

Correlations of Receptor Binding and Metabolic and Mitogenic Potencies of Insulin Analogs Designed for Clinical Use

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In recent years, analogs of human insulin have been engineered with the aim of improving therapy for people with diabetes. To ensure that the safety profile of the human hormone is not compromised by the molecular modifications, the toxico-pharmacological properties of insulin analogs should be carefully monitored. In this study, we compared the insulin and IGF-I receptor binding properties and metabolic and mitogenic potencies of insulin aspart (B28Asp human insulin), insulin lispro (B28Lys,B29Pro human insulin), insulin glargine (A21Gly,B31Arg,B32Arg human insulin), insulin detemir (NN304) [B29Lys(ϵ -tetradecanoyl),desB30 human insulin], and reference insulin analogs. Receptor affinities were measured using purified human receptors, insulin receptor dissociation rates were determined using Chinese hamster ovary cells overexpressing the human insulin receptor, metabolic potencies were evaluated using primary mouse adipocytes, and mitogenic potencies were determined in human osteosarcoma cells. Metabolic potencies correlated well with insulin receptor affinities. Mitogenic potencies in general correlated better with IGF-I receptor affinities than with insulin receptor off-rates. The 2 rapid-acting insulin analogs aspart and lispro resembled human insulin on all parameters, except for a slightly elevated IGF-I receptor affinity of lispro. In contrast, the 2 long-acting insulin analogs, glargine and detemir, differed significantly from human insulin. The combination of the B31B32diArg and A21Gly substitutions provided insulin glargine with a 6- to 8-fold increased IGF-I receptor affinity and mitogenic potency compared with human insulin. The attachment of a fatty acid chain to LysB29 provided insulin detemir with reduced receptor

affinities and metabolic and mitogenic potencies but did not change the balance between mitogenic and metabolic potencies. The safety implications of the increased growth-stimulating potential of insulin glargine are unclear. The reduced in vitro potency of insulin detemir might explain why this analog is not as effective on a molar basis as human insulin in humans. *Diabetes* 49:999–1005, 2000

Over the last decade, a large number of insulin analogs have been designed and examined with the aim of tailoring the time-action profile of injected insulin to the need of the diabetic patient (1,2). Insulin analogs also constitute a unique tool to unravel structure-function relationships in insulin biochemistry and insulin action. Thus, recombinant insulin analogs, together with naturally occurring insulins, have been important in mapping the putative receptor binding domain(s) of the insulin molecule (3–6) and have also been used in studies aimed at elucidating the specificity of pathways leading to the metabolic and mitogenic effects of the hormone (7–10).

Structure-function studies have indicated that amino acids essential for binding to the insulin receptor include A1Gly, A2Ile, A3Val, A19Tyr, B6Leu, B12Val, B23Gly, B24Phe, and B25Phe (3,4,11). The B26–B30 region is not particularly critical for insulin receptor recognition and has been a preferred site for structural modifications aimed at modifying the pharmacokinetic profile of the insulin molecule (1,6). However, the COOH-terminal part of the insulin B-chain appears to be implicated in insulin binding to the IGF-I receptor. Studies with a large series of insulin analogs indicated that the IGF-I receptor affinity of insulin was altered by amino acid substitutions at positions B28–B32 (10). In particular, the number and position of basic residues in this region appeared to be important for IGF-I receptor binding.

Furthermore, Sliker et al. (10) demonstrated that the relative affinity of the tested insulin analogs for the IGF-I receptor correlated highly with the potency in stimulating growth of human mammary epithelial cells (HMECs), suggesting that the growth-promoting activity of the analogs was mediated via the IGF-I receptor. In a similar study of receptor binding characteristics and metabolic and mitogenic effects of insulin analogs, Hansen et al. (9) found a distinct correlation between mitogenic potential and occupancy time at the insulin receptor. Thus, analogs dissociating very slowly from

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BHK, baby hamster kidney; BSA, bovine serum albumin; CHO, Chinese hamster ovary; CHO-hIR cells, Chinese hamster ovary cells overexpressing the human insulin receptor; DMEM, Dulbecco's modified Eagle's medium; EC₅₀, concentration required for half-maximal effect; FCS, fetal calf serum; hIGF-IR, human IGF-I receptor; hIR, human insulin receptor; HMEC, human mammary epithelial cell; HSA, human serum albumin; insulin aspart, B28Asp human insulin; insulin detemir (NN304), B29Lys(ϵ -tetradecanoyl),desB30 human insulin; insulin glargine, A21Gly,B31Arg,B32 Arg human insulin; insulin lispro, B28Lys,B29Pro human insulin; k_d , dissociation rate constant; sIGF-IR, soluble IGF-I receptor; sIR, soluble insulin receptor; TBS, Tris-buffered saline.

the insulin receptor (with >3-fold lower dissociation rates than human insulin) showed a disproportionate increase in mitogenic activity compared with metabolic activity. An increased duration of the insulin signal at the receptor level therefore seemed to result in a shift toward mitogenic effects of insulin, indicated by an increased ratio between mitogenic and metabolic potencies. This finding suggests that the mitogenic activity of insulin analogs can alternatively be mediated via a sustained activation of the insulin receptor. This hypothesis is supported by studies showing that the mitogenic response to insulin requires a continuous stimulation with the hormone for a period of >14.5 h (12).

Insulin analogs can potentially provide diabetic patients with a more efficient, reproducible, and convenient therapy. However, for patients in life-long treatment with insulin, it is imperative that the benefits of novel insulin analogs are not associated with increased safety risks. The finding of an increased tumorigenic potential in female Sprague-Dawley rats of the insulin analog insulin B10Asp compared with human insulin indicated that a single substitution in the amino acid sequence of the insulin molecule might have significant toxicological implications (8). The molecular mechanisms responsible for the tumor-promoting activity of insulin B10Asp are not clear. Like human insulin, insulin B10Asp is not genotoxic. However, insulin B10Asp is significantly more potent in stimulating DNA synthesis than human insulin *in vitro* (9). It is therefore reasonable to assume that the increased tumor-promoting activity of insulin B10Asp was coupled with its increased mitogenic potency. The lesson learned from insulin B10Asp is that an assessment of molecular pharmacological properties, including insulin and IGF-I receptor binding and metabolic and mitogenic potency, is of significant importance in the toxico-pharmacological evaluation of novel insulin analogs.

The aim of the present study was to investigate the relationships between insulin structure, insulin receptor and IGF-I receptor binding, and metabolic and mitogenic potency of a series of insulin analogs that have been designed for clinical use: the rapid-acting analogs insulin lispro (B28Lys,B29Pro human insulin) and insulin aspart (B28Asp human insulin), which have already been approved for clinical use, and the long-acting analogs insulin glargine (A21Gly,B31Arg,B32Arg human insulin) and insulin detemir (NN304) [B29Lys(*ε*-tetradecanoyl),desB30 human insulin], which are currently undergoing clinical investigation. Insulin B10Asp was included in the study as a reference compound, whereas insulin A21Gly and insulin B31B32diArg were included to differentiate the effect of the individual amino acid modifications of insulin glargine. The results provide a basis for discussing the risk-to-benefit ratio of introducing specific amino acid modifications into the insulin molecule.

RESEARCH DESIGN AND METHODS

Materials. Human insulin, insulin aspart, insulin lispro, insulin B10Asp, insulin A21Gly, and insulin detemir were produced by recombinant DNA techniques and purified essentially as previously described (13,14). Insulin glargine and insulin B31B32diArg were synthesized by enzymatic semisynthesis. Briefly, Thr-Arg-Arg-Ome was coupled with A21Gly,desB30 insulin or desB30 insulin by the use of the lysine-specific Achromobacter Lyticus Protease in 70% dimethyl formamide. The methyl ester group was removed by treatment with 0.1 mol/l NaOH at 4°C for 15 min, and the insulin analog was purified by reverse-phase high-performance liquid chromatography. TyrA14^[125I]-labeled human insulin and analogs and Tyr31^[125I]-labeled IGF-I were prepared as previously described (15,16). IGF-I was obtained from Chi-

ron (Emeryville, CA) or from Novo Nordisk. Human serum albumin (HSA) was from Behringwerke (Marburg, Germany), bovine serum albumin (BSA) from Sigma (Steinheim, Germany), and culture media from Gibco/Life Technologies (Paisley, Scotland, U.K.). Radiochemicals were from Amersham (Little Chalfont, U.K.). Other chemicals were of reagent grade.

Determination of insulin receptor binding. Human insulin receptor (hIR) (isoform without exon 11) was isolated from transfected baby hamster kidney (BHK) cells by solubilization and partial purification on a wheat germ agglutinin column. For binding experiments, hIR was incubated with 3 pmol/l TyrA14^[125I] human insulin and various concentrations of unlabeled human insulin or insulin analog in a binding buffer containing 0.1 mol/l HEPES, 0.1 mol/l NaCl, 0.01 mol/l MgSO₄, 0.5% HSA, 0.2% γ -globulin, and 0.025% Triton X-100, pH 7.8, for 42 h at 4°C. Bound tracer was isolated by precipitation with 400 μ l 25% PEG 8000 and washing with 1 ml 15% PEG 8000. The data were fitted to a 4-parameter logistic function where B_{max} and B_{min} were fixed and slope and concentration required for half-maximal effect (EC_{50}) varied. The relative affinity of an insulin analog was calculated as the ratio between the EC_{50} value for human insulin and that of the insulin analog.

Determination of IGF-I receptor binding. IGF-I receptor affinities were determined as described for insulin receptor affinities, except that the human IGF-I receptor (purified from transfected BHK cells) was used and Tyr31^[125I]-IGF-I was used as the tracer.

Determination of receptor binding of insulin detemir. The conventional binding assays were not applicable for insulin detemir because of the lipophilicity and tight binding to albumin of this analog. Binding experiments with insulin detemir were therefore done using albumin-free buffers and a modified procedure. The binding assays used were microtiter plate antibody capture assays, essentially as described in the literature (17). Microtiter plates were coated with affinity-purified goat anti-mouse IgG antibody (Pierce, Rockford, IL) (50 μ l/well of 20 μ g/ml solution in Tris-buffered saline [TBS]: 0.01 mol/l Tris, pH 7.5, 100 mmol/l NaCl). Plates were incubated for 1 h at room temperature before washing twice with TBS and blocking with 250 μ l Superblock (Pierce). Then, a suitable dilution of receptor-specific antibody was added: antibody 18–44 against soluble insulin receptor (sIR) and 24–31 against soluble IGF-I receptor (sIGF-IR) (antibodies from Neo Markers, Union City, CA). For this and all subsequent dilutions, binding buffer was used (100 mmol/l HEPES, pH 8.0, 100 mmol/l NaCl, 10 mmol/l MgCl₂, 0.02% Triton X-100). Plates were incubated for 2 h before washing 3 times with binding buffer. Then, a suitable dilution of sIR or sIGF-IR was added and plates were again incubated for 2 h before washing 3 times with binding buffer. Binding experiments were performed by adding a total volume of 150 μ l binding buffer to 8–10 pmol/l of tracer (A14Tyr^[125I]-insulin or Tyr31^[125I]-IGF-I) and varying concentrations of insulin, IGF-I, or insulin detemir. After 16 h at 4°C, unbound ligand was removed by washing once with cold binding buffer, and the tracer bound in each well was counted in a γ -counter. The binding data were fitted using the nonlinear regression algorithm in GraphPad Prism 2.01 (GraphPad Software, San Diego, CA).

Dissociation from the insulin receptor. Dissociation constants for A14Tyr^[125I]-insulins were determined as previously described using Chinese hamster ovary (CHO) cells overexpressing the human insulin receptor (CHO-hIR cells) (9). The cells were cultured at 37°C in a 5% CO₂ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS), 1% nonessential amino acids, 50 μ g/ml streptomycin, 50 U/ml penicillin, and 1 μ mol/l methotrexate. Cells were subcultured at a 1:5 split ratio every 3–4 days for maintenance. For dissociation studies, cells were seeded on 24-well plates at a density of 5×10^4 cells/well in DMEM containing 10% FCS, 1% nonessential amino acids, 20 mmol/l HEPES, 0.1% HSA, and 0.1% bacitracin (pH 7.4). Cells were used at confluence and were incubated with 50 pmol/l A14Tyr^[125I]-insulin or analog for 3 h at 4°C. Cells were washed quickly twice with ice-cold HEPES-buffered DMEM (pH 7.4), and the dissociation of radioactivity was measured after the addition of HEPES-buffered DMEM containing 0.1 μ mol/l unlabeled human insulin to measure the maximal accelerated dissociation rate. Cell-associated radioactivity was measured as a function of time, and the dissociation rate constant (k_d) was calculated from the fitted monoexponential dissociation profiles using the following equation: $B = B_{min} + (B_{max} - B_{min}) \times \exp(-k_d \times t)$, where B represents cell-associated radioactivity.

Metabolic potency. The potency of the insulin analogs for stimulating lipogenesis was determined using isolated primary mouse adipocytes according to Moody et al. (18) with minor modifications. In brief, male mice younger than 4 weeks of age were killed, and the epididymal fat pads were removed and placed in a degradation buffer containing collagenase at 37°C for 1 h under vigorous shaking. The cell suspension was filtered, and adipocytes were washed twice and resuspended in an incubation buffer (Krebs buffer, 1% HSA). The cell suspension (0.5 ml) containing $\sim 10^6$ cells was incubated in 5-ml vials with the test solutions and D-[³H]glucose in an atmosphere of 95% O₂ and 5% CO₂ for 2 h. The incubation was stopped by adding 3 ml toluene (with 5 g Permablend/l) and

counted. Some experiments were performed using a 96-well format in which a 100- μ l cell suspension was incubated with test compound in an incubation buffer (Krebs buffer, 25 mmol/l HEPES, pH 7.8, 1% HSA) and stopped after 2 h with 150 μ l MicroScint-E (Packard, Groningen, the Netherlands). Dose-response profiles were generated, and EC₅₀ values were determined using a 4-parameter logistic function (19). The metabolic potency of the insulin analogs relative to human insulin was calculated as the ratio between the EC₅₀ values of human insulin and of the analog, assuming parallelism of the dose-response curves.

Mitogenic potency. The human osteosarcoma cell line Saos/B10 (20), a subclone of Saos-2 (ATCC HTB-85), was used in this study. B10 cells express ~30,000 IGF-I receptors and <1,000 insulin receptors per cell (W. Moritz, M. Böni-Schnetzler, unpublished data). Cells were cultured in DMEM/F12 supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin. During the entire experiment, cells were grown and kept at 37°C in a humidified atmosphere with 5% CO₂. For experiments, cells were trypsinized, plated in 24-well dishes at ~75–100 \times 10³ cells per well, and cultured for 16 h. Growth medium was then removed and replaced with test buffer (DMEM/F12 without FCS but with 0.5% BSA), and the cells were starved for 4 h. Then, the medium was withdrawn, and cells were incubated with test buffer containing 0.8 pmol/l to 0.8 mmol/l insulin analog for 16 h at 37°C before addition of [³H]methyl-thymidine. [³H]thymidine incorporation was allowed to take place for 4 h at 37°C. The cells were then washed 3 times with ice-cold phosphate-buffered saline and lysed in 10% trichloroacetic acid for 15 min under constant gentle rocking at 4°C. The soluble free [³H]thymidine was removed by 3 consecutive washes in ice-cold 10% trichloroacetic acid. The high molecular weight part of the cell lysates was resolubilized in 1 mol/l KOH and neutralized with an equal volume of 1 mol/l HCl, and DNA-incorporated [³H]thymidine was measured in a β -counter. Each concentration was assayed in triplicate for each experiment. The experiments were repeated 3 times. To compare the mitogenic potential of the insulin analogs with that of human insulin, log-dose response profiles were generated, and EC₅₀ values were estimated using a 4-parameter logistic function (19). Relative mitogenic potency was calculated as the ratio between the estimated EC₅₀ values (EC_{50,H1}/EC_{50,analog}), assuming parallelism of the log-dose response profiles.

RESULTS

We have determined the insulin and IGF-I receptor binding properties and metabolic and mitogenic potencies of a series of clinically relevant insulin analogs.

Insulin receptor binding. Relative insulin receptor binding affinities were (apart from in the case of insulin detemir) determined using solubilized hIR, to which human insulin bound with an affinity of ~0.01 nmol/l. The results are shown in Table 1.

Insulin aspart and insulin lispro were equally potent to human insulin in binding to the insulin receptor, in accordance

with the B26–B30 region being of little importance for insulin receptor recognition. The insulin receptor affinity of insulin glargine was also similar to that of human insulin and appeared to be determined by the combined effect of the A21Gly and B31B32diArg substitutions.

Insulin detemir was less potent than human insulin in binding to the insulin receptor. The relative receptor affinity of insulin detemir determined in this study was lower than the 46% found in a previous study (14). This difference is ascribed to the use of different assay systems in the 2 studies. It is emphasized that receptor binding assays with insulin detemir are complicated by the lipophilicity and albumin binding of the analog and that modified assay procedures therefore have to be applied.

IGF-I receptor binding. Relative IGF-I receptor affinities (apart from in the case of insulin detemir) were determined using solubilized human IGF-I receptor (hIGF-IR), to which human insulin bound with an affinity of ~200 nmol/l. The results are shown in Table 1.

Insulin aspart showed an IGF-I receptor affinity similar to or slightly lower than that of human insulin. In contrast, insulin lispro was 1.5-fold and insulin glargine 6.5-fold more potent in binding to the IGF-I receptor than human insulin. Similar to binding to hIR, the relative IGF-I receptor affinity of insulin glargine appeared to reflect the combined effect of the A21Gly and B31B32diArg substitutions. Thus, the 6.5-fold increase in IGF-I receptor affinity of insulin glargine corresponded roughly to the combined effect of a 2.6-fold reduction provided by A21Gly and a 20-fold increase provided by B31B32diArg.

Insulin detemir was >5-fold less potent than human insulin in binding to the IGF-I receptor in the applied assay system. Relative to human insulin, insulin detemir was slightly less potent in binding to hIGF-IR than in binding to hIR.

Insulin receptor dissociation. Time courses for dissociation of insulin analogs from the insulin receptor were determined using CHO-hIR cells, and k_d values were calculated from monoexponential dissociation profiles. Examples of dissociation curves are shown in Fig. 1.

The k_d for human insulin was $5.2 \pm 0.4 \times 10^{-3} \text{ min}^{-1}$. As shown in Table 1, insulin aspart and insulin lispro dissociated

TABLE 1
Receptor binding and metabolic and mitogenic potencies of insulin analogs relative to human insulin

Analog	Insulin receptor affinity (%)	Insulin receptor off-rate (%)	Metabolic potency (lipogenesis) (%)	IGF-I receptor affinity (%)	Mitogenic potency (%)
Human insulin	100	100	100	100	100
B10Asp	205 \pm 20	14 \pm 1*	207 \pm 14	587 \pm 50	975 \pm 173
Aspart	92 \pm 6	81 \pm 8*	101 \pm 2	81 \pm 9	58 \pm 22
Lispro	84 \pm 6†	100 \pm 11†	82 \pm 3	156 \pm 16	66 \pm 10
Glargine	86 \pm 3	152 \pm 13	60 \pm 3	641 \pm 51	783 \pm 132
A21Gly	78 \pm 10	162 \pm 11	88 \pm 3	42 \pm 11	34 \pm 12
B31B32diArg	120 \pm 4	75 \pm 8	75 \pm 5	2,049 \pm 202	2,180 \pm 390†
Detemir	46 \pm 5‡/18 \pm 2§	204 \pm 9	ca 27	16 \pm 1§	ca 11

Data are means \pm SE, except for metabolic potency data, which are means \pm 95% confidence limits. *From Hansen et al. (9); †from Sliker et al. (10); ‡from Markussen et al. (14). §Binding assays for insulin detemir were done in albumin-free buffer systems using sIR and siGF-IR to which human insulin bound with affinities of 0.1 and 93 nmol/l, respectively. ||Estimated potencies of free (not albumin-bound) insulin detemir. Free insulin detemir was estimated using the binding constants of 1×10^5 l/mol for HSA and 4×10^5 l/mol for BSA (21). Measured relative metabolic potency (in 1% HSA) was $1.7 \pm 0.3\%$, and measured relative mitogenic potency (in 0.5% BSA) was $0.36 \pm 0.06\%$. Attempts to use albumin-free buffer systems for determination of metabolic and mitogenic potency of insulin detemir were not successful.

from the insulin receptor with a rate similar to that of human insulin. The A21Gly substitution accelerated the dissociation rate ~1.6-fold compared with human insulin, whereas a B31B32diArg substitution slightly reduced the dissociation rate. The combined effect of these substitutions provided a slightly faster dissociation of insulin glargine from the insulin receptor when compared with human insulin.

Insulin detemir dissociated 2-fold faster from the insulin receptor than human insulin, suggesting that an increased off-rate from the insulin receptor contributes in reducing the hIR affinity of this analog.

Metabolic potency. The metabolic potencies of the insulin analogs were compared by generating dose-response profiles for stimulation of lipogenesis in primary mouse adipocytes. Human insulin stimulated lipogenesis ~12-fold over the basal level, with an EC₅₀ value of ~45 pmol/l. The relative metabolic potencies of the analogs are summarized in Table 1.

In general, the metabolic potencies correlated with the hIR binding affinities. However, the B31B32diArg analog had a slightly lower metabolic potency compared with its hIR affinity.

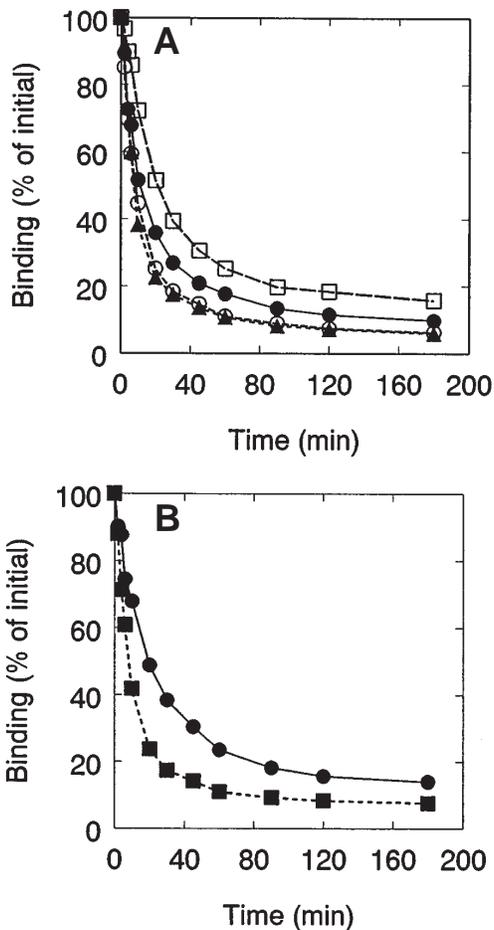


FIG. 1. Examples of time courses for dissociation of insulin analogs from the insulin receptor. *A*: Insulin A21Gly (▲), insulin glargine (○), insulin B31B32diArg (□), and human insulin (●). *B*: Insulin detemir (■) and human insulin (●). Each data point is the average of 2 determinations. All experiments were performed in triplicate. The resulting dissociation constants relative to human insulin are listed in Table 1.

The measured metabolic potency of insulin detemir was ~50-fold lower than that of human insulin. However, insulin detemir binds to HSA with an affinity of $\sim 1 \times 10^5$ l/mol (22), from which it can be calculated that insulin detemir is 93.7% albumin bound in the assay buffer. Assuming that only free insulin detemir is biologically active, the metabolic potency of insulin detemir relative to human insulin corrected for albumin binding was ~27%.

Mitogenic potency. The mitogenic potential of the insulin analogs was evaluated using human osteosarcoma cells (Saos/B10). Examples of parallel dose-response profiles that were used to determine mitogenic potencies of analogs relative to human insulin are shown in Fig. 2. The Saos/B10 cells showed a maximum insulin growth response of >10-fold over basal and did not differ between analogs. The EC₅₀ value for stimulation of mitogenesis by human insulin was typically 4 nmol/l. IGF-I was included as a positive control in all studies and stimulated growth with an EC₅₀ value of ~50 pmol/l.

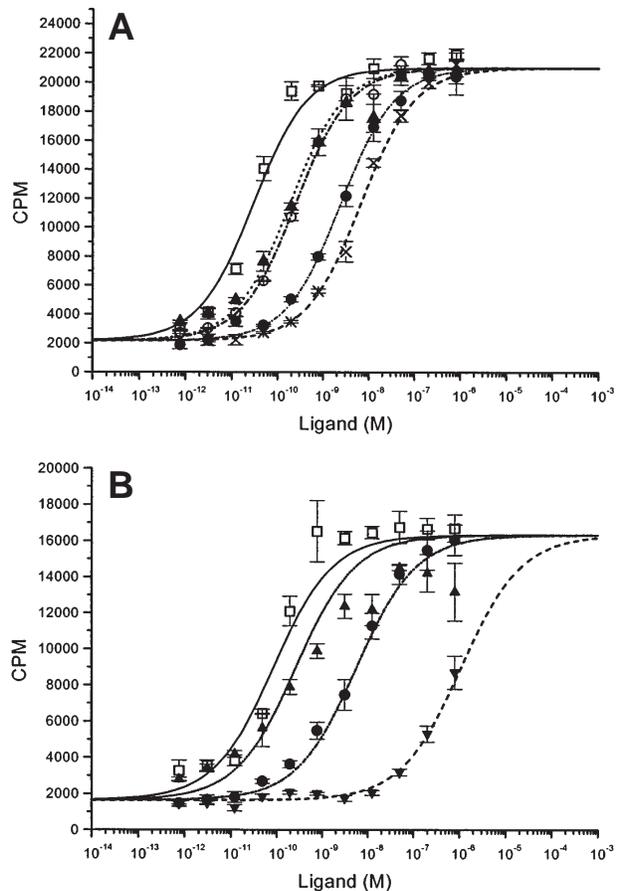


FIG. 2. Dose-response profiles for stimulation of [³H]thymidine incorporation into DNA in Saos/B10 cells. *A*: IGF-I (□), human insulin (●), insulin B10Asp (▲), insulin glargine (○), and insulin A21Gly (×). *B*: IGF-I (□), human insulin (●), insulin B10Asp (▲), and insulin detemir (▼). It is noted that in the case of insulin detemir, the maximum level of DNA synthesis was not reached within the tested concentration range because of the low mitogenic potential and high degree of albumin binding of this analog. However, the data do allow for calculating the mitogenic potency of insulin detemir relative to human insulin under the assumption of parallel log-dose response profiles. Each data point is the mean of 3 determinations. All experiments were performed in triplicate. The resulting mitogenic potencies for all analogs relative to human insulin are listed in Table 1.

Both insulin aspart and insulin lispro were slightly less potent than human insulin in stimulating growth of Saos/B10 cells. The 2 analogs had equal mitogenic potencies, despite the slightly higher IGF-I receptor affinity of insulin lispro than of insulin aspart. This finding is in agreement with previous data obtained using CHO-K1 cells (9) and HMECs (10). In contrast, insulin glargine was ~8-fold more potent than human insulin in stimulating DNA synthesis in Saos/B10 cells. This increase corresponded to the combined effect of the A21Gly and B31B32diArg substitutions that provided a 3-fold lower and 22-fold higher mitogenic potency than human insulin, respectively. It is noted that the result for B31B32diArg was determined in a previous study using HMECs. Saos/B10 cells have similar numbers of insulin and IGF-I receptors per cell as HMECs (23).

The measured mitogenic potency of insulin detemir was >250-fold lower than that of human insulin. However, insulin detemir binds to BSA with an affinity of $\sim 4 \times 10^5$ l/mol (21), from which it can be calculated that insulin detemir is 96.8% albumin-bound in the assay buffer. Assuming that only free insulin detemir is biologically active, the mitogenic potency of insulin detemir relative to human insulin corrected for albumin binding was ~11%.

The correction for albumin binding is associated with some uncertainty. The mitogenic potency of insulin detemir has, however, also been determined in CHO-K1 cells at low albumin concentrations (0.02% HSA) (A.S., unpublished data). At this albumin concentration, insulin detemir is estimated to be 77% free (not albumin-bound). In this assay, the mitogenic potency of insulin detemir was determined to be 5.1% (95% confidence limits 4.0–6.4%, $n = 7$) compared with human insulin, and the corresponding calculated mitogenic potency of free insulin detemir was 7%. The agreement between this result and that found in the present study provides support for the validity of the applied correction procedure.

DISCUSSION

Insulin binds and activates its cognate receptor with subnanomolar affinity. Insulin also binds the structurally related IGF-I receptor but with ~1,000-fold lower affinity than the insulin receptor and also with ~1,000-fold lower affinity than IGF-I. At normal physiological insulin concentrations, the IGF-I receptor therefore does not play any role in mediating the effects of insulin. Accordingly, it has been demonstrated that both metabolic and mitogenic effects of the hormone can be mediated via the insulin receptor (24). However, at supra-physiological insulin concentrations, insulin might also exert mitogenic effects via the IGF-I receptor, which transmits growth stimuli more efficiently than the insulin receptor (24). **Insulin receptor binding and metabolic potency.** The analogs examined in this study confirmed that amino acid modifications in the insulin B-chain beyond position B25 are not essential for insulin receptor binding (3,4). However, insulin detemir had a reduced insulin receptor affinity although modified only in this region of the molecule. It is likely that the C14 fatty acid attached to the B29Lys side chain makes hydrophobic contacts to one or more of the aromatic amino acids in positions B24–B25 and thereby partly shields these residues from recognition by the insulin receptor. The small reduction of the insulin receptor affinity provided by the A21Gly substitution may be due to a minor structural change caused by the loss of the salt bridge between A21Asn and B22Arg (5).

The relative metabolic potency of the analogs correlated well with the hIR affinities, as expected from previous studies that have shown that binding and activation of the insulin receptor are the primary events leading to the metabolic response of the hormone (6,8).

The relationship between *in vitro* metabolic potency and biological activity *in vivo* was not examined in this study. However, it is interesting to note that insulin detemir, which showed a relatively low insulin receptor binding affinity and metabolic potency in this study, has been reported to be less effective than human insulin when given in equimolar doses to healthy volunteers (25,26). It was previously found that insulin analogs with receptor binding affinities and metabolic potencies ranging from 20 to 300% had equivalent *in vivo* biological activity in pigs, probably because elimination of these analogs was almost exclusively receptor mediated (27). However, it is possible that the low receptor binding affinity of insulin detemir combined with the markedly changed pharmacokinetic properties of this analog (14,25,26) could contribute to reduce the biological activity *in vivo*, e.g., by allowing a greater fraction of insulin detemir than of human insulin to undergo nonreceptor-mediated clearance.

IGF-I receptor binding and mitogenic potency. Insulin glargine was 6.5-fold more potent than human insulin in binding to the IGF-I receptor and slightly more potent than insulin B10Asp. The B31B32diArg substitution has previously been found to confer increased affinity for the IGF-I receptor. Thus, an analog containing both a B31B32diArg substitution and a B10Asp substitution was almost 90-fold more potent than human insulin in binding to the IGF-I receptor (10). The 2 arginine residues at position B31 and B32 probably mimic the positively charged Arg37Arg38 sequence in the C domain of IGF-I, which has previously been shown to contribute in mediating IGF-I receptor selectivity (28). Similarly, the loss of a positive charge in position B29 as a consequence of the acylation of the side-chain amino group of B29Lys might contribute to reducing the IGF-I receptor affinity of insulin detemir. The 3-fold reduction of IGF-I affinity caused by the A21Gly substitution is difficult to explain considering that native IGF-I contains an Ala residue in the corresponding position.

Previous structure-function studies using insulin analogs have shown that analogs having either an increased affinity for the IGF-I receptor and/or a reduced rate of dissociation from the insulin receptor possess a relatively higher mitogenic potency than metabolic potency compared with human insulin. In this study, the relative mitogenic potencies were determined using the human osteosarcoma cell line Saos/B10, which shows a marked growth response on insulin stimulation (10- to 20-fold). The mitogenic potency of the tested insulin analogs correlated in most cases with the relative IGF-I receptor binding affinities, suggesting that the growth response of insulin in this cell type is mediated via the IGF-I receptor. This is particularly clear for the 2 analogs carrying a B31B32diArg substitution. Thus, insulin glargine and insulin B31B32diArg were markedly more potent than human insulin in IGF-I receptor binding and stimulation of mitogenesis but had insulin receptor off-rates comparable to that of human insulin. The low mitogenic potency of insulin lispro, despite the slightly elevated IGF-I receptor affinity, indicates that a minor increase of IGF-I affinity is not sufficient to provide a mitogenic stimulus of this cell line. A similar result was found in studies using human mammary epithelial cells (10).

However, a sustained activation of the insulin receptor might also contribute to provide the mitogenic response of the insulin analogs in the applied cell line. Thus, for insulin B10Asp, which dissociated slowly from the insulin receptor and was also more potent than human insulin in binding to the IGF-I receptor, the relative mitogenic potency was higher than expected from the IGF-I receptor affinity, suggesting that the decreased insulin receptor dissociation rate was involved. Similarly, the more rapid dissociation of insulin detemir than of human insulin from the insulin receptor might contribute to the low mitogenic potential of this analog. However, to resolve the relative contribution of IGF-I receptor binding and sustained signaling from the insulin receptor to stimulation of Saos/B10 cell growth, we would need a slowly dissociating analog that binds the IGF-I receptor with the same affinity as human insulin.

The 6- to 8-fold increased IGF-I receptor affinity and mitogenic potency of insulin glargine found in this study is somewhat greater than previously reported. Thus, Bähr et al. (29) found that glargine was only slightly more potent than human insulin in binding to the IGF-I receptor and stimulating DNA synthesis of H9 myotubes. Berti et al. (30) found similar growth-stimulating effects of glargine and human insulin using Rat-1 fibroblasts. However, none of these studies allowed for exact calculation of mitogenic potencies. The observed differences are most likely due to different IGF-I and/or insulin receptor densities in the applied cell lines.

It is interesting to note that the dose-response profiles for IGF-I receptor binding and stimulation of mitogenesis of Saos/B10 cells do not superimpose. Thus, the EC_{50} value of human insulin for stimulation of DNA synthesis is ~ 4 nmol/l, whereas the IC_{50} value for insulin binding to the IGF-I receptor is ~ 100 -fold greater (data not shown). A similar relationship between potencies for IGF-I receptor binding and stimulation of cell growth was observed using human mammary epithelial cells (10). This relationship implies that only a small fraction of IGF-I receptors need to be activated to fully activate mitogenesis. Alternatively, it can be speculated that hybrid insulin/IGF-I receptors (31–33) might be implicated in mediating the mitogenic response of insulin in these cell lines. **Toxicopharmacological implications.** An elevation of IGF-I receptor affinity and mitogenic potency compared with human insulin can potentially lead to undesired toxicological effects of insulin analogs. However, it is not easy to assess to what extent an IGF-I receptor-mediated effect of insulin can add to the biological response of endogenous IGF-I. The total plasma levels of endogenous IGF-I are high (20–80 nmol/l), but IGF-I is almost completely bound (>95%) to specific binding proteins, and the concentration of free IGF-I in the bloodstream is therefore much lower than the total concentration (34,35). Furthermore, insulin-resistant type 2 diabetic patients show plasma levels of insulin that are severalfold greater than normal physiological insulin concentrations. Hence, it cannot be excluded that insulin analogs with a relatively high affinity for IGF-I receptors can have IGF-I receptor-mediated effects in vivo.

IGF-I has a variety of physiological effects on growth and differentiation and is implicated in mediating the effect of growth hormone (34,36). An increased IGF-I receptor activation could precipitate pathological effects. Thus, stimulation of the growth hormone/IGF-I axis has been implicated in various pathological conditions, including mammary, ovarian,

and bone tumor development (37,38) and development of retinopathy (39–42) and diabetic nephropathy (43). Accordingly, in the clinical trials of IGF-I that have now been terminated, these potential side effects were given special attention (44,45). Interestingly, a recent clinical case report suggested that insulin lispro, which has a slightly increased IGF-I receptor affinity compared with human insulin, might enhance the development of diabetic retinopathy. However, because this was a case report, there was no control group, and the observed development of retinopathy might have been due to other factors, e.g., a more rapid tightening of glycemic control after treatment with lispro (46).

Conclusion. After more than 50 years of treatment with animal insulins, recombinant DNA technologies and advanced protein chemistry (47–49) made human insulin preparations available in the early 1980s. Over the last decade, a number of insulin analogs have been constructed with the aim of providing the diabetic patient with the most efficient, reproducible, and convenient therapy possible. It is imperative that these benefits are not achieved at the cost of the safety profile of the native human hormone.

The results presented in this study show that the amino acid modifications in the 2 rapid-acting insulin analogs, lispro and aspart, had no significant influence on metabolic or mitogenic potency. In contrast, the 2 long-acting analogs, insulin detemir and insulin glargine, both showed in vitro properties that differed significantly from human insulin. The clinical safety implications of the elevated IGF-I receptor affinity and mitogenic potency of insulin glargine are not clear. In contrast, insulin detemir was less potent than human insulin in binding to the IGF-I receptor and stimulating mitogenesis as well as in binding to the insulin receptor and stimulating lipogenesis. Hence, the in vitro profile of insulin detemir did not cause any safety concerns. The relatively low in vitro potency of insulin detemir may, however, contribute to explanations of why this analog is not as effective on a molar basis as human insulin in humans.

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