Insulin Analogues with Modifications at Position B26. Divergence of Binding Affinity and Biological Activity †

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Insulin Analogues with Modifications at Position B26. Divergence of Binding Affinity and Biological Activity[†]

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ABSTRACT: In this study, we prepared several shortened and full-length insulin analogues with substitutions at position B26. We compared the binding affinities of the analogues for rat adipose membranes with their ability to lower the plasma glucose level in nondiabetic Wistar rats in vivo after subcutaneous administration, and also with their ability to stimulate lipogenesis in vitro. We found that [NMeHisB26]-DTI-NH_2 and [NMeAlaB26]-DTI-NH_2 were very potent insulin analogues with respect to their binding affinities (214 and 465%, respectively, compared to that of human insulin), but they were significantly less potent than human insulin in vivo. Their full-length counterparts, [NMeHisB26]-insulin and [NMeAlaB26]-insulin, were less effective than human insulin with respect to binding affinity (10 and 21%, respectively) and in vivo activity, while [HisB26]-insulin exhibited properties similar to those of human insulin in all of the tests we carried out. The ability of selected analogues to stimulate lipogenesis in adipocytes was correlated with their biological potency in vivo. Taken together, our data suggest that the B26 residue and residues B26−B30 have ambiguous roles in binding affinity and in vivo activity. We hypothesize that our shortened analogues, [NMeHisB26]-DTI-NH_2 and [NMeAlaB26]-DTI-NH_2, have different modes of interaction with the insulin receptor compared with natural insulin and that these different modes of interaction result in a less effective metabolic response of the insulin receptor, despite the high binding potency of these analogues.

The hormone insulin binds to a specific transmembrane receptor. This interaction is a key and stimulating step for a complex signaling pathway, which leads to the transport of glucose across the cell membrane in muscle and adipose cells.

In general, research on insulin analogues is still important for several reasons. New insulin analogues with faster onsets of activity and shorter durations of action and analogues with a sustained action along with a more flat time−action profile are required for better control of glycemia, and the maintenance of normoglycemia in diabetic patients. Hundreds of insulin analogues that vary in binding and biological properties have been synthesized (*1*). However, only five insulin analogues (fast-acting lispro, aspart, glulisine, long-acting glargine, and detemir) are available for clinical use (*2*). Moreover, insulin "superagonists" may be of great importance since the use of highly potent insulin analogues may reduce the quantity of synthesized insulin required for patient needs. An analogue which is more potent in vitro and in vivo could also be useful for noninjectable methods of delivering insulin, such as pulmonary administration (*3, 4*) or nasal (*5*), where the bioavailability of insulin is very low. Insulin analogues with different binding properties and biological potencies may help provide a better understanding of the subtleties of the structure−function relationships between insulin and the insulin receptor. A better understanding of these complex interactions may help in the design of new analogues and thus improve the treatment of diabetes and many other diseases related to insulin action.

The insulin receptor is a membrane glycoprotein that belongs to the superfamily of transmembrane receptor tyrosine kinases, along with insulin-like growth factor 1 and 2 (IGF-1 and IGF-2),[1] and epidermal growth factor receptors (*6*). The 480 kDa

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[1] Abbreviations: COSY, correlation spectroscopy; DCM, dichloromethane; DIPEA, *N*,*N*-diisopropylethylamine; DOI, des-octapeptide(B23−B30)-insulin; DPI, des-pentapeptide(B26−B30)-insulin; DPI-NH_2, des-pentapeptide(B26−B30)-insulin-B25-amide; DTI, des-tetrapeptide(B27−B30)-insulin; DTI-NH_2, des-tetrapeptide(B27−B30)-insulin-B26-amide; HI, human insulin; HMBC, heteronuclear multiple-bond correlation spectroscopy; HSQC, heteronuclear single-quantum correlation spectroscopy; IC_{50}, concentration required for 50% inhibition; NMP, 1-methyl-2-pyrrolidinone; PA, penicillin G acylase, penicillin amidase, or penicillin G amidohydrolase (EC 3.5.1.11); Pac, phenylacetyl; PI, porcine insulin; PMSF, phenylmethanesulfonyl fluoride; PyBroP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; ROESY, rotating frame Overhauser enhanced spectroscopy; RT, room temperature; TIS, triisopropylsilane; TOCSY, total correlation spectroscopy; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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insulin receptor is composed of two monomers, each of which contains one α and one β chain. The 135 kDa α subunit is extracellular, whereas the 95 kDa β subunit contains an extracellular portion, a single transmembrane sequence, and an intracellular tyrosine kinase domain. Recently, Ward et al. (*7, 8*) determined the crystal structure of the soluble ectodomain of isoform A of the insulin receptor in a complex with four Fab monoclonal antibodies. However, despite enormous scientific efforts, the structure of a complex of insulin with its receptor is still unknown. The main reasons for this may be the difficulty of crystallizing the wild-type insulin receptor and also the low binding affinity of insulin for the engineered soluble insulin receptor ectodomain (*9*).

Studies on the biological activities of insulin analogues, comparison of the amino acid sequences of insulin from different animals (*10, 11*), and alanine-scanning mutagenesis experiments (*12*) strongly suggest that the regions responsible for interaction with the insulin receptor mainly involve invariant amino acids located on the surface of the insulin monomer. The three-dimensional structure of insulin shows that this region may include both A-chain residues, GlyA1, IleA2, ValA3, GlnA5, TyrA19, and AsnA21, and adjacent B-chain residues, LeuB6, ValB12, TyrB16, GlyB23, PheB24, and PheB25 (*10, 11, 13, 14*). Furthermore, residues outside of this "classical binding site", such as SerA12, LeuA13, GluA17, HisB10, GluB13, or LeuB17, are also involved in receptor binding (*6, 15*).

Recently, much attention has been directed toward the movements of insulin molecules in the proximity of the insulin receptor. A commonly held view is that insulin must undergo conformational changes upon binding to its receptor. These conformational changes transform the insulin molecule into the "active conformation", which still remains uncharacterized (*16–19*). It seems that after the insulin molecule makes first contact with the insulin receptor via the C-terminus of the B-chain (possibly the side chain of PheB25), this part of the insulin molecule is detached from the central α-helix (*18, 20*). The detachment of the B-chain β-strand reorganizes exposed highly conserved side chains (IleA2 and ValA3) at the protein surface in the N-terminal α-helix of the A-chain. These hydrophobic side chains are now accessible to make direct contact with the insulin receptor. It is clear that the T-state of insulin is inactive, but the transition between the T- and R-states may play an important role in the formation of the active conformation (*19*).

The amino acids in the C-terminus of the B-chain, especially hydrophobic residues PheB24 and PheB25, involved in recognition by the insulin receptor have been the subject of a number of structure−activity studies (*18, 21–26*). The C-terminus of the B-chain, and in particular residues B24 and B26−B28 (*11*), are also essential for dimer formation. The importance of the B-chain C-terminal residues in receptor binding was also demonstrated by their critical role in negative cooperativity (*27, 28*).

The C-terminal pentapeptide B26−B30 is not needed for potent binding affinity in vitro, provided that the C-terminal carboxylate is amidated (*29, 30*). Despentapeptide[B26−B30]-insulin-B25-NH_2 (DPI-amide) displays full binding potency, while DPI has only 20−25% binding affinity (*30, 31*). In contrast, although carboxamidation of B26 in DTI-amide is not necessary (95% of that of DTI), it enhances the binding affinity (110% of that of DTI-amide) (*25*). The diminished importance of residues B26−B30 is demonstrated well by alanine-scanning mutagenesis (*1, 12*). This part of insulin is also tolerant of substitutions, since [GlyB27,GlyB28,GlyB29, GlyB30]-insulin exhibits 86% binding potency (*32*). The exact role of residues B26−B30 in biological activity is still unclear.

Although residues B26−B30 are not needed for potent binding, substitution of B26 may significantly alter the binding affinity of analogues. DTI-amides often yield highly potent insulin analogues; however, full-length analogues with the same substitutions are usually less potent than insulin. [D-TyrB26]-DTI-NH_2 has 203% binding affinity (*25*), but [D-TyrB26]-insulin has only 63% (*33*). [AlaB26]-DTI-NH_2 has 273% binding affinity (*32*), but [AlaB26]-insulin has, according to different measurements, only 54 (*34*), 82 (*32*), or 36% (*12*). Kurapkat et al. (*35*) reported the synthesis and properties of an extremely potent shortened insulin analogue, [D-AlaB26]-DTI-NH_2, which displayed 1252% binding potency when compared to human insulin. They also prepared [D-AlaB26]-insulin, which had only 18% binding potency. Leyer et al. (*32*) published details of another extremely potent analogue, [SarB26]-DTI-NH_2 (1100%), revealing that N-methylation of the B25−B26 peptide bond does not negatively alter binding. This is consistent with our previous study on a series of B26 N-methylated DTI-amides (*36*). In contrast, the [PheB25-ψ(CH_2NH)-PheB26]-DTI-NH_2 analogue, which has a reduction in the B25−B26 peptide bond, displayed only 0.36% binding potency (*37*), suggesting that the respective carbonyl oxygen plays an important role in the insulin−receptor interaction.

As mentioned above, we have recently studied a series of shortened analogues (*36*). We found that the destetrapeptide [B27−B30]-shortened analogue, [NMeHisB26]-DTI-NH_2, was extremely potent at binding to isolated rat adipocyte membranes and stimulating transport of glucose into adipocytes. This analogue exhibited more than 5000% binding affinity for rat adipocyte membranes compared to human insulin. Recently, the same analogue has been synthesized and characterized by Richard DiMarchi's group (*38*). Surprisingly, they found that this analogue is weaker (80%) at binding to membranes from engineered cells that overexpress the human insulin receptor when compared to human insulin. In this study, we decided to investigate this discrepancy in detail.

The results of Kurapkat et al. (*35*) and Leyer et al. (*32*) have inspired us to synthesize and investigate binding and/or in vivo properties of the new shortened insulin analogues, [NMeAlaB26]-DTI-NH_2 and [NMe-D-AlaB26]-DTI-NH_2, which we describe in this study. We also incorporated NMeHis and NMeAla at the B26 position of full-length insulin and compared their properties with those of shortened analogues. Here, we present the data regarding the binding affinities of these novel analogues and several previously published analogues for the insulin receptor in membranes from rat adipocytes, together with their in vivo abilities at lowering plasma glucose levels in nondiabetic Wistar rats after subcutaneous administration and their abilities to stimulate lipogenesis in rat adipocytes in vitro.

EXPERIMENTAL PROCEDURES

Materials. 2-Chlorotrityl and Sieber amide resins, protected amino acids, and reagents for solid-phase synthesis of peptides were purchased from Novabiochem (Laufelfingen, Switzerland), with the exception of Fmoc-NMe-His(Trt)-OH, which was from Anaspec (San Jose, CA). Fmoc-Lys(Pac)-OH was prepared as described previously (*39*). TPCK-treated trypsin was purchased from Sigma and PA from Fluka. Human [^{125}I]monoiodotyrosylA14-insulin was purchased from Amersham Biosciences (Piscataway, NJ), and porcine [^{125}I]monoiodotyrosylA14-insulin was from NEN-Perkin-Elmer (Waltham, MA). All other chemicals and solvents were obtained from Sigma-Aldrich-Fluka.

In general, solid-phase synthesis, enzymatic semisynthesis of analogues, purification of analogues, preparation of rat adipose membranes, and binding assays were performed as described in detail previously (*39*).

Solid-Phase Synthesis of Peptides. The octapeptides and tetrapeptides were synthesized on 2-chlorotrityl (*40*) and Sieber amide resins, respectively, using Fmoc-protected amino acids with *t*Bu protection of Thr and Tyr side chains and Trt protection of the His side chain (*41*). A similar synthetic protocol was used for both peptides. Coupling was carried out using HBTU/DIPEA reagents in NMP. Coupling after Fmoc-NMe-His(Trt)-OH or Fmoc-NMe-Ala-OH was performed with PyBrop/DIPEA reagents. The peptides were cleaved from the 2-chlorotrityl resin with a mixture of AcOH, TFE, and DCM (2:2:6, v/v/v) for 2 h and from Sieber amide resin with a 2% TFA/DCM mixture (v/v). The residue was evaporated to dryness and treated with a mixture of TFA, TIS, DCM, and H_2O (50:2:48:2, v/v/v/v) for 30 min. The mixture was evaporated in vacuo and treated with diethyl ether. The solid residue after diethyl ether extraction was dissolved in 10% AcOH, and peptide was purified using RP-HPLC (Phenomenex Luna C-18 column, 250 mm × 21.2 mm, 10 μm) with a linear gradient of acetonitrile in water with 0.1% TFA (v/v). The purity of the peptides was controlled using an analytical Nucleosil 120-5 C-18 column (250 mm × 4 mm, Watrex Praha, Czech Republic). The identity of peptides was confirmed with MALDI-TOF mass spectrometry. The identity of peptides with N-methylated peptide bonds was also confirmed using NMR spectroscopy.

NMR Spectroscopy. The NMR spectra (data not shown) of N-methylated peptides were recorded on a Varian UNITY-500 and/or Bruker AVANCE-500 spectrometer (^{1}H at 500 MHz and ^{13}C at 125.7 MHz) in DMSO-d_6. ^{1}H and ^{13}C signals were structurally assigned by means of the series of one-dimensional ([^{1}H] and [^{13}C]APT) and two-dimensional NMR spectra (^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{1}H ROESY, ^{1}H−^{13}C HMQC, and ^{1}H−^{13}C HMBC). Detailed analysis of NMR spectra confirmed the structure and purity of the prepared peptides and allowed the determination of the ratio of the isomers with cis and trans peptide bonds of the N-methylated residue.

Enzymatic Semisynthesis of Insulin Analogues. The semisyntheses of full-length insulin DTI-NH_2 analogues were performed following the method of Zakova et al. (*39*). Typically, octapeptide or tetrapeptide (150 mM) and DOI (30 mM) were dissolved in a solution (total volume of 200 μL) containing 55% aqueous dimethylformamide, 20 mM calcium acetate, and 4.7 mg of TPCK-trypsin (enzyme: substrate molar ratio of 1:30). The pH value was adjusted with *N*-methylmorpholine (usually 0.5−6 μL) to 6.9−7.0 and checked with an ISFET pH Meter IQ125 (IQ Scientific Instruments, Carlsbad, CA) or pH testing paper. The resulting reaction mixture was incubated at RT (20−25 °C). After 7−24 h, the reaction, monitored by analytical RP-HPLC, was stopped by the addition of acetone (4 °C). The sediment was dissolved in 10% acetic acid, and the product was separated from trypsin, DOI, and side products by preparative RP-HPLC using a gradient of acetonitrile. Insulin analogues were purified by RP-HPLC (Nucleosil 120-5 C-18 column, 250 mm × 8 mm). The purity of the analogues was verified using analytical RP-HPLC (Nucleosil 120-5 C-18 column, 250 mm × 4 mm). The identity of the insulin analogues was confirmed with MALDI-TOF mass spectrometry (Reflex IV, Bruker).

Enzymatic Deprotection of Full-Length Insulin Analogues. All of our full-length insulin analogues contained Lys(Pac) at the B29 position, and the Pac protecting groups were cleaved using PA. Insulin analogues (5 mg) were dissolved in 1 mL of 50 mM potassium phosphate buffer (pH 7.5), and soluble PA (10 units in 6.6 μL of buffer) was added. The reaction proceeded at RT (22 °C), and the progress of the reaction was followed with RP-HPLC. It was necessary to increase the temperature to 37 °C for the complete deprotection of [NMeAlaB26,Lys(Pac)B29]-insulin. After completion of the deprotection (12−24 h), the insulin analogues were purified and characterized as described above.

Isolation of Rat Adipose Tissue Plasma Membranes. Plasma membranes (*42*) were prepared from epididymal adipose tissue of adult male Wistar rats weighing 210−250 g. The adipose tissue (5−6 g) was homogenized using an ultraturrax 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 using the Bradford method (*43*).

Receptor Binding Studies. Plasma membranes (50 μg of protein) were incubated in 5 mL tubes with [^{125}I]insulin at a concentration of 5×10^{-11} M (~57000 dpm) and various concentrations (10^{-13}−10^{-5} M) of insulin or insulin analogues in a 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 (Wizard 1470 Automatic Gamma Counter, Perkin-Elmer, Wellesley, MA). The total binding (the binding in the absence of the competitor) was 8−10% of the total radioactivity. The nonspecific binding (the binding in the presence of 10^{-5} M insulin) was determined to be 30% of the total binding.

Analysis of Binding Data. Competitive binding curves were plotted using GraphPad (San Diego, CA) Prism 3, comparing the best fits for single-binding site models. Half-

maximal inhibition values of binding of [^{125}I]insulin to the receptor (IC_{50}) were obtained from nonlinear regression analysis.

In Vivo Experiments in Rats. For in vivo studies, we used adult male rats of the Wistar strain (body weight, 199 ± 3 g) fed a standard laboratory diet. Rats were housed in an air-conditioned animal facility and allowed free access to food and water until the study.

All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997) and were approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (Prague, Czech Republic).

Insulin and analogues were dissolved in a mixture of 0.1% acetic acid and physiological saline (1:9, v/v). Insulin or analogues were injected subcutaneously (sc) at a dose of 4 units/kg of body weight, and blood was drawn from the tail without anesthesia before insulin administration (0 min time point) and 30, 60, 120, 180, and 220 min after administration. Blood glucose levels were measured with a glucometer (Medisence Optium Plus). One unit (U) of insulin or analogue is defined as 6 nmol of peptide (~34 μg).

Lipogenesis in Vitro. Male Wistar rats weighing 220−240 g were used. After decapitation, epididymal adipose tissue was cut into small pieces and isolated adipocytes were prepared by a modification of the method of Rodbell (*44*). Mixed tissue fragments were incubated at 37 °C for 1 h in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 4% (w/v) albumin (fraction V, fatty acid free), 3 μmol/mL glucose, and 1 mg/mL collagenase (type II, Sigma). The digested cell suspension was passed through a nylon filter and washed five times. After the final rinse, the adipocytes were resuspended in the incubation buffer, and aliquots containing 40−50 μmol of triglycerides were added to incubation vessels. The cells were incubated for 2 h at 37 °C in a gas phase of 95% O_2 and 5% CO_2 in Krebs-Ringer bicarbonate buffer (pH 7.4) that contained 5.5 mM unlabeled glucose, 0.1 μCi/mL [U-^{14}C]glucose, and 3 mg/mL BSA (fraction V), with or without 50−1250 μU/mL insulin or analogue. All incubations were performed at 37 °C in sealed vials in a shaking water bath. After a 2 h incubation, the adipocytes were removed from the incubation medium, rinsed with saline, and immediately placed in chloroform. Methanol was then added [chloroform/methanol (2:1, v/v)], and the lipids were extracted at 4 °C overnight. The next day, KH_2PO_4 was added, the solution centrifuged, and the clear organic phase removed for further analysis. One aliquot was evaporated under an atmosphere of nitrogen and reconstituted in scintillation liquid, and the radioactivity was measured by scintillation counting. The second aliquot was evaporated, the resulting pellet dissolved in isopropyl alcohol, and the triglyceride content determined by enzymatic assay (Pliva-Lachema, Brno, Czech Republic). Dose−response curves for incorporation of [U-^{14}C]glucose into triglycerides were plotted using GraphPad Prism 3. EC_{50} values, i.e., concentrations giving half the maximal effect, were obtained using a nonlinear regression analysis.

RESULTS

Using trypsin-catalyzed semisynthesis (*39*), we prepared four novel insulin analogues, namely, [NMe-D-AlaB26]-DTI-NH_2, [NMeAlaB26]-DTI-NH_2, [NMeAlaB26]-insulin, and [NMeHisB26]-insulin, and we compared their biological properties with those of the previously published [NMe-HisB26]-DTI-NH_2, [NMePheB26]-DTI-NH_2 (*36*), and [HisB26]-insulin analogues (*38, 39*). We determined the binding affinities of analogues for the insulin receptor in membranes from rat adipose tissue. We used two different radiotracers, human [^{125}I]monoiodo-TyrA14-insulin and also porcine [^{125}I]monoiodo-TyrA14-insulin. We compared the binding affinities of analogues with those of human and porcine insulin. The data are summarized in Table 1, and the binding curves are shown in Figures 1A−C and 2A−C.

Table 1: Values of IC_{50} and Relative Receptor Binding Affinity of Human Insulin, Porcine Insulin, and Shortened and Full-Length Insulin Analogues[a]

peptide	IC_{50}[b] ± standard error of the mean (nM) (*n*), potency[c] (%)	
	[^{125}I]HI	[^{125}I]PI
human insulin	0.79 ± 0.19 (5), 100	0.93 ± 0.15 (3), 100
porcine insulin	0.58 ± 0.12 (3), 136	0.72 ± 0.13 (3), 129
[NMeHisB26]-DTI-NH_2	0.37 ± 0.06 (4), 214	0.53 ± 0.01 (3), 175
[NMeAlaB26]-DTI-NH_2	0.17 ± 0.04 (5), 465	0.29 ± 0.03 (3), 321
[NMe-D-AlaB26]-DTI-NH_2	0.34 ± 0.03 (5), 232	nd[d]
[NMePheB26]-DTI-NH_2	2.18 ± 0.27 (3), 36.2	3.22 ± 0.40 (3), 28.9
[NMeHisB26]-insulin	7.90 ± 0.86 (3), 10.0	12.9 ± 1.86 (3), 7.2
[NMeAlaB26]-insulin	3.73 ± 0.55 (4), 21.2	4.06 ± 0.43 (3), 22.9
[HisB26]-insulin	0.92 ± 0.21 (4), 85.7	2.24 ± 0.37 (3), 41.5

[a] The experimental values were determined with either human [^{125}I]monoiodotyrosylA14-insulin ([^{125}I]HI) or porcine [^{125}I]monoiodotyrosylA14-insulin ([^{125}I]PI). [b] IC_{50} values represent concentrations of insulin or the analogue that cause half-maximal inhibition of binding of human [^{125}I]monoiodotyrosylA14-insulin ([^{125}I]HI) or porcine [^{125}I]monoiodotyrosylA14-insulin ([^{125}I]PI) to the insulin receptor. Each value represents the mean ± the standard error of the mean of multiple determinations (*n*). [c] Relative receptor binding affinity defined as (IC_{50} of human insulin/IC_{50} of analogue) × 100. [d] Not determined. For details, see Experimental Procedures.

The data in Table 1 reveal that porcine insulin has a slightly higher affinity for the insulin receptor in rat adipose membranes than human insulin. The consequence of this phenomenon is that all analogues have subtly higher IC_{50} values if determined using porcine [^{125}I]insulin than if measured with human [^{125}I]insulin. The percent of binding measured with porcine [^{125}I]insulin as a radioligand was in good agreement with the percent of binding obtained with human [^{125}I]insulin for most analogues. To simplify the discussion, only the binding data obtained with human [^{125}I]insulin will be addressed.

By using human [^{125}I]insulin as a radiotracer, we found that [NMeHisB26]-DTI-NH_2 had 214% binding affinity when compared to human insulin. This finding was very different from our previously published results, when we reported more than 5000% binding affinity for rat adipocyte membranes (*36*). The probable reason for this discrepancy will be discussed below. In addition to [NMeHisB26]-DTI-NH_2, we also re-evaluated [NMePheB26]-DTI-NH_2, which displayed 80% binding affinity in the previous study (*36*). In the study presented here, we find that this analogue had 36% binding affinity. The new shortened analogues, [NMeAlaB26]-DTI-NH_2 and [NMe-D-AlaB26]-DTI-NH_2, had 465 and 232% binding potency, respectively, and are more potent than [NMeHisB26]-DTI-NH_2. The 465% binding affinity ranks [NMeAlaB26]-DTI-NH_2 among the most potent insulin analogues. Although both of the shortened analogues are

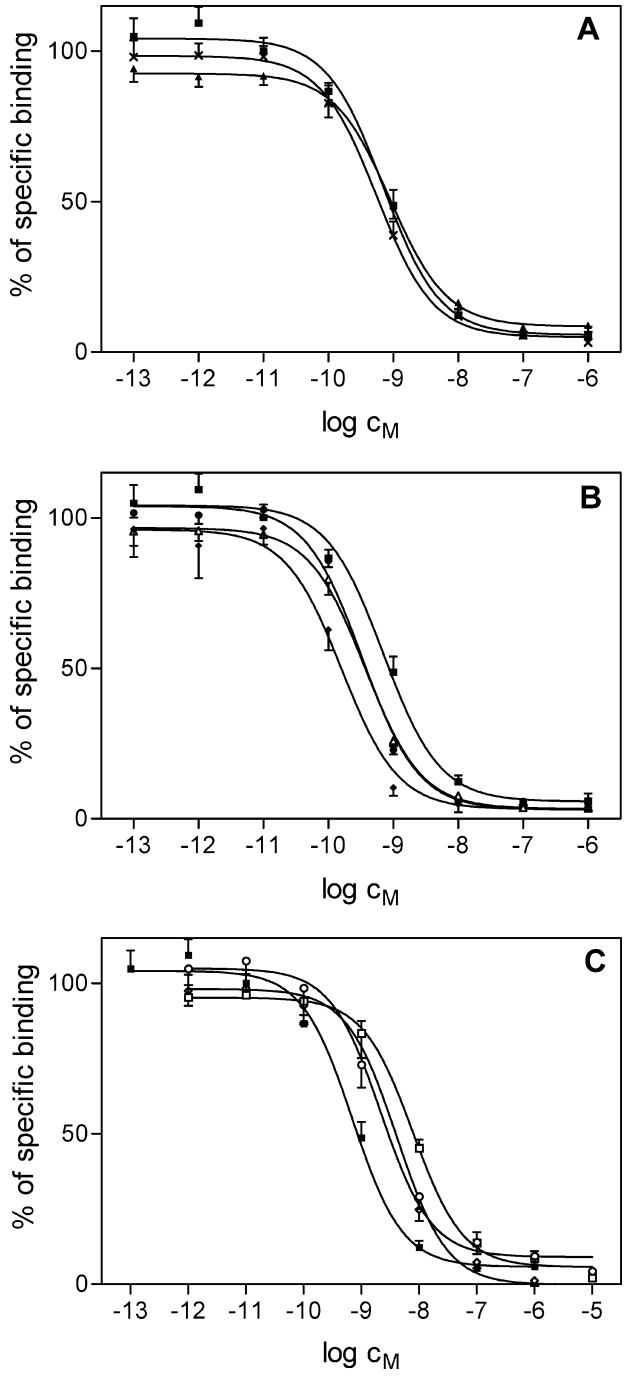

FIGURE 1: Inhibition of binding of human [^{125}I]insulin to adipose plasma membranes by insulin and insulin analogues: (A) human insulin (■), porcine insulin (×), and [HisB26]-insulin (▲), (B) human insulin (■), [NMeHisB26]-DTI-NH_2 (△), [NMeAlaB26]-DTI-NH_2 (◆), and [NMe-D-AlaB26]-DTI-NH_2 (●), and (C) human insulin (■), [NMePheB26]-DTI-NH_2 (○), [NMeAlaB26]-insulin (◇), and [NMeHisB26]-insulin (□). Quantitative information is provided in Table 1. See Experimental Procedures for details.

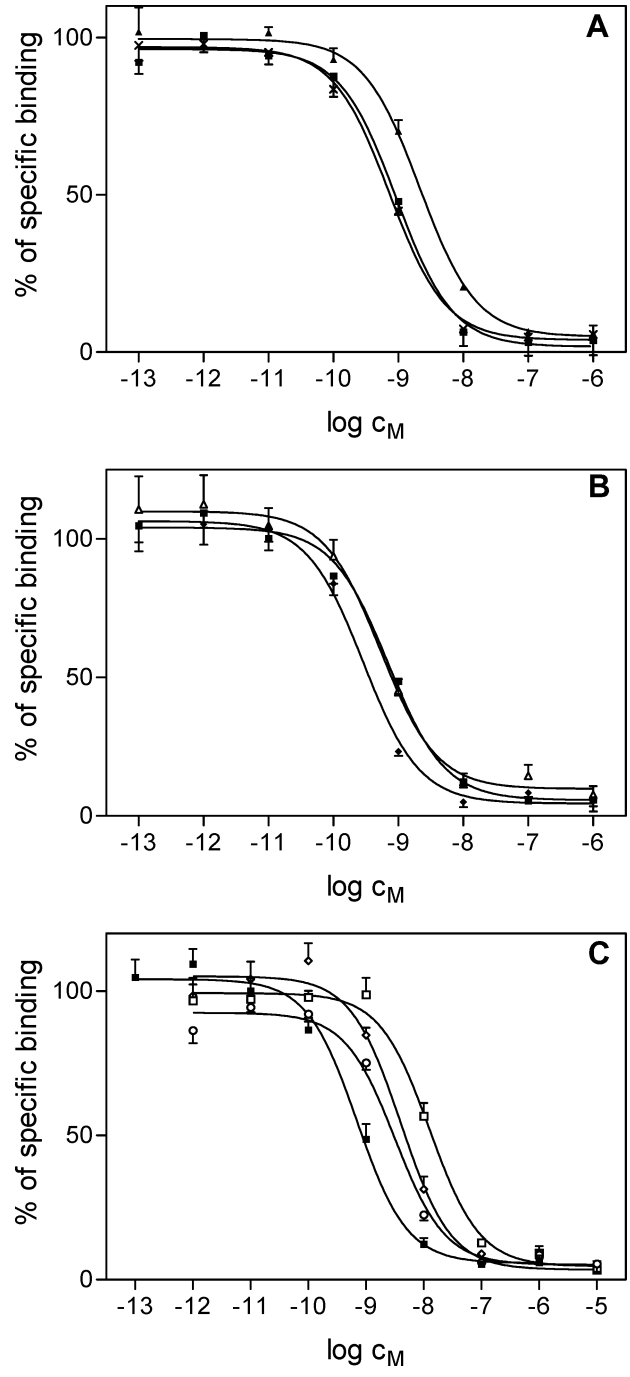

FIGURE 2: Inhibition of binding of porcine [^{125}I]insulin to adipose plasma membranes by insulin and insulin analogues: (A) human insulin (■), porcine insulin (×), and [HisB26]-insulin (▲), (B) human insulin (■), [NMeHisB26]-DTI-NH_2 (△), and [NMeAlaB26]-DTI-NH_2 (◆), and (C) human insulin (■), [NMePheB26]-DTI-NH_2 (○), [NMeAlaB26]-insulin (◇), and [NMeHisB26]-insulin (□). Quantitative information is provided in Table 1. See Experimental Procedures for details.

more potent than human and porcine insulin, this is not true for their full-length counterparts. The N-methylated analogues [NMeHisB26]-insulin and [NMeAlaB26]-insulin have only 10 and 21.2% binding affinity, respectively, compared to human insulin. [HisB26]-Insulin displays 86% binding affinity, and this result is in agreement with our recently published data (78%) (*39*), and also with data from Smiley at al. (75%) (*38*). We also prepared and determined the binding potency (see the Supporting Information) of the previously published analogue, [D-AlaB26]-insulin (*35*). We found that this analogue exhibited 17% binding potency compared to human insulin, which is in close agreement with the previously published binding affinity of 18% (*35*).

In the second part of our study, we investigated the ability of analogues to influence the concentration of plasma glucose in vivo in normal nondiabetic male Wistar rats after sc administration. The rats were treated subcutaneously with 4 U of insulin or analogue/kg of body weight. The concentra-

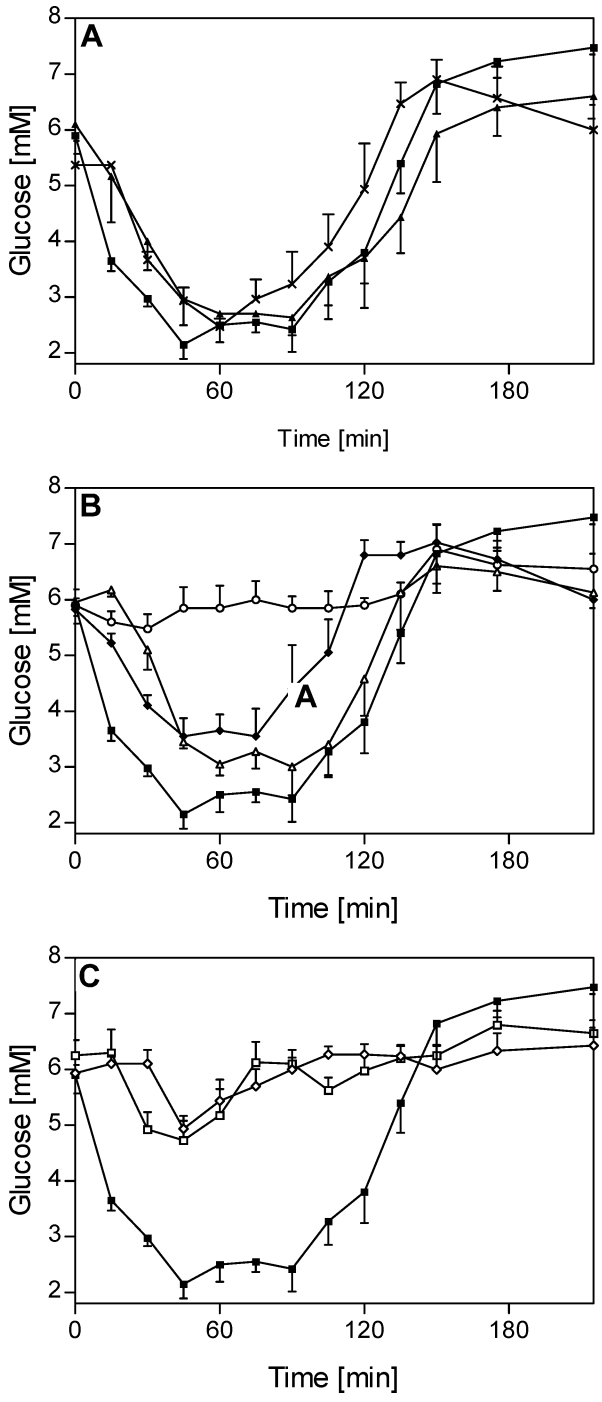

FIGURE 3: (A) Time course of blood plasma glucose levels in Wistar rats after sc injection of 4 U/kg of insulin or insulin analogues. The data are shown as the means ± the standard error of the mean. See Experimental Procedures for details: (A) human insulin (■; $n = 4$), porcine insulin (×; $n = 3$), and [HisB26]-insulin (▲; $n = 3$), (B) human insulin (■; $n = 4$), [NMeHisB26]-DTI-NH_2 (△; $n = 4$), [NMeAlaB26]-DTI-NH_2 (◆; $n = 4$), and [NMePheB26]-DTI-NH_2 (○; $n = 4$), and (C) human insulin (■; $n = 4$), [NMeAlaB26]-insulin (◇; $n = 3$), and [NMeHisB26]-insulin (□; $n = 4$).

tion of glucose in the blood was measured periodically during a 220 min period. The results are shown in Figure 3A−C.

Human and porcine insulin and [HisB26]-insulin display similarly potent abilities to lower plasma glucose levels in rats. This observation seems to be in good agreement with their respective binding potencies. Shortened insulin analogues, [NMeHisB26]-DTI-NH_2 and [NMeAlaB26]-DTI-NH_2 that have enhanced binding affinities (214 and 465%, respectively), are less active in lowering plasma glucose levels than human insulin, especially during the first part of the response. The third shortened analogue, [NMePheB26]-DTI-NH_2, was completely inactive in vivo despite its reduced but still adequate binding affinity (36%). Both of the N-methylated full-length analogues, [NMeHisB26]-insulin and [NMeAlaB26]-insulin, which are only slightly potent in binding, are equally slightly active with only short and shallow lowering of glucose concentration but are more active than [NMePheB26]-DTI-NH_2.

Table 2: Values of EC_{50} and in Vitro Biological Activity Expressed as the Ability of Human Insulin and Insulin Analogues To Stimulate Incorporation of [^{14}C]Glucose into Lipids

peptide	EC_{50}[a] ± standard error of the mean (nM) (n), potency[b] (%)
human insulin	0.77 ± 0.21 (4), 100
[NMeAlaB26]-DTI-NH_2	1.37 ± 0.24 (4), 56.2
[NMePheB26]-DTI-NH_2	>10 (3), <8
[HisB26]-insulin	1.12 ± 0.27 (2), 68.8

[a] EC_{50} values represent concentrations of insulin or the analogue that cause half the maximal response of insulin or insulin analogue. Each value represents the mean ± the standard error of the mean of multiple measurements (n). [b] Relative potency at stimulating incorporation of [^{14}C]glucose defined as (EC_{50} of human insulin/EC_{50} of analogue) × 100. For details, see Experimental Procedures.

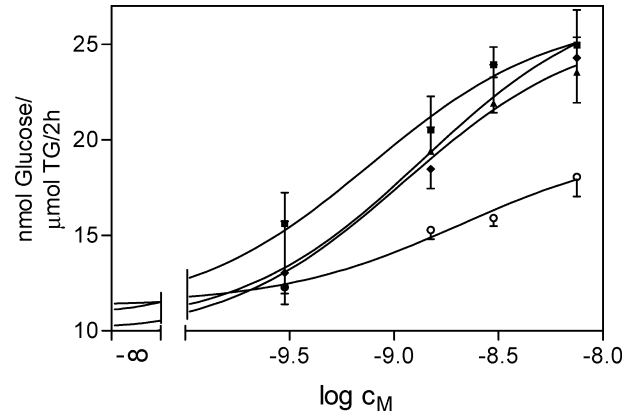

FIGURE 4: Stimulation of incorporation of [^{14}C]glucose into lipids in isolated rat adipocytes by insulin and insulin analogues. The data are expressed as nanomoles of [^{14}C]glucose per micromole of triglycerides (TG) per 2 h. See Experimental Procedures for details. Quantitative information is provided in Table 2: human insulin (■), [HisB26]-insulin (▲), [NMeAlaB26]-DTI-NH_2 (◆), and [NMePheB26]-DTI-NH_2 (○).

Finally, in the third part of our study, we investigated the ability of human insulin, [NMeAlaB26]-DTI-NH_2, [NMePheB26]-DTI-NH_2, and [HisB26]-insulin to stimulate lipogenesis in isolated rat adipocytes in vitro. The data are summarized in Table 2, and the curves are shown in Figure 4. The relative ability of these analogues to stimulate lipogenesis is in agreement with their biological activity in vivo. The high-affinity binding analogue [NMeAlaB26]-DTI-NH_2 is less active in vivo than human insulin and stimulates lipogenesis with 56% potency compared to human insulin. Analogue [NMePheB26]-DTI-NH_2 has significantly lower biological activity in vitro (<8%) compared to its binding affinity (36%). This analogue was inactive in vivo at 4 U/kg but fully active at 60 U/kg in diabetic rats (data not shown), which indicates that [NMePheB26]-DTI-NH_2 is not an antagonist. Analogue [HisB26]-insulin stimulates lipogenesis with a potency (69%) similar to its binding affinity (75−86%).

This analogue was equipotent with human insulin at lowering plasma glucose levels in rats.

DISCUSSION

This study investigated the role of the B26 position in shortened and full-length insulin analogues. This position, naturally occupied by tyrosine, was substituted with alanine, histidine, or phenylalanine in the natural, N-methylated, and/or D-conformations. These modifications were introduced to investigate both the role of the B26 position and the role of the last four amino acids (B27−B30) of the insulin B-chain during the complex interaction of insulin with its receptor.

Our previous study concerning shortened insulin analogues (*36*) reported the high-affinity binding of the [NMeHisB26]-DTI-NH_2 analogue (more than 5000% compared to human insulin). However, this result was not in agreement with data produced by Smiley et al. (*38*), who reported that this analogue displaced only 80% binding potency to membranes from engineered cells that overexpressed the human insulin receptor, or with independent measurements made by P. De Meyts (Hagedorn Research Institute, Gentofte, Denmark), who found that this analogue bound with 175% affinity to the insulin receptor from IM-9 lymphocytes (personal communication). The previous study (*36*) was performed with noncommercial, "homemade", porcine [^{125}I]monoiodo-TyrA14-insulin, which was not purified using RP-HPLC. The possibility that the use of this homemade radiotracer could cause erroneous results has not been excluded. To eliminate the possible influence of species differences between porcine and human insulin, we performed the binding experiments presented here with two different commercial radiotracers, human [^{125}I]monoiodo-TyrA14-insulin (Amersham) and porcine [^{125}I]monoiodo-TyrA14-insulin (NEN). We have now found that [NMeHisB26]-DTI-NH_2 has 214 or 175% binding potency, depending upon the radiotracer used (Table 1), to the insulin receptor from rat adipose membranes. The less potent analogue [NMePheB26]-DTI-NH_2 displayed a binding affinity of 36% in this study and 80% in the previous study (*36*), which is a less significant difference than in the case of the higher-affinity ligand, [NMeHisB26]-DTI-NH_2.

Binding Studies. All of our shortened analogues, with the exception of [NMePheB26]-DTI-NH_2, are stronger in binding than HI. One of the structural consequences of the B-chain truncation is better exposure of the N-terminal residues of the A-chain (*26, 45, 46*). Moreover, the truncation could positively affect the detachment of the C-terminal β-strand from the central α-helix of the B-chain of insulin, which is the basic requirement for high-affinity binding (*18, 20, 47, 48*).

The [NMeAlaB26]-DTI-NH_2 and [NMe-D-AlaB26]-DTI-NH_2 analogues are very potent with 465 and 232% binding affinities, respectively (Table 1). Although they are among the most potent analogues that have been described, they do not reach the binding potency of [SarB26]-DTI-NH_2 [1100% (*32*)] or [D-AlaB26]-DTI-NH_2 [1252% (*35*)]. Other previously published shortened analogues with amino acids that have small side chains in the B26 position, such as [AlaB26]-DTI-NH_2 and [GlyB26]-DTI-NH_2, display 273 and 280% binding affinities, respectively (*32*). It is rather surprising that DTI-NH_2 analogues with similar small side chain amino acids, such as Ala, NMe-D-Ala, Gly, and NMe-Ala, in the B26 position have relatively similar affinities (237−465%), but the DTI-NH_2 analogues with Sar and D-Ala are extremely potent. The NMR study (*35*) of [D-AlaB26]-DTI-NH_2 did not reveal any significant structural changes compared to HI. These data reveal that the influence of simple methyl groups in the above-mentioned small amino acids in the B26 position in DTI-NH_2 analogues is governed by rather complicated and subtle rules, which are not easy to understand.

The substitution of the amide hydrogen of a peptide bond with a methyl group has many implications. First, N-methylation of a peptide bond can affect the local flexibility of the main chain and enable the possibility of cis−trans isomerism and the loss of hydrogen bonding in N-methylated analogues. Second, N-methylation results in the elimination of the ability to form intermolecular or intramolecular hydrogen bonds, which are important for stability and correct folding of the insulin monomer, dimer formation, and 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 (*32, 49, 50*).

The NH group of B26 together with the NH group of B24 plays an important role in the formation of hydrogen bonds in the insulin dimer (*14*). A similar interaction is supposed to be important in the binding of insulin to its receptor. N-Methylation of one of these amides can destabilize and disturb these interactions. Since the ability to form a dimer is weakened in shortened DTI-NH_2 and DPI-NH_2 analogues and the binding affinity is preserved, it follows that the B25−B26 amide is not necessary for binding. On the contrary, the N-methylation of the respective amide can in some cases contribute to very strong binding of shortened analogues, e.g., [SarB26]-DTI-NH_2 (*32*) or [NMeAlaB26]-DTI-NH_2 (this study). We suppose that the incorporation of the N-methyl group into the B25−B26 peptide bond can change the conformation of the backbone and thus move the B26 residue into a more favorable position, provided that the B26 residue is small (e.g., Gly or Ala). Nevertheless, the high potency of [D-AlaB26]-DTI-NH_2 reveals that N-methylation of the B25−B26 bond is not necessary for strong binding. Overall, all of the above-mentioned small amino acids, in L-, D-, or N-methylated forms, are very well tolerated in the B26 position.

On the other hand, the substitution of the B26 position with N-methylated amino acids with bulky side chains can yield different results. Our [MePheB26]-DTI-NH_2 analogue has only 36% binding affinity, but [MeHisB26]-DTI-NH_2 had 214%. This result indicates the importance of the side chain of the N-methylated amino acid in the B26 position. We suppose that the ability of the side chain to form a hydrogen bond plays a significant role. This is in agreement with our previous study, where histidine was more active than tyrosine and tyrosine than phenylalanine in both N-methylated and nonmethylated shortened analogues (*36*). In 1991, an interesting series of B26-substituted DTI analogues was published in the doctoral thesis of D. Sievert from Aachen (*51*). [ThrB26]-DTI-NH_2 had 130% binding affinity, but [SerB26]-DTI-NH_2 had 306% and [GluB26]-DTI-NH_2 even 449%. On the other hand, [LysB26]-DTI-NH_2 displayed only 76% binding affinity. These results further support the important role of the side chain of the

B26 residue in the interaction of DTI-NH_2 analogues with the receptor.

N-Methylation of the B25−B26 peptide bond in full-length insulin analogues gives results different from those in the case of shortened analogues. Full-length analogues with NMeAla and NMeHis in the B26 position exhibited very weak binding, 21% and 10%, respectively, with respect to human insulin. On the other hand, the nonmethylated analogue [HisB26]-insulin was almost equipotent to human insulin in binding affinity (Table 1). This could imply that the N-methylation of the B25−B26 peptide bond induces a different position or orientation of residues B26−B30 in such analogues compared to nonmethylated analogues. This putative different orientation is highly unfavorable for the effective interaction with the insulin receptor. However, in addition to the N-methylated amino acid in the B26 position, the chirality in this position can also affect the interaction with the receptor; [D-AlaB26]-insulin has 18% binding affinity (*35*), while [AlaB26]-insulin with 34−82% binding affinity is better tolerated (*12, 32, 34*). These and other results indicate that the side chain of the B26 residue is important and the resulting analogues may significantly differ in binding affinity; [TrpB26]-insulin has 18% (*52*), [ThrB26]-insulin 58% (*53*), [HisB26]-insulin 86% (this study), and [GluB26]-insulin 125% (*54*) binding affinity. Whereas analogues [HisB26]-insulin and [ThrB26]-insulin are tolerated well due to the appropriate size and functional groups in their B26 side chains, the low binding affinity of [TrpB26]-insulin is not surprising due to the bulky side chain of Trp, which is difficult to accommodate because the B26 side chain is oriented toward the central α-helix of insulin. On the other hand, the enhanced affinity of [GluB26]-insulin is rather surprising because the formation of the insulin dimer requires a hydrophobic residue at B26. These data indicate that the role of the B26 residue is not negligible despite the fact that residues B26−B30 are not necessary for full binding (*55*).

Biological Activity Studies. All analogues, with the exception of [NMe-D-AlaB26]-DTI-NH_2, were tested for their ability to lower plasma glucose levels in rats in vivo, and three selected analogues, [NMeAlaB26]-DTI-NH_2, [NMePheB26]-DTI-NH_2, and [MeHisB26]-insulin, were tested for their ability to stimulate lipogenesis in isolated rat adipocytes in vitro.

All three full-length nonmethylated analogues, human, porcine insulin, and [HisB26]-insulin, display similar and potent responses at lowering blood glucose levels in vivo (Figure 3A). This finding is in agreement with their binding affinities. [HisB26]-Insulin stimulated lipogenesis in vitro with 69% potency (Table 2), which is relatively close to its binding affinity: 86% in this study, 78% in ref *39*, and 75% in ref *38*. Both full-length N-methylated analogues, [MeHisB26]-insulin and [MeAlaB26]-insulin, which display very weak binding affinities, are also very weak in vivo (Figure 3C).

In contrast, [NMeAlaB26]-DTI-NH_2 and [NMeHisB26]-DTI-NH_2, which are both very potent at binding, are significantly less potent in vivo, especially during the first 90 min of the response (Figure 3B). [NMeAlaB26]-DTI-NH_2 also displays only 56% potency at stimulating lipogenesis in vitro (Table 2 and Figure 4). Therefore, the [NMeAlaB26]-DTI-NH_2 analogue is less potent than human insulin in both of the biological activities we tested, despite its very high binding affinity (465%). This result may indicate that the [NMeAlaB26]-DTI-NH_2 analogue binds strongly to the insulin receptor; however, this binding does not induce an equivalent biological response.

In addition, the [NMePheB26]-DTI-NH_2 analogue, with 36% binding potency, is completely inactive in vivo at 4 U/kg (Figure 3B), despite its higher binding potency with respect to [MeAlaB26]-insulin and [MeHisB26]-insulin (Table 1). Nevertheless, in diabetic rats, [NMePheB26]-DTI-NH_2 at 60 U/kg gives full biological activity compared to human insulin (data not shown), which shows that this analogue is not an antagonist but only a very weak agonist in vivo. This weak biological ability at lowering plasma glucose levels is in agreement with our previous study, where the ability of [NMePheB26]-DTI-NH_2 to stimulate glucose transport was very weak (*36*), and correlates well with our present finding that this analogue has also very weak ability to stimulate lipogenesis in vitro [<8% (Table 2)].

Overall, the inability to lower blood glucose levels versus the high binding affinities of shortened analogues is surprising and raises questions about the role of residues B26−B30 in the biological action of insulin in vivo.

There are few examples of insulin analogues with discrepancies between binding affinity and biological activity. Several analogues with weaker binding affinity have higher-than-expected in vivo activities (*56, 57*), and this has also been shown in patients with inherited insulin mutations (*58, 59*). Ribel et al. (*60*) have reported that there is an equal rate of glucose utilization for both low- and high-affinity binding analogues. In contrast, Emdin et al. (*61*) found that hagfish insulin has 23% binding affinity, but only 4.6% ability to stimulate lipogenesis. Burke et al. (*62*) showed that [AsnA21-amide]-insulin had 64% binding affinity and 12−15% biological activity in vitro; however, in vivo, biological activity was comparable (72%) to binding affinity. They concluded that the substitution disrupts the interaction between the carboxylate of AsnA21 and ArgB22 and that it (i) reduced the binding affinity of the analogues for the receptor by distorting the B24−B29 segment and (ii) impaired the efficiency of the analogue−receptor complex to initiate the physiological response. The previously mentioned DTI-NH_2 analogues that were described by D. Sievert (*51*) have interesting properties when their binding affinities are compared with their ability to stimulate lipogenesis in vitro. All of the DTI analogues with Ser, D-Ser, His, or Glu at the B26 position were more potent at binding than at stimulating lipogenesis. In contrast, DTI analogues with Thr, Lys, or Arg at the B26 position were more potent at stimulating lipogenesis than in their binding affinity. However, the divergence between the binding affinity and biological activity of the described analogues is smaller than that observed for our analogues, [NMeAlaB26]-DTI-NH_2 and [NMeHisB26]-DTI-NH_2, which are more potent at binding (465 and 214%, respectively), and significantly less potent in vivo, and [NMeAlaB26]-DTI-NH_2 is also less potent at stimulating lipogenesis when compared to HI.

DTI-NH_2 was shown to have 110% binding affinity and 129% in vitro biological activity (*25*). Likewise, DPI-NH_2, which is equipotent to insulin at binding (100−115%) (*29, 30, 63, 64*), retained full in vivo hypoglycemic activity after

intravenous injection into rats (*65*). These data and the results relating to analogues with specific substitutions at the B26 position do not support the hypothesis that residues B26−B30 are important for biological activity, and instead, we suggest that the observed discrepancy between binding affinity and biological activity of the shortened analogues could be caused by N-methylation of the B25−B26 peptide bond and specific substitutions at the B26 position. It is possible that our shortened analogues have different modes of interaction with the insulin receptor, which may lead to differential metabolic responses of the insulin receptor. This may indicate that there are two distinct regions within the insulin molecule: one primarily associated with binding and the other with the expression of the cellular response (*57, 66, 67*). Similar distinct regions or sites may also exist within the insulin receptor (*68, 69*). However, we cannot exclude the possibility that the reason for the discrepancy between both activities may lie in the different behavior of our analogues and insulin after sc administration, e.g., differential clearance from the circulation, different physical and proteolytic stability of analogues, or a tendency to aggregate in tissues or bind plasma proteins, such as serum albumin.

Taken together, the data suggest that the B26 residue and the C-terminus of the B-chain play ambiguous roles in insulin binding. It is interesting to note that even if the B26 position is not involved in a direct interaction with the insulin receptor, replacement of the tyrosine with various amino acids results in analogues with a wide range of binding affinities, from the less active [NMePheB26]-DTIA (this study and ref *36*) to the "superpotent" [D-AlaB26]-DTI-NH_2 (*35*) or [SarB26]-DTI-NH_2 (*32*). Moreover, our data show that shortened analogues that are highly potent at binding may be significantly less potent in vivo. The elucidation of the crystal structure of our N-methylated full-length and DTIA-NH_2 analogues could help in the clarification of the structural implications of the observed data. These experiments are currently in progress.

SUPPORTING INFORMATION AVAILABLE

Data on the binding affinity of [D-AlaB26]-insulin. This material is available free of charge via the Internet at http://pubs.acs.org.

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