Originals

In vitro activity of biosynthetic human diarginylinsulin

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Summary. In diarginylinsulin two arginine residues are located at the C-terminal end of the B-chain (Arg^{B31} and Arg^{B32}). This accounts for a shift of the isoelectric point from pH 5.4 in native insulin to pH 7.0 in diarginylinsulin leading to pharmacodynamic characteristics of an intermediate acting insulin when administered s.c. as pH 4.0–5.0 solution. We have investigated insulin receptor binding and biological activity of biosynthetic human diarginylinsulin in human adipocytes and compared to native insulin and proinsulin. Association- and dissociation studies of insulin receptor binding revealed no differences for diarginylinsulin and native insulin. In competition studies under steady-state binding conditions, half-maximal displacement of tracer occurred at 352 ± 33 pmol/l, 337 ± 32 pmol/l and 3640 ± 480 pmol/l for diarginylinsulin, insulin and proinsulin, respectively. The biologic potency of human diarginylinsulin was evaluated by

the ability to stimulate D-glucose transport and by the assessment of the antilipolytic activity. Activation of D-glucose transport was half-maximal at 49.6 ± 5.4 pmol/l (diarginylinsulin), 44.8 ± 5.8 pmol/l (insulin) and at 476.7 ± 134.3 pmol/l (proinsulin). Half-maximal inhibition of lipolysis occurred at 13.9 ± 3.4 pmol/l, 15.4 ± 2.9 pmol/l and 138.4 ± 38.6 pmol/l, respectively. In conclusion, diarginylinsulin has almost identical insulin receptor binding characteristics and full biological activity in vitro compared to native insulin. This pharmacodynamically intermediate acting insulin preparation is therefore of potential therapeutical value.

Key words: Intermediate acting insulin, biosynthetic human diarginylinsulin, insulin receptor binding, glucose transport, anti-lipolysis, protamine allergy, proinsulin.

In the bioconversion process of proinsulin to insulin several intermediate products physiologically occur. Diarginylinsulin is one of these intermediate metabolites and differs from native insulin by two remaining arginine residues at the C-terminal end of the B-chain [1–6] (Fig. 1). The two arginine residues at the C-terminal end of the B-chain in diarginylinsulin account for a shift of the isoelectric point from pH 5.4 in native insulin to pH 7.0 in diarginylinsulin [4].

Diabetic patients receiving daily s.c. injections of insulin containing protamine appear to have a 40 to 50-fold increase in the risk of life-threatening reactions when given protamine i.v. for reversal of heparin anticoagulation during vascular surgery, cardiac catheterization or dialysis [8, 9].

Attempts have been made to find an insulin analogue which retains full biological activity and has an intermediate or long-lasting action without the addition of a retardant. Due to the shift of the isoelectric point to pH 7.0 diarginylinsulin is least soluble at a physiological pH. When this insulin analogue is injected s.c. as pH 4.0–5.0

solution it has the pharmacodynamic characteristics of an intermediate acting NPH-insulin preparation.

However, previous studies revealed a reduced biological activity of porcine diarginylinsulin [7, 10, 11]. By application of recombinant DNA-technology biosynthetic human diarginylinsulin became available. The in vitro bi-

Fig. 1. Scheme of the proinsulin molecule. Diarginylinsulin occurs in the bioconversion process of proinsulin to insulin and differs from native insulin by two remaining arginine residues at the C-terminal end of the B-chain

ological activity of the human diarginylinsulin has not yet been investigated in isolated human adipocytes. In this study we describe the *in vitro* characteristics of this insulin analogue with respect to insulin receptor binding, degradation, activation of D-glucose transport and inhibition of catecholamine stimulated lipolysis. Biosynthetic human diarginylinsulin is compared to native human insulin and biosynthetic human proinsulin.

Subjects, materials and methods

Materials

Crystalline human diarginylinsulin and native human insulin as well as the respective ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-monoiodinated insulin preparations (specific activity 340 mCi/mg) were provided by Hoechst AG (Frankfurt, FRG). Biosynthetic human proinsulin and radiolabelled ($[^{125}\text{I}]\text{Tyr}^{\text{B9}}$)-proinsulin (specific activity 216 mCi/mg) were a gift from the Eli Lilly Laboratories (Indianapolis, Ind., USA). Proinsulin has a different numerical sequence of the carboxyterminal amino acids than insulin because of the connecting peptide (C-peptide). In fact the iodination of the tyrosine residue at position 79 in proinsulin is equivalent to the iodinated tyrosine in position A14 after cleavage of the C-peptide [12, 13]. Iodination of the tyrosine residue in position A14 (diarginylinsulin, native insulin) or 79 (proinsulin) does not influence receptor binding characteristics or biological activity [14–16].

Potency and purity of all investigated insulins were checked by HPLC according to methods described in the United States pharmacopoeia (USP XXI). No cocrystallization was detectable by HPLC.

Crude collagenase was obtained from Sigma (type II, lot 102 F-6805). Human serum albumin was purchased from Behringwerke (Marburg, FRG). U- ^{14}C -D-glucose (specific activity 270 mCi/mmol) was obtained from Amersham (Braunschweig, FRG), phloretin from Sigma (St. Louis, Mo., USA), silicone oil (density 0.97 g/ml) from Serva Feinbiochemica (Heidelberg, FRG). All other chemicals were purchased from Merck (Darmstadt, FRG).

Preparation of isolated adipocytes

Subcutaneous adipose tissue (about 4–5 g) was obtained from the abdomen of patients undergoing elective gastroenterological surgery. No subject suffered from any endocrine or metabolic disorder or was taking drugs known to affect metabolism. Impaired glucose tolerance was excluded by measuring the fasting blood glucose concentration and the 2 h value after a 75 g oral glucose load according to World Health Organisation criteria [17]. The study was approved by the local ethical committee in accordance with the Declaration of Helsinki and informed consent was obtained from the patients.

The adipose tissue was finely chopped and incubated for 90 min at 37° C in a HEPES buffer (pH 7.4), containing human serum albumin (25 g/l) and collagenase (0.5 g/l). The isolated adipocytes were subsequently washed five times in a HEPES buffer containing 50 g/l human albumin [18, 19]. The diameters of adipocytes ($n = 200$) were measured at 200-fold magnification using an eyepiece micrometer. Surface area and volume were calculated for every cell diameter. The cell diameter of the isolated fat cells averaged $102 \pm 7 \mu\text{m}$.

Insulin receptor binding and degradation

Insulin receptor binding studies to isolated human adipocytes (300 μl cell suspension with an adipocyte volume fraction of 0.05, i.e. about 1×10^5 cells/ml cell suspension) were performed in a

HEPES buffer (10 mmol/l HEPES, 50 g/l human serum albumin, pH 7.4) at 37° C. Each ^{125}I -labelled ligand (final concentration 20 pmol/l) was incubated with and without increasing amounts of unlabelled diarginylinsulin, native insulin or proinsulin, respectively. The reaction was stopped by adding 10 ml of chilled 0.154 mol/l NaCl and subsequent centrifugation through silicone oil [18, 19]. Non-specific binding was measured by incubating tracer in the presence of a large excess of unlabelled insulin. Non-specific binding accounted for $9.7 \pm 1.6\%$ (diarginylinsulin), $9.7 \pm 1.4\%$ (native insulin) and $24.4 \pm 2.6\%$ (proinsulin) of the cell-bound fraction at tracer concentration.

For association studies the ^{125}I -labelled ligands were incubated for the time indicated (1 min to 240 min) and the reaction was terminated as described above. At each time point the non-specific binding was measured and subsequently subtracted from the corresponding data for total binding.

Dissociation rates were determined by first preincubating isolated human adipocytes at 37° C with either ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-diarginylinsulin or ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-insulin for 90 min to achieve steady-state binding conditions. Each incubation mixture was then centrifuged for 60 s and the infranatant removed. The adipocytes were rapidly washed twice by diluting to the original volume with buffer at 4° C and repeating the centrifugations and aspirations. After the third aspiration, the cells were diluted to the original volume either with buffer alone or buffer to which either diarginylinsulin, native insulin or proinsulin at a final concentration of 0.2 $\mu\text{mol/l}$ was added (22° C). At this hormone concentration a maximal effect of ^{125}I -insulin dissociation is reported [20, 21]. The reaction was stopped at the times indicated (10 min to 180 min) and cell-associated radioactivity was determined [21]. Results are expressed as percentage specific binding per 10 cm^2 plasma membrane surface area [22, 23].

Total degradation (cellular and extracellular) of ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-diarginylinsulin, ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-insulin or ($[^{125}\text{I}]\text{Tyr}^{\text{A14}}$)-proinsulin was assessed by the trichloroacetic acid (12% weight/volume) precipitation method [24]. Extracellular degradation (as assessed in the presence of 0.1 $\mu\text{mol/l}$ insulin accounted for less than 15% of the total degradation for all three investigated insulins.

Measurement of insulin stimulated glucose uptake

Initial D-glucose uptake was measured using the method described by Pedersen and Gliemann [25] and modified by Zeuzem and Taylor [26]. Human adipocytes (40 μl cell suspension with an adipocyte volume fraction of 0.40, i.e. about 0.8×10^6 cells/ml) were preincubated with and without increasing concentrations of diarginylinsulin, native insulin or proinsulin for 180 min. The incubation was started by a direct injection of 12 μl U- ^{14}C -D-glucose into the cell suspension (final concentration 20 $\mu\text{mol/l}$) and then stopped by the addition of 3 ml of 154 mmol/l NaCl, containing 0.3 mmol/l phloretin, pH 7.4. Silicone oil was layered on the surface and the tubes were centrifuged within 2 min for 90 s at 3000 g. The cell pellet was then immediately harvested and added to 5 ml scintillation fluid.

Extracellular trapped radioactivity was measured by adding 3 ml of the phloretin solution to the cell suspension before incubation with U- ^{14}C -D-glucose and subsequently subtracted from all observed values. Results are expressed as pmol D-glucose taken up per 10 cm^2 plasma membrane surface area per 20 s [23]. The coefficient of variation of the assay was 9.0% for basal and 11.4% for maximal uptake.

Measurement of the antilipolytic insulin effect

Human adipocytes (500 μl cell suspension with an adipocyte volume fraction of 0.15, i.e. about 0.3×10^6 cells/ml) were preincubated with and without increasing concentrations of diarginylinsulin, native insulin and proinsulin for 180 min at 37° C to achieve steady-state binding conditions. Lipolysis was stimulated by noradrenaline

Fig. 2. Association kinetics. Time course of ^{125}I -diarginylininsulin ($\circ\text{---}\circ$), ^{125}I -insulin ($\times\text{---}\times$) and ^{125}I -proinsulin ($\Delta\text{---}\Delta$) binding ($n = 4$). Isolated human adipocytes were incubated at 37°C and the reaction was stopped at the indicated time points. All data are corrected for non-specific binding

(final concentration $1.7\text{ }\mu\text{mol/l}$) and caffeine (final concentration 0.3 mmol/l) and the suspension was further incubated for 60 min. Subsequently the cells were centrifuged through silicone oil and the infranatant was sampled. Glycerol release into the medium was measured using an enzymatic method [27]. Results are expressed as nmol glycerol released per 100,000 cells per 60 min [23].

Statistical analysis

All results are presented as mean $\pm$ SEM. Statistical analysis was performed using Student's paired t -test.

Results

Steady-state binding

To determine when steady-state binding conditions were achieved, the time course of binding for ^{125}I -diarginylininsulin, ^{125}I -insulin and ^{125}I -proinsulin (each concentration 20 pmol/l) were studied in parallel at 37°C . In isolated human adipocytes internalization and degradation of the insulin is minimal even at physiological temperatures [18]. These binding studies therefore reflect primarily the association of each insulin to the cell surface receptors.

^{125}I -diarginylininsulin reached half-maximal binding at $19.3 \pm 3.1\text{ min}$ compared to $19.8 \pm 2.1\text{ min}$ for native insulin and $40.0 \pm 4.1\text{ min}$ for proinsulin. ^{125}I -diarginylininsulin reached 87% of its maximal binding at 60 min and maximal binding at 90 min. Maximal binding was maintained at least up to 4 h. Native ^{125}I -insulin reacted similarly, reaching maximal binding also at 90 min. However, human proinsulin attained steady-state binding conditions much slower than diarginylin- and native insulin. Under identical conditions, ^{125}I -proinsulin reached 47% of its maximal binding at 60 min and maximal binding at 180 min (Fig. 2). For all competition studies of insulin receptor binding we therefore used an incubation period of 180 min to ensure steady-state conditions for all investigated insulins.

Fig. 3. Association rate constants. Second order rate plot of the data shown between 1 and 4 min in Figure 2 (R = receptor number, L = ligand concentration, X = % specific binding at times indicated). The association rate constants (k_a) were $1.9 \times 10^8\text{ mol/l}^{-1}\text{min}^{-1}$, $1.3 \times 10^8\text{ mol/l}^{-1}\text{min}^{-1}$ and $0.01 \times 10^8\text{ mol/l}^{-1}\text{min}^{-1}$ for diarginylininsulin ($\circ\text{---}\circ$), native insulin ($\times\text{---}\times$) and proinsulin ($\Delta\text{---}\Delta$), respectively

Kinetic analysis

The calculated association rate constant [28] k_a was $1.9 \times 10^8\text{ mol/l}^{-1}\text{min}^{-1}$ for diarginylininsulin compared to $k_a = 1.3 \times 10^8\text{ mol/l}^{-1}\text{min}^{-1}$ (insulin) and $k_a = 0.01 \times 10^8\text{ M}^{-1}\text{min}^{-1}$ (proinsulin) (Fig. 3). The difference between diarginylininsulin and insulin was not significant ($p > 0.1$).

The effect of unlabelled diarginylininsulin, insulin and proinsulin on the dissociation of (^{125}I)-diarginylininsulin (Fig. 4) and (^{125}I)-insulin (data not shown) were investigated. The dissociation curves of diarginylininsulin and native insulin were almost identical with a half-time of dissociation of $29.8 \pm 4.2\text{ min}$ and $28.6 \pm 5.6\text{ min}$, respectively. The k_d for dissociation were $2.5 \times 10^{-2}\text{ min}^{-1}$ and $2.7 \times 10^{-2}\text{ min}^{-1}$, respectively. Unlabelled diarginylininsulin and native insulin accelerated the dissociation of the corresponding ^{125}I -labelled ligand when compared to the dissociation due to dilution only. Half-maximal disso-

Fig. 4. Dissociation kinetics. Time course of ^{125}I -diarginylinulin dissociation. Human adipocytes were preincubated at 37°C with ^{125}I -diarginylinulin for 90 min to achieve steady-state binding conditions ($n = 5$). Subsequently the cells were rapidly washed at 4°C and resuspended to the original volume with either buffer alone ($\square$ --- $\square$) or buffer to which either diarginylinulin ($\bullet$ — $\bullet$), insulin ($\blacklozenge$ — $\blacklozenge$) or proinsulin ($\blacktriangle$ — $\blacktriangle$) at a final concentration of $0.2\ \mu\text{mol/l}$ was added (22°C). The reaction was stopped at the times indicated, the data corrected for non-specific binding and expressed as percentage of maximal binding

Fig. 5. Insulin receptor binding. Specific insulin binding to isolated human adipocytes incubated with $20\ \text{pmol/l}$ monoiodinated tracer at 37°C in absence or presence of diarginylinulin ($\circ$ — $\circ$), native insulin ($\times$ --- $\times$) and proinsulin (Δ --- Δ) in increasing concentrations ($n = 7$)

ciation occurred at $178.0 \pm 17.1\ \text{min}$ (dilution only) and at $29.8 \pm 4.2\ \text{min}$ (diarginylinulin). Proinsulin dissociated ^{125}I -diarginylinulin and ^{125}I -insulin much slower with a half-time of $69.0 \pm 14.5\ \text{min}$ and $78.1 \pm 5.4\ \text{min}$, respectively.

Insulin receptor binding

Competition studies were performed by incubating isolated human adipocytes with human (^{125}I)-Tyr A14 -diarginylinulin, (^{125}I)-Tyr A14 -insulin and (^{125}I)-Tyr 79 -proinsulin and increasing concentrations of unlabelled diarginylinulin, native insulin and proinsulin, respectively for 180 min at 37°C . At equilibrium $1.21 \pm 0.10\%$ of ^{125}I -diarginylinulin was bound per $10\ \text{cm}^2$ plasma membrane surface area compared to $1.09 \pm 0.13\%$ (^{125}I -insulin) and $0.10 \pm 0.03\%$ (^{125}I -proinsulin). The difference in maximal

binding between diarginylin- and native insulin was not significant ($p > 0.1$).

The displacement curves for diarginylin- and native insulin were similar. Half-maximal displacement of tracer occurred at $352 \pm 33\ \text{pmol/l}$, $337 \pm 32\ \text{pmol/l}$ and $3640 \pm 480\ \text{pmol/l}$ for diarginylinulin, insulin and proinsulin, respectively (Fig. 5). Scatchard analysis [29] of the binding data revealed curvilinear plots for all three investigated insulins. There were no differences between diarginylinulin and insulin concerning the slopes of the high affinity portions of the plots. As anticipated, the abscissal intercepts (computer modelled, Dr. Schloos, Department of Pharmacokinetics, University of Frankfurt, FRG) yielded similar numbers of receptor sites per cell membrane surface area (7.2 , 7.7 and $8.2\ \text{fmol}$ per $10\ \text{cm}^2$ cell membrane surface area, respectively), indicating that the investigated three ligands bind to a similar number of receptors. The differences between diarginylinulin and proinsulin were most marked for the high affinity portions of the plots, expressing the marked decrease of the receptor binding affinity of proinsulin.

Degradation

As measured by solubility in trichloroacetic acid 1.5% of (^{125}I)-Tyr A14 -diarginylinulin, 1.3% of (^{125}I)-Tyr A14 -insulin and 0.8% of (^{125}I)-Tyr 79 -proinsulin was degraded per 1×10^5 cells/ml per 60 min.

Glucose transport

In isolated human adipocytes basal glucose uptake was $0.80 \pm 0.09\ \text{pmol} \cdot 10\ \text{cm}^2 \cdot 20\ \text{s}^{-1}$. Initial D-glucose uptake was half-maximally stimulated at $49.6 \pm 5.4\ \text{pmol/l}$ (diarginylinulin), at $44.8 \pm 5.8\ \text{pmol/l}$ (native insulin) and at $476.7 \pm 134.3\ \text{pmol/l}$ (proinsulin). Rates of D-glucose uptake achieved by maximal stimulation with diarginylinulin, insulin and proinsulin were similar (1.46 ± 0.17 , 1.45 ± 0.19 and $1.41 \pm 0.17\ \text{pmol} \cdot 10\ \text{cm}^2 \cdot 20\ \text{s}^{-1}$, respectively). This results in an almost identical dose-response

Fig. 6. D-glucose transport. Initial rates of D-glucose uptake by human adipocytes incubated at 37° C in absence or presence of diarginylinsulin (○—○), insulin (×—×) and proinsulin (Δ—Δ) in increasing concentrations ($n = 6$)

curve for diarginylinsulin and native insulin. The dose-response curve of proinsulin is shifted to the right, indicating a reduced sensitivity but no decrease in responsiveness (Fig. 6).

Anti-lipolysis

The glycerol release for noradrenaline and caffeine stimulated lipolysis was 1086 ± 122 nmol per 100,000 cells per 60 min. Half-maximal inhibition of lipolysis occurred at 13.9 ± 3.4 pmol/l (diarginylinsulin), 15.4 ± 2.9 pmol/l (insulin) and 138.4 ± 38.6 pmol/l (proinsulin). Maximal inhibition of lipolysis was observed at 814 ± 229 pmol/l, 943 ± 307 pmol/l and $75,714 \pm 19,615$ pmol/l, respectively. No significant differences were found for the maximal inhibition of lipolysis ($59.4 \pm 6.9\%$, $61.3 \pm 8.8\%$ and $54.5 \pm 8.3\%$) nor the paradoxical effect of the insulins at high concentrations in stimulating lipolysis (Fig. 7).

Fig. 7. Anti-lipolysis. Anti-lipolysis by human adipocytes incubated at 37° C in absence or presence of diarginylinsulin (○—○), insulin (×—×) and proinsulin (Δ—Δ) in increasing concentrations ($n = 7$)

Discussion

The polypeptide chain of proinsulin is ordered as follows: NH_2 – B-chain – Arg – Arg – C-peptide – Lys – Arg – A-chain – COOH (Fig. 1). The conversion of proinsulin to insulin in the B-cells of the pancreas occurs by a branched pathway involving the formation of Arg⁶⁵/Gly⁶⁶-split proinsulin and Arg³²/Glu³³-split proinsulin, the latter being the preferential cleavage pathway (about 3 to 1) [5, 30]. The next step in the bioconversion process is the formation of des-Lys⁶⁴, Arg⁶⁵-proinsulin and des-Arg³¹, Arg³²-proinsulin (des-dipeptide proinsulin intermediates). Cleavage of either intermediate by a trypsin-like endoprotease at the remaining paired dibasic amino acid conversion site, followed by the action of a carboxypeptidase B-like exoprotease to remove COOH-terminal basic residues, yields free lysine and arginine. During the last conversion steps of the des-Lys⁶⁴, Arg⁶⁵-proinsulin diarginyl- and monoarginylinsulin occur as intermediates [3, 12].

The biological activities of several derivatives (conversion intermediates) of human proinsulin containing peptide bond cleavages or peptide deletions in the connecting peptide region have been investigated by Peavy et al. [31]. At high concentrations each derivative produced the same maximal response. Differences in the relative potency of these analogues, as indicated by the concentration required to produce a half-maximal response, generally agreed with the differences in receptor binding affinity. Interestingly, an apparent discrepancy was described for the des-[Glu³³-Leu⁵⁶]-proinsulin. This analogue demonstrated a receptor binding affinity 27 times that of human proinsulin, but a biological activity only nine times that of proinsulin [31]. Des-[Glu³³-Leu⁵⁶]-proinsulin differs from diarginylinsulin only by nine amino-acids, which remain at the NH_2 -terminal end of the A-chain (Ala⁵⁷ to Arg⁶⁵). In the present study, however, no differences were observed between receptor binding affinity and biological potency for the diarginylinsulin. The relative binding affinity of human proinsulin was 9.7% compared to diarginylinsulin, while the relative biological potencies for stimulating glucose-transport and inhibiting lipolysis were 10.4% and 10.0%, respectively.

The proposed insulin receptor binding region of the insulin molecule comprises the amino acids at position Gly¹, Gln⁵, Tyr¹⁹ and Asn²¹ of the A-chain and the position Val¹², Tyr¹⁶, Arg²², Gly²³, Phe²⁵ and Tyr²⁶ of the B-chain [34, 35]. The almost identical receptor binding affinity of biosynthetic human diarginylinsulin and native insulin implies that the substitution of Arg^{B31} and Arg^{B32} does not affect the binding region and makes conformational changes of this region unlikely.

Kinetic studies showed no differences in the association rate between diarginylinsulin and native insulin. Both ligands were capable of accelerating the dissociation of either ¹²⁵I-diarginylinsulin or ¹²⁵I-insulin. These data suggest that diarginylinsulin is able to exhibit negative cooperative effects to a similar extent as native insulin. Studies with modified insulin analogues have defined the C-terminal end of the B-chain and the position Asn^{A21} as most important for the potency of an insulin analogue to induce negative cooperative effects [36]. The substitution of two further amino-acids at the C-terminal B-chain as in the diarginylinsulin did not influence the negative cooperative properties.

Various chemical substitutions at the COOH-terminal end of the B-chain, e.g. with acetyl residues or butyloxycarbonyl (BOC) derivatives, were reported to cause a 25–56% decrease in potency [33]. In previous studies the biological activity of porcine diarginylinsulin was found to be reduced by 11 to 60% compared to native insulin [7, 10, 11, 32].

The results of biosynthetic human diarginylinsulin in this study are in contrast to these previously reported data of porcine diarginylinsulin. However, most of the studies on the biological activity of porcine diarginylinsulin were performed ten or more years ago. The possibility exists that a small amount of contamination with proinsulin or partially cleaved proinsulin had been present, therefore explaining the reduced activity of porcine diarginylinsulin found in these studies. Native porcine and human insulin differ only by one amino acid (alanine and threonine in position B30). The biological activity of both insulins is equivalent. Therefore species differences as an explanation of the described differences appear to be unlikely. However, the two arginine residues are substituted at the position B30. It remains questionable whether the addition of two arginine residues at Ala^{B30} instead of Thr^{B30} can lead to a reduced biological activity.

The reported relative potencies of human proinsulin in vitro range between 1 and 16% that of native insulin [10, 12, 20, 37, 38]. In this study, using isolated human adipocytes, we observed a relative potency of 9.3% for receptor binding, 9.4% and 11.1% for activation of D-glucose transport and inhibition of lipolysis, respectively.

Measurement of total degradation showed no difference between ¹²⁵I-diarginylinsulin and ¹²⁵I-insulin. The first step of insulin degradation may be mediated by a "insulin-specific protease" [12]. No difference in substrate activity of an insulin-specific protease was reported for porcine diarginylinsulin and insulin, the ability of the enzyme to degrade proinsulin, however, was negligible [39]. In our study the degradation of ¹²⁵I-proinsulin was reduced in comparison to diarginylinsulin and native in-

sulin. Studies of ¹²⁵I-proinsulin internalization and degradation in rat fat cells by Podlecki et al. have suggested that internalization of receptor bound proinsulin proceeds to a greater degree than does insulin, but once proinsulin is internalized the rate of degradation was also found markedly reduced compared to insulin [20].

In comparison to insulin, little attention has been paid to the immunogenicity of retardants. Protamine, a commonly used retardant of insulin absorption, is a basic polypeptide. Sera from patients treated with different types of protamine-insulin showed a high prevalence of circulating IgG-antibody to protamine. Rosenthal found that some protamine-insulin treated patients had responded immunologically to protamine as judged by the lymphocyte proliferation test [41]. In diabetic patients treated with protamine-insulins, the increased risk of serious reactions when i.v. protamine was given appeared to be caused largely by antibody-mediated mechanisms [8]. In a study by Kurtz et al. a relationship between insulin and protamine antigenicity was shown, suggesting that the protamine-insulin complex is itself immunogenic [40]. In several reports local and systemic reactions specifically to protamine and zinc have been described [42–46]. However, the actual percentage of diabetic patients in whom additives, such as protamine or zinc, are a cause of "insulin" allergy is unknown.

In conclusion, biosynthetic human diarginylinsulin and native insulin have almost identical in vitro bioactivities in isolated human adipocytes. Further investigations concerning the metabolic activity and the immunogenicity of diarginylinsulin in vivo as well as detailed absorption kinetic studies are required. By application of recombinant DNA technologies, it is possible to prepare large quantities of human diarginylinsulin. Thus, biosynthetic diarginylinsulin is a potential candidate for an intermediate acting insulin preparation containing no retarding substances.

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