin analogue. We suppose that B25−B26 N-methylation may result in a different orientation of a residue in the position B26 and thus may enable a more favorable exposure of B26 side chain to form a hydrogen bond with the insulin receptor. Overall, these results are very consistent with each other and convey several clear implications depending on the modifications used.

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