Effect of thyroid hormone binding proteins on insulin receptor binding of B1-thyronine-insulin analogues

Fariba SHOJAEE-MORADIE*[1], Michelle P. Y. CHAN*, Micayla A. TELFER*, Dietrich BRANDENBURG†, Erik SUNDERMANN†, Heike ECKEY†, Jens KLEINJUNG‡, Achim SCHÜTTLER† and Richard H. JONES*

*Department of Diabetes, Endocrinology and Internal Medicine, St. Thomas' Hospital, Guy's, King's and St Thomas' School of Medicine, King's College, London SE1 7EH, U.K., †Deutsches Wollforschungsinstitut, Aachen, Germany, and ‡Bioinformatics Unit, Faculty of Sciences, Free University of Amsterdam, Amsterdam, The Netherlands

Certain thyronine-insulin analogues, which form non-covalent complexes with plasma proteins, have been shown to act preferentially in the liver. We hypothesized that this property may be dependant on the ability of the analogue to bind to the insulin receptor without prior dissociation from the binding protein. $N^{\alpha B1}$-L-thyroxyl-insulin, $N^{\alpha B1}$-3,3′,5′-triiodothyronine-insulin, $N^{\alpha B1}$-D-thyroxyl-insulin and $N^{\alpha B1}$-L-thyroxyl-aminolauroyl-insulin were compared with insulin for their capacity to inhibit the binding of [^{125}I]Tyr^{A14}-insulin to rat liver plasma membrane in albumin-free buffer. Effective doses at 50 % maximum inhibition of binding (ED_{50}) were calculated with and without addition of the thyroid hormone binding proteins transthyretin, thyroxine binding globulin and human serum albumin. The binding of thyronine-insulin analogues to insulin receptors was inhibited in a dose-dependant manner by the addition of thyroid hormone binding proteins at concentrations in the physiological range. Complexes of thyronine-insulin analogues with thyroid hormone binding proteins exhibit impaired insulin receptor binding affinities compared with those of the analogues in their free form. Hepatoselectivity *in vivo* may not depend on binding of the intact complexes to hepatocytes. These results have implications for the physiological role of hormone binding proteins and the *in vivo* properties of other insulin analogues which bind to plasma proteins.

Key words: hepatoselective, insulin analogues, insulin receptor binding, liver plasma membrane, protein binding, receptor binding affinity.

INTRODUCTION

Pancreatic insulin is released into the hepatic portal circulation and delivered directly to the liver. Thus freshly secreted insulin both exerts direct hormonal effects on the liver and is metabolized by hepatocytes. In this 'first pass' the liver extracts up to 60 % of the insulin delivered to it [1] and the remainder enters the larger systemic plasma volume, before redistribution through the arterial tree. In consequence, after physiological insulin release, hepatocytes are exposed to insulin concentrations approximately three times higher than exist in the capillaries of muscle and adipose tissue, the other major target organs for insulin [2,3].

Conventional insulin therapy is administered subcutaneously and is absorbed from injection sites directly into the peripheral circulation such that hepatocytes and peripheral tissues are exposed to almost identical profiles of insulin concentration. As a result, the balance of insulin action is disturbed with relatively increased activation of insulin-dependent pathways in muscle and adipose tissue, and under-insulinization of the liver. This imbalance can potentially be implicated in several pathogenic mechanisms.

The hepatic portal vein is generally not accessible for therapeutic insulin delivery in man, but data are available from studies with implantable insulin pumps [4]. Crossover studies with intraperitoneal insulin therapy have shown a reduction in the incidence of hypoglycaemia, particularly of severe episodes, when compared with intensified subcutaneous regimes, despite similar degrees of glycaemic control [5,6]. Lactate, alanine and pyruvate levels, and glucose carbon cycling, have been shown to be lower with intravenous insulin delivery than when insulin is administered to the intraperitoneal space during euglycaemic clamps [7].

Intraperitoneal insulin therapy has been shown also to improve the lipid profile in diabetes. In a study of 10 patients with Type I diabetes, treatment for 9 months decreased total plasma cholesterol, the triglyceride:apoB ratio in very-low-density lipoprotein and the cholesterol:apo B ratio in low-density lipoprotein. An increase in the activity of hepatic lipase was proposed as the main determinant of the improved lipid profile [8]. Cholesterol ester transfer protein has been shown to be increased in conventionally treated Type I diabetic patients, but restored to normal by treatment with intraperitoneal insulin [9]. Thus restoration of the hepatic/peripheral insulin gradient could potentially reduce the risk of macrovascular complications of diabetes [4].

Loss of the normal hepatic/peripheral insulin concentration gradient disturbs the IGF-I (insulin-like growth factor 1)/growth hormone axis and could contribute to the development of diabetic microvascular complications [10,11,12]. One hypothesis based on the available data suggests that in the absence of adequate intrahepatic insulin levels IGF-I synthesis and secretion by the liver is reduced at normal plasma growth-hormone concentrations. The compensatory increase in growth-hormone secretion may result in locally increased cellular exposure to IGF-I or other growth-hormone-dependent growth factors via autocrine or paracrine effects [10].

We have designed and synthesized insulin analogues intended to exhibit preferential effects on the liver even when injected subcutaneously and, which we have therefore described as

Abbreviations used: ANSA, 8-anilino-1-naphthalenesulphonic acid; B1-L-T4-Ins, $N^{\alpha B1}$-L-thyroxyl-insulin; B1-D-T4-Ins, $N^{\alpha B1}$-D-thyroxyl-insulin; B1-rT3-Ins, $N^{\alpha B1}$-3,3′,5′-triiodothyronine-insulin; B1-L-T4-AL-Ins, $N^{\alpha B1}$-L-thyroxyl-aminolauroyl-insulin; Boc, t-butoxycarbonyl; DCC, dicyclohexylcarbodi-imide; HGO, hepatic glucose output; H-Ins, human insulin; HSA, human serum albumin; IGF, insulin-like growth factor; KRP-A, Krebs Ringer phosphate, pH 7.8; LPM, liver plasma membrane; Msc-T4, Msc-thyroxine; RPE, relative potency estimate; TBG, thyroxine-binding protein; THBP, thyroid hormone binding protein, TIA, thyronine-insulin analogue; TTR, transthyretin.

[1] To whom correspondence should be addressed (e-mail fariba.shojaee-moradie@kcl.ac.uk).

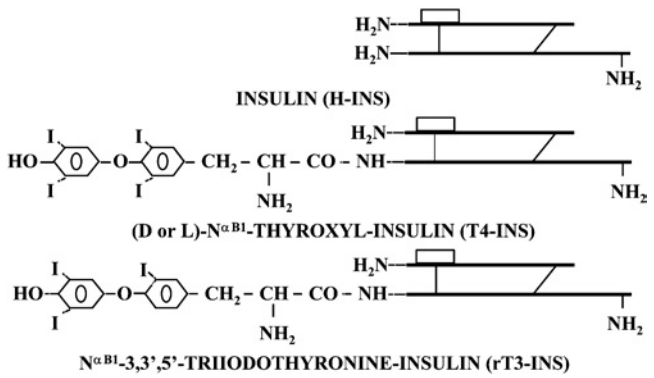

Figure 1 Schematic representation of TIAs

Thyronine covalently attached by a peptide link to the ε-amino group of Phe^{B1}. The spacer chain in B1-L-T4-AL-Ins is an amino lauryl (12 carbon chain).

'hepatoselective' [13,14]. To achieve this we have chosen to exploit differences in the nature of the capillary endothelium. In the liver there is effectively no barrier between hepatocytes and all constituents of blood that circulate freely around them. In contrast, in adipose tissue and muscle the capillary endothelium acts as a barrier between blood and extracellular fluid preventing access of larger plasma protein molecules to the cell surface. Thyronine-insulin analogues (TIAs) consist of an insulin moiety covalently linked to a thyronine group capable of binding to thyroid hormone binding proteins (THBPs). We have shown that one of these analogues $N^{\alpha B1}$-L-thyroxyl-insulin (B1-L-T4-Ins) is indeed hepatoselective in man [13]. It is not clear whether the analogue exhibited this property by binding to hepatocyte insulin receptors while still bound into complexes with one or more of the THBPs or whether dissociation from the THBP is required for receptor binding and hence activation.

In human subjects three plasma proteins are involved in the transport of thyroid hormones, thyroxine-binding protein (TBG; M_r 54000), transthyretin (TTR; M_r 54000) and human serum albumin (HSA; M_r 65000). All three are of sufficient magnitude to inhibit their passage through the peripheral capillary endothelial barrier.

The present study was designed to explore directly the effects of association with each of these proteins on the affinity of the analogues for an *in vitro* preparation of insulin receptors.

MATERIALS AND METHODS

Materials

Human insulin (H-Ins) and four of its semi-synthetic analogues were studied. Crystalline semi-synthetic mono-component H-Ins (M_r 5807) was obtained from Biobrás (Monte Claros, Brazil). TBG, TTR and thyroxine were purchased from Sigma. The four novel TIAs to be studied were: B1-L-T4-Ins, $N^{\alpha B1}$-D-thyroxyl-insulin (B1-D-T4-Ins), $N^{\alpha B1}$-3,3′,5′-tri-iodothyronine-insulin (B1-rT3-Ins), and $N^{\alpha B1}$-L-thyroxyl-aminolauroyl-insulin (B1-L-T4-AL-Ins) (Figure 1). B1-L-T4-Ins (M_r 6566) was synthesized with modifications to methods reported previously [13,14]. The synthesis and chemical characterization of the other three analogues will be reported elsewhere (E. Sundermann, J. Kleinjung, H. Eckey, A. Schüttler and D. Brandenburg, unpublished work).

Briefly, L- or D-thyroxine and the $N^{\alpha A1}$ and $N^{\varepsilon B29}$ amino groups of H-Ins were acylated with methylsulphonylethoxycabonyl-*N*-hydroxy-succinimide ester to yield Msc-thyroxine (Msc-T4) and $N^{\alpha A1}$, $N^{\varepsilon B29}$-bis-Msc-insulin respectively. The L- or D-Msc-thyroxine was coupled to the free amino group of the diprotected insulin in the presence of dicyclohexylcarbodi-imide/*N*-hydroxy-succinimide (DCC/NHS). Deprotection with dioxane/NaOH yielded B1-L-T4-Ins or B1-D-T4-Ins.

B1-L-T4-AL-Ins was prepared by first protecting the amino group of aminolauric acid using the t-butoxycarbonyl (Boc) group. $N^{\alpha A1}$-$N^{\varepsilon B29}$-bis-Msc-insulin was reacted with N-terminally protected aminolauric acid in the presence of DCC and hydroxybenzotriazole. The Boc group was then selectively cleaved with trifluoroacetic acid. Msc-T4 was coupled to $N^{\alpha A1}$,$N^{\varepsilon B29}$-bis-Msc, $N^{\alpha B}$-lauroyl-insulin as above, and the resulting intermediate was deprotected with dioxane/NaOH.

After purification with HPLC, the yields for B1-L-T4-Ins, B1-D-T4-Ins, B1-rT3-Ins, B1-L-T4-AL-Ins were 35%, 33%, 10%, and 27% respectively.

METHODS

In vitro studies

Equilibrium binding experiments

Binding studies were carried out for determination of the binding characteristics of (a) the thyronine moiety to THBPs (using size exclusion chromatography) and (b) the insulin moiety to the insulin receptor (using rat liver plasma membranes).

Size exclusion chromatography

H-Ins or analogue (27.6 pM) were incubated with a near physiological concentration of a THBP (0.238 μM TBG, 757 μM HSA and normal human serum) in 1 ml of PBS, pH 7.2. Normal fasting human serum was also used as a source of binding proteins. In those experiments 1 ml of human serum was incubated with H-Ins or analogue (27.6 nmol in 100 μl). Following incubation, the characteristics of binding were evaluated *in vitro* using FPLC (Amersham Biosciences, Little Chalfont, Bucks., U.K).

RIA

A modified double-antibody RIA for immunoreactive insulin [15] was used to determine the concentrations of H-Ins or analogue in each FPLC fraction. ^{125}I-labelled (monoiodinated at tyrosine position A14) H-Ins tracer (Amersham Biosciences) was made up in a buffer [0.05 mol/l barbitone, pH 7.8/0.2% (w/v) HSA] with 2 mg/ml 8-anilino-1-naphthalenesulphonic acid (ANSA; Aldrich, Poole, Dorset, U.K.). Both barbitone and ANSA function to displace the thyronine moieties of the analogues from the THBPs, thereby allowing unhindered antibody binding to the insulin moiety of the analogues. The antibody-binding behaviour of the novel analogues was also tested against standard H-Ins (0–6.9 nmol/l).

Isolation of rat liver plasma membrane (LPM)

Rat LPM was isolated from Corworth–Sprague–Dawley rats to be used as the source of insulin receptors in the equilibrium binding assays by differential and density gradient centrifugation [16].

Equilibrium binding assay

A series of standard solutions of H-Ins and the analogues at different concentrations (0–1132 nmol/l) were prepared in Krebs Ringer phosphate, pH 7.8 (KRP-A) in the presence of γ globulin (1% w/v) modified from [17]. Each standard solution (100 μl) was incubated with ^{125}I-labelled H-Ins tracer (100 μl) (9000–13000 c.p.m.) and 20 μl of LPM was diluted in KRP-A (protein 400 μg/ml) at 30 °C for 60 min to allow equilibrium. The incubation tubes were then placed on ice. Duplicate samples

(100 μl) were taken from each tube and transferred into separate microfuge tubes chilled previously at 4 °C. These were centrifuged (MicroCentaur; Sanyo, Uxbridge, Middx., U.K.) at 15 850 ***g***, at 4 °C for 4 min to separate the LPM-bound and -free moieties. The supernatant was aspirated and discarded. The pellets, containing the bound analogue–insulin receptor complexes, were washed with 100 μl of cold KRP, re-centrifuged, and the supernatant was discarded. These steps were carried out in the cold room. The radioactivity of the final pellet was counted to quantify the amount of ^{125}I-labelled H-Ins tracer bound to the LPM in a γ counter (1277 Gammamaster; Wallac, Milton Keynes, Bucks., U.K.). In each assay, tubes containing tracer alone were counted to allow calculation of percentage bound.

Data analysis

FPLC fractionation

Percentage bound (5.5–15 ml) and free (15.5–20 ml) TIAs were calculated as a percentage of the sum of immunoreactive insulin concentration for all fractions (5.5–25 ml), minus the background.

Equilibrium binding assay

The bound fraction of ^{125}I-labelled H-Ins was calculated for each sample as a percentage of the total counts. The duplicates in each set were averaged and normalized against the binding in the absence of analogue (which was taken as 100 %). Equilibrium binding curves were constructed for each assay using the SigmaPlot5.0 software program (SPSS Inc., Chicago, IL, U.S.A.). Plots of normalized bound ^{125}I-labelled insulin against analogue concentration were constructed using a logarithmic scale for concentration. A regression programme for sigmoidal curves (using Hill's regression equation) was used to construct the 'best fit' curve. The effective dose of unlabelled analogue needed to displace 50 % of ^{125}I-labelled H-Ins from LPM (ED_{50}) for each equilibrium binding curve was calculated by SigmaPlot5.0.

Statistical analysis

The significance of differences between two means was tested using Fisher's least squares, using the Number Cruncher Statistical System software (Hintze, Kaysville, Utah, U.S.A.). All values are arithmetic means $\pm$ S.E.M., with P values < 0.05 considered statistically significant.

Relative potency estimates (RPEs) of the analogues (in inhibition of binding) relative to a standard were calculated using PARLIN computer software (Finney, Edinburgh, U.K.). The RPEs in the absence of THBP were calculated relative to H-Ins, and the RPE in the presence of THBP was calculated relative to the same analogue without THBP. A 95 % fudicial limit for RPE was also derived by PARLIN.

The PARLIN program analyses biological assays based on responses that show linear regression on the logarithm of dose. Therefore, only data from the linear portions of the equilibrium binding curves were used. RPE is only a valid comparison if two curves are parallel and are homoscedastic (homogeneous in variances). The program performed the Bartlett χ^2 test to test for significant heteroscedasticity, and the F test to determine significance in non-parallelism. A value of $P < 0.05$ is taken as significant for both tests.

RESULTS

Size-exclusion chromatography

Figure 2 illustrates the elution profile of immuno-reactive analogue concentrations after overnight incubation with TBG.

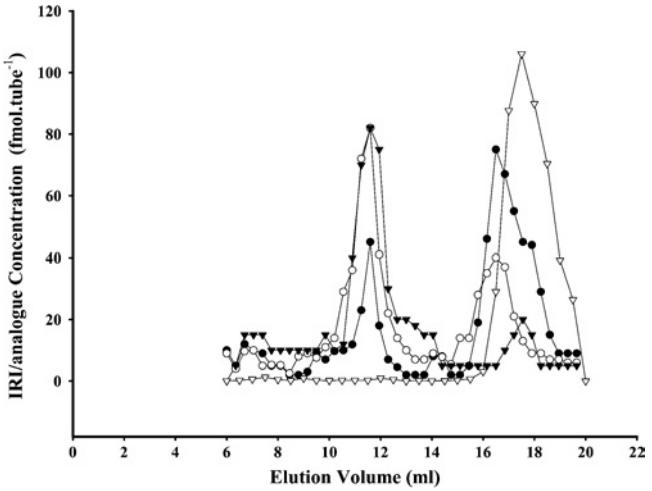

Figure 2 FPLC elution profile of analogues

FPLC elution profile of analogues [(▽) H-Ins, (○) B1-L-T4-Ins, (●) B1-rT3-Ins and (▼) B1-D-T4-Ins] after overnight incubation with TBG. A Superose 12 HR 10/30 column (Pharmacia FPLC system, Pharmacia LKB Ltd, U.K.) was equilibriated with 0.1 mol/l phosphate buffered saline (PBS) buffer, pH 7.2, and eluted with it at 0.5 ml · min^{-1}, under a column pressure of 1.2 MPa. Fractions of 0.5 ml from eluate 5.5 ml–25 ml were collected by a programmable fraction collector (Pharmacia LKB Ltd, U.K.). The column was standardized by eluting a series of marker proteins of known molecular mass. Analogue concentration in the eluate fractions was measured by RIA. Bound (fractions eluting from to 5.5 to 15 ml) (%) and unbound (fractions eluting from 15.5–25 ml) (%) components of the analogues was calculated. The proportion of each analogue bound to the THBPs was calculated from the RIA concentrations in the eluate fractions.

Table 1 Characteristics of bound and free moieties of thyronine-insulin analogues by FPLC analysis

Characteristics of analogues bound to THBPs (fractions from 5.5–15 ml) and unbound or free analogue (fractions from 15.5–25 ml) determined by FPLC separation. The results are means $\pm$ S.E.M., $n = 3$.

Analogue and THBP	Bound (%)	Free (%)
H-Ins		
Normal human serum	1.6 $\pm$ 0.5	98.4
HSA	9.0 $\pm$ 3.1	91.0
TBG	9.9 $\pm$ 2.1	90.2
B1-L-T4-Ins		
Normal human serum	63.2 $\pm$ 0.1	36.8
HSA	72.4 $\pm$ 2.3	27.6
TBG	73.7 $\pm$ 3.4	26.3
B1-rT3-Ins		
Normal human serum	32.9 $\pm$ 0.9	67.1
HSA	32.7 $\pm$ 1.0	67.3
TBG	35.4 $\pm$ 3.1	65.4
B1-D-T4-Ins		
Normal human serum	77.8 $\pm$ 2.4	22.2
HSA	77.1 $\pm$ 1.9	22.9
TBG	84.0 $\pm$ 2.6	16.0
B1-L-T4-AL-Ins		
Normal human serum	75.6 $\pm$ 2.9	24.4
HSA	86.3 $\pm$ 2.1	13.7
TBG	74.3 $\pm$ 1.8	25.7

Fractions eluted between 5.5 and 15 ml and 15.5 and 20 ml contained bound analogue ($M_r > 66\,000$ Da) and free analogue ($M_r < 66\,000$ Da) respectively. The calculated bound fraction (%) and free fraction (%) values are included in Table 1. All

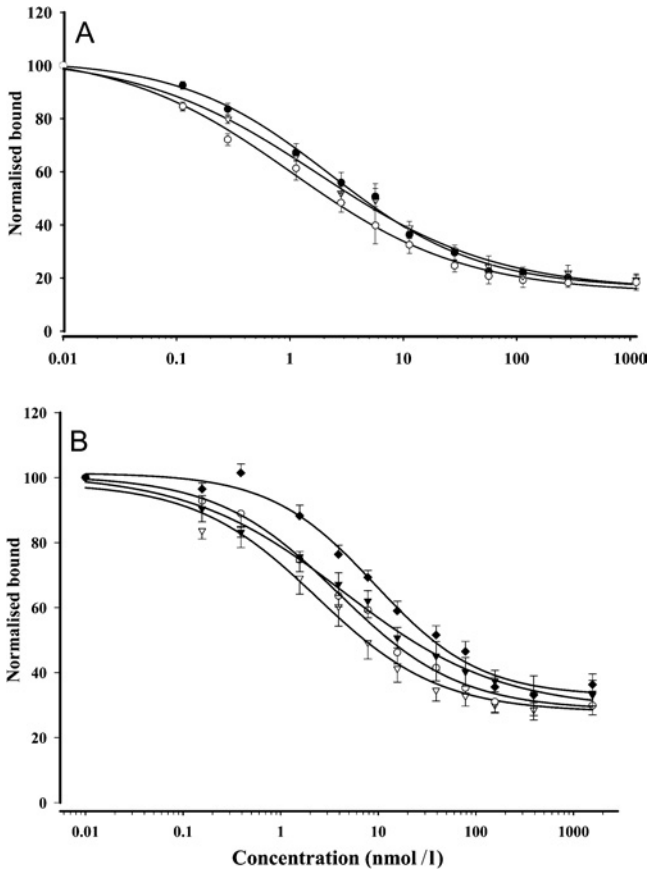

Figure 3 Equilibrium binding curves of the TIAs to LPM in the absence of thyroid hormone binding proteins

Inhibition of binding of [^{125}I]Tyr^{A14} monoiodinated H-Ins to rat LPM by the analogues in KRP buffer pH 7.8, containing γ globulin (1 % w/v) in the absence of THBPs. (**A**) (○) B1-L-T4-Ins, (●) B1-rT3-Ins and (▽) H-Ins, and (**B**) (▽) H-Ins, (○) B1-L-T4-Ins, (▼) B1-D-T4-Ins and (◆) B1-L-T4-AL-Ins.

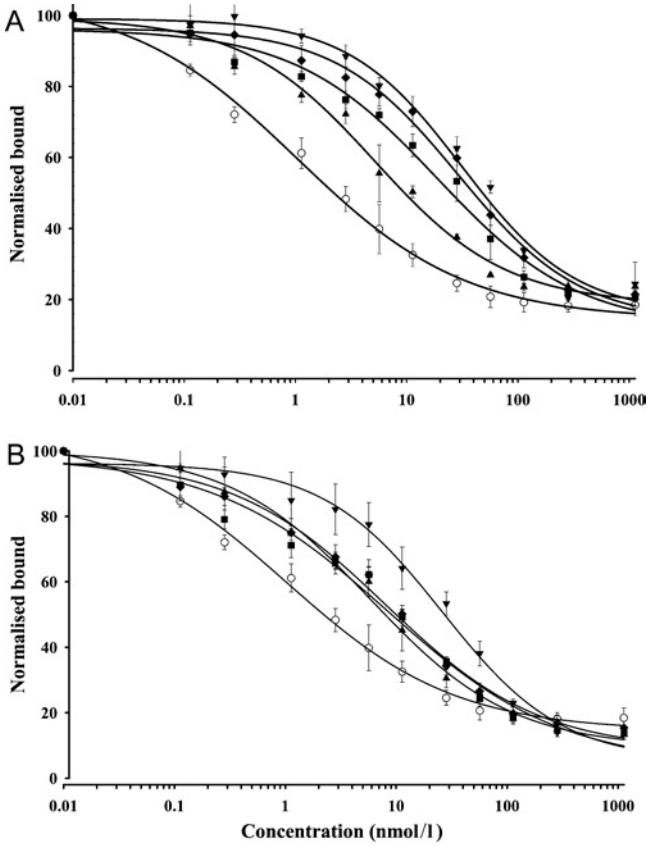

Figure 4 Equilibrium binding curves of the B1-L-T4-Ins to LPM in the presence of thyroid hormone binding proteins

Inhibition of binding of [^{125}I]Tyr^{A14} monoiodinated H-Ins to rat LPM by (○) B1-L-T4-Ins in KRP buffer pH 7.8, containing γ globulin (1 % w/v) in the presence of increasing concentration of TBG [(▲) 0.023 μmol/l, (■) 0.068 μmol/l, (◆) 0.135 μmol/l and (▼) 0.27 μmol/l] (**A**) and in the presence of increasing concentration of HSA [(▲) 55 μmol/l, (■) 110 μmol/l, (◆) 275 μmol/l and (▼) 550 μmol/l] (**B**).

analogues bound to the binding proteins, some more readily than others. For example binding of B1-L-T4-Ins to TBG was (73 % ± 3.4) compared with B1-rT3-Ins (35.4 % ± 3.1). These results were confirmed using ^{125}I-labelled H-Ins tracer and ^{125}I-labelled thyronine analogues. The elution profiles of the iodinated analogues bound to human serum, HSA or TBG were similar to the un-iodinated analogues measured by RIA. The radioactive counts in the fractions collected between the peaks were as low as the background counts in fractions collected at the beginning of the elution, suggesting that the bound analogue complex did not dissociate during the separation.

Equilibrium binding

Equilibrium binding curves are shown for H-Ins, L-T4-Ins, B1-D-T4-Ins, B1-rT3-Ins and B1-L-T4-AL-Ins in Figures 3 and 4. Analysis of the ED_{50}s calculated for the curves in the absence of the binding proteins showed that the inhibition of binding of ^{125}I-labelled H-Ins tracer by the insulin analogues (L-T4-Ins, B1-D-T4-Ins, B1-rT3-Ins) to the insulin receptor were similar to that of H-Ins standard (3.0 ± 0.7 nmol/l) (Table 2). The ED_{50} calculated for B1-L-T4-AL-Ins (9.6 ± 1.4 nmol/l) was significantly different from that of H-Ins and the other analogues, $P < 0.05$.

The equilibrium binding studies were also carried out in the presence of THBPs. Both B1-L-T4-Ins and B1-rT3-Ins have been studied extensively. In the presence of TBG (0, 0.02, 0.07, 0.14 and 0.27 μmol/l) (Figure 4), the equilibrium binding curves for B1-L-T4-Ins were displaced to the right of the standard curve in a dose-dependent manner. This shift to the right as judged by the ED_{50} values was significant, $P < 0.05$ (Table 2). Displacement of the binding curves to the right was also observed in the presence of HSA (55, 110, 275 and 550 μmol/l), $P < 0.05$ (Table 2).

Table 2 Calculated ED_{50} of inhibition of binding of ^{125}I-labelled H-Ins in the absence and presence of THBPs

Calculated ED_{50} of ^{125}I-labelled H-Ins (nmol/l) in the absence and presence of THBPs TTR (4.6 μmol/l), TBG (0.27 μmol/l) and HAS (385 μmol/l). Human serum was used at concentrations similar to the THBPs experiments. Results are means ± S.E.M., n was at least 4. *$P < 0.05$, significantly different from human insulin standard; †$P < 0.05$, significantly different from respective analogue alone. NA, transthyretin was not available; ND, not determined.

THBP	H-Ins	B1-LT4-Ins	B1-rT3-Ins	B1-D-T4-Ins	B1-L-T4-AL-Ins
No protein	3.0 ± 0.7	3.1 ± 0.8	2.5 ± 0.4	4.2 ± 1.3	9.6 ± 1.4*
+ Albumin	2.6 ± 0.6	34.0 ± 5.0†	7.2 ± 1.4†	10.4 ± 0.9†	17.1 ± 3.6†
+ TBG	2.9 ± 0.6	29.5 ± 4.4 †	3.2 ± 0.2	22.9 ± 0.01†	78.9 ± 8.1†
+ TTR	3.1 ± 0.5	2.9 ± 0.3	1.8 ± 0.6	NA	NA
+ Serum	3.0 ± 0.9	24.6 ± 5.8†	ND	41.2 ± 3.1†	50.7 ± 6.8†

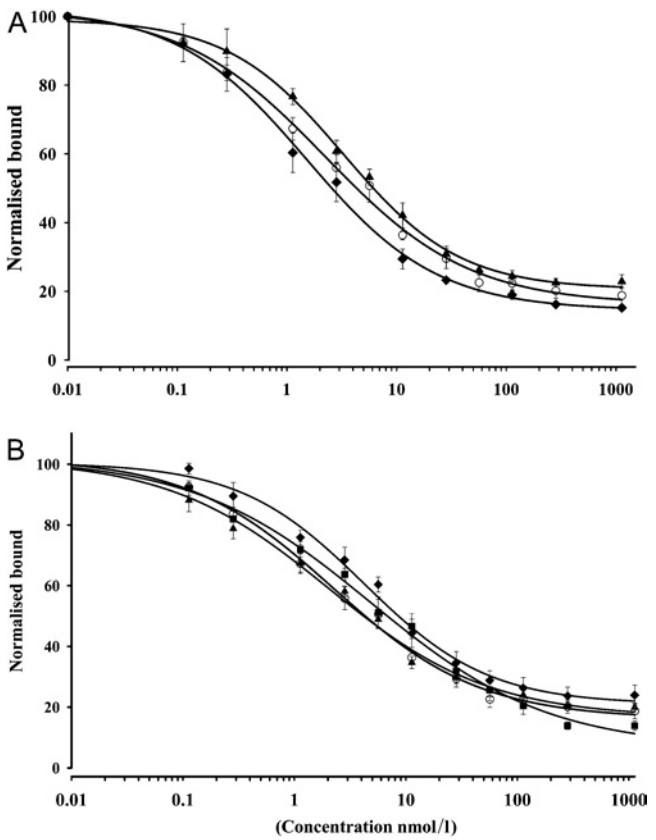

Figure 5 Equilibrium binding curves of the B1-rT3-Ins to LPM in the presence of thyroid hormone binding proteins

Inhibition of binding of [^{125}I]Tyr^{A14} monoiodinated H-Ins to rat LPM by (○) B1-rT3-Ins in KRP buffer pH 7.8, containing γ globulin (1 % w/v) in the presence of (▲) TBG (0.135 μmol/l) and (◆) TTR (4.6 μmol/l) (**A**) and in the presence of increasing concentration of HSA HSA [(▲) 55 μmol/l, (◆) 110 μmol/l, (■) 275 μmol/l] (**B**).

Addition of TTR (4.6 μmol/l) had no significant effect on the inhibition of binding of the tracer to the LPM by B1-L-T4-Ins.

In the presence of HSA (55, 110, 275 and 550 μmol/l), the equilibrium binding curves for B1-rT3-Ins were displaced to the right of the standard curve in a dose-dependent manner (Figure 5). This shift to the right, as judged by ED_{50} values, was significant, $P < 0.05$ (Table 2). Addition of TBG (0.135 μmol/l) or TTR (4.6 μmol/l) to the equilibrium buffer had no significant effect on the inhibition of binding of the tracer to the LPM by B1-rT3-Ins.

In the presence of TBG (0.27 μmol/l), the equilibrium binding curves for B1-D-T4-Ins and B1-L-T4-AL-Ins were shifted significantly to the right of the appropriate standard curves, $P < 0.05$. The calculated ED_{50}s were 22.9 ± 0.01 and 78.9 ± 8.9 nmol/l versus 4.2 ± 1.3 and 9.6 ± 1.4 respectively, $P < 0.05$ (Table 2). Addition of HSA (550 μmol/l) to the buffer also displaced the equilibrium curves of B1-D-T4-Ins and B1-L-T4-AL-Ins significantly to the right of the appropriate standard curves, $P < 0.05$. Calculated ED_{50}s were 10.4 ± 0.9 and 17.1 ± 3.6 nmol/l versus 4.2 ± 1.3 and 9.6 ± 1.4 respectively, $P < 0.05$ (Table 2).

Addition of human serum was associated with significant displacement of the equilibrium binding curves of all the analogues to the right of the appropriate standard curve. Calculated ED_{50}s for all the analogues with addition of human serum were significantly different from the standard, $P < 0.05$ (Table 2).

Table 3 Potency estimates of the TIAs relative to H-Ins

Potency estimates, with 95 % fiducial limits (in parentheses) of the analogues relative to H-Ins in the absence of THBPs, and potency estimates of the analogues in the presence of binding proteins relative to the analogue in the absence of THBPs. TTR (4.6 μmol/l), TBG (0.27 μmol/l) and HSA (385 μmol/l). Concentrations of the THBPs were equal to the concentrations found in normal serum. Results are means $\pm$ S.E.M., $n = 4$.

	Potency (fiducial limit)
H-Ins	100
B1-LT4-Ins	93 % (46–183)
B1-rT3-Ins	94 % (56–157)
B1-D-T4-Ins	45 % (28–70)
B1-L-T4-AL-Ins	23 % (14–34)
B1-L-T4-Ins	100
HSA	35 % (19–60)
TTR	33 % (20–54)
TBG	5 % (2–9)
B1-rT3-Ins	100
HSA	119 % (80–178)
TTR	183 % (111–306)
TBG	76 % (54–107)
B1-D-T4-Ins	100
HSA	67 % (38–115)
TBG	25 % (16–38)
B1-L-T4-AL-Ins	100
HSA	93 % (67–129)
TBG	24 % (13–36)

Potency estimates

The potency estimates of the analogues relative to H-Ins and the analogues in the presence of THBPs relative to the analogues in the absence of THBP are summarized in Table 3. All values showed insignificant heteroscedasticity (Bartlett test, $P < 0.05$), but some showed significant non-parallelism.

DISCUSSION

In the present study, we have shown that four TIAs are full agonists with respect to insulin receptor binding and that they compete with insulin tracer for association to antibodies raised against H-Ins. These analogues also bind to THBPs and, in the presence of such association, binding to insulin receptors is hindered.

Insulin binds and activates its receptor with high affinity and specificity. It has been demonstrated that certain other modifications at the Phe^{B1} position of the insulin molecule do not affect the biological properties of insulin analogues *in vivo* or *in vitro* [18]. In the absence of THBPs we have now demonstrated that addition of a thyronyl group to the N-terminus of the B chain of the insulin molecule does not affect binding to its receptor on the liver plasma membrane. However, the presence of THBPs impairs the binding process *in vitro*.

The free hormone hypothesis proposed by Robbins and Rall in 1957 [19] assumed that hormones must dissociate from their binding proteins in order to be able to bind to their receptors. This will clearly be the case for endocrine systems with intracellular receptors but may not apply to ligand systems involving peptide hormones with cell surface receptors. TIAs were designed with that in mind by choosing the Phe^{B1} position for substitution with the thyronine moieties. We also chose to explore the possible need for a molecular 'spacer arm' between the thyronine and insulin moieties to reduce the likelihood that receptor binding of the complexed analogues could be impaired by steric hindrance due to close proximity of the THBP to the insulin receptor. The

results presented in this study demonstrate that neither strategy has resulted in unimpaired receptor association kinetics. Our data could be explained by steric hindrance reducing the affinity of the complexes for the receptor, possibly effectively to zero, with competition for the tracer confined to analogue molecules in the free form. *In vivo* data support this explanation. In the studies with T4-Ins in human subjects the degree of suppression of glucose production was less than would be predicted from the very high levels of bound analogue present in the circulation, suggesting that the complex itself was inactive even in the liver [13].

At equilibrium, the ratios of TIA in the free form and bound to the various THBPs will depend on the concentrations of the reactants and the relative binding affinities. These factors acting jointly will determine the ratio of free to bound analogue in the arterial supply. However, in a capillary bed other considerations are important. During the time-course of capillary passage, free TIA will be generated by dissociation from the complexes at a rate dependant on the dissociation rate constant of the complex. The free TIA may re-associate with the binding protein (at a rate dependant on the relevant association rate constant) or pass through the endothelial membrane of the capillary into the interstitial space in which it has access to cell surface receptors. Thus in tissues with a capillary barrier the potentially available free TIA for receptor interaction depends not on the affinity for binding proteins as defined by the ratio of 'on-rate' to 'off-rate' but on the absolute values of these rate constants. For example, if both on and off rates are relatively rapid more free TIA will be generated during the capillary residence time than if both the on and off rates are relatively slow. It follows that delivery of free TIA to cell surface receptors will be defined quantitatively by the dissociation rate constant, the association rate constant (which will remove free TIA), the mean transit time through the capillary and the rate of diffusion of free TIA through the endothelial barrier. In the liver the last variable is absent from the equation as there is no endothelial barrier. However, reported mean capillary transit times are approximately 10-fold higher in the liver compared with skeletal muscle [20,21]. In view of this, more time is available during transit through the liver for generation by dissociation of free analogue available for receptor binding. Furthermore, the open architecture of the hepatic sinusoids will facilitate rapid access of free hormone to cell surface receptors before rebinding to the binding protein can occur. Both of these factors are likely to lead to hepatoselectivity of protein-bound hormones.

From data reported previously it turns out that, *in vivo*, the balance of these factors does indeed result in more delivery to hepatic insulin receptors than to insulin receptors in muscle and adipose tissue [13]. Further work to include direct measurement of association and dissociation rates to each of the binding proteins, as opposed to estimates of equilibrium binding, will be required accurately to predict the likely *in vivo* properties of protein-bound insulins with respect to their degree of hepatoselectivity.

Data with other insulin analogues which bind to plasma proteins are available. ε-Lys^{B29}-tetradecanoyl,des-B30-H-Ins, which binds to human albumin, has been infused intravenously into dogs and the time courses of the approach to equilibrium separately examined for HGO (hepatic glucose output) and rate of glucose disposal [22]. Although at equilibrium this analogue demonstrated full agonism, there were delays in reaching steady state in relation to plasma (and interstitial) concentrations both with insulin and the analogue. With the analogue this delay was particularly marked with respect to rate of glucose disposal, suggesting limitation of access to the receptor sites in peripheral tissues. It has been reported [23] that ε-Lys^{B29}-tetradecanoyl,des-B30-H-Ins has an hypoglycaemic potency in man of only 24–36 % that of NPH (neutral protamine hagedorn). Our data offer an explanation for these observations. At low doses insulin acts to reduce HGO. At insulin concentrations above which HGO is reduced to zero and at euglycaemia, insulin action in muscle and adipose tissue increases glucose flux by approx. 6-fold above basal values [24]. It follows that a fully hepatoselective insulin analogue with reduced effect on peripheral tissues will exhibit equivalent potency with insulin only at low concentrations. We propose that the apparently reduced biopotency of ε-Lys^{B29}-tetradecanoyl,des-B30 compared with H-Ins [22] may be due to previously unrecognized hepatoselectivity of this analogue.

In conclusion, the data presented here demonstrate that TIAs bind to the insulin receptor with affinities similar to that of H-Ins and exhibit substantial binding to the THBPs via their thyronine moieties. Binding to THBPs impairs their binding to the insulin receptor on liver plasma membranes. In spite of this these analogues have been shown to exhibit hepatoselectivity *in vivo*. The peripheral route for insulin administration is likely to remain the primary method of treatment for the majority of diabetic patients for whom insulin is most appropriate. Insulin analogues which bind to plasma proteins offer opportunities for a more physiological and potentially beneficial distribution of insulin action in these patients than is currently available.

This study was supported in part by the Institute for Pharmaceutical Discovery, Branford, CT, U.S.A.

REFERENCES

1 Field, J. B. (1973) Extraction of insulin by liver. Annu. Rev. Med. **24**, 309–314
2 Chap, Z., Ishida, T., Chou, J., Hartley, C. J., Entman, M. L., Brandenburg, D., Jones, R. H. and Field, J. B. (1987) First-pass hepatic extraction and metabolic effects of insulin and insulin analogues. Am. J. Physiol. **252**, E209–E217
3 Jones, R. H. (1988) Insulin action and metabolism. In Clinical Diabetes: An Illustrated Text (Besser, G. M., Bodansky, H. J. and Cudworth, A. G., eds.), pp. 5.1–5.16, Raven Press
4 Ruotolo, G., Micossi, P., Galimberti, G., Librenti, M. C., Petrella, G., Marcovina, S., Pozza, G. and Howard, B. V. (1990) Effects of intraperitoneal versus subcutaneous insulin administration on lipoprotein metabolism in Type I diabetes. Metabolism **39**, 598–604
5 Olsen, C. L., Liu, G., Iravani, M., Nguyen, S., Khourdadjian, K., Turner, D. S., Waxman, K., Selam, J. L. and Charles, M. A. (1993) Long-term safety and efficacy of programmable implantable insulin delivery systems. Int. J. Artif. Organs **16**, 847–854
6 Lahtela, J. T., Mustonen, J. and Pasternack, A. (1995) Comparison of intraperitoneal and subcutaneous insulin administration on insulin sensitivity and serum lipids in Type I diabetic patients on continuous ambulatory peritoneal dialysis treatment. Clin. Sci. **88**, 427–432
7 Monti, L. D., Piatti, P. M., Home, P. D., Tomson, C. and Alberti, K. G. (1992) The effect of intraperitoneal insulin delivery on carbohydrate metabolism in Type I (insulin-dependent) diabetic patients. Diabetes Res. Clin. Pract. **15**, 237–244
8 Ruotolo, G., Parlavecchia, M., Taskinen, M. R., Galimberti, G., Zoppo, A., Le, N. A., Ragogna, F., Micossi, P. and Pozza, G. (1994) Normalization of lipoprotein composition by intraperitoneal insulin in IDDM. Role of increased hepatic lipase activity. Diabetes Care **17**, 6–12
9 Bagdade, J. D., Dunn, F. L., Eckel, R. H. and Ritter, M. C. (1994) Intraperitoneal insulin therapy corrects abnormalities in cholesteryl ester transfer and lipoprotein lipase activities in insulin-dependent diabetes mellitus. Arterioscler. Thromb. **14**, 1933–1939
10 Sonksen, P. H., Russell-Jones, D. and Jones, R. H. (1993) Growth hormone and diabetes mellitus. A review of sixty-three years of medical research and a glimpse into the future? Horm. Res. **40**, 68–79
11 Poulsen, J. E. (1966) Diabetes and anterior pituitary insufficiency. Final course and post-mortem study of a diabetic patient with Sheehan's syndrome. Diabetes **15**, 73–77
12 Poulsen, J. E. (1953) Houssay phenomenon in man: Recovery from retinopathy in a case of diabetes with Simmond's disease. Diabetes **2**, 7–12
13 Shojaee-Moradie, F., Powrie, J. K., Sundermann, E., Spring, M. W., Schüttler, A., Sonksen, P. H., Brandenburg, D. and Jones, R. H. (2000) Novel hepatoselective insulin analog: studies with a covalently linked thyroxyl-insulin complex in humans. Diabetes Care **23**, 1124–1129
14 Shojaee-Moradie, F., Eckey, H., Jackson, N. C., Schüttler, A., Brandenburg, D., Sonksen, P. H. and Jones, R. H. (1998) Novel hepatoselective insulin analogues: studies with covalently linked thyroxyl-insulin complexes. Diabetic Medicine **15**, 928–936

15 Sonksen, P. H. (1976) Double antibody technique for the simultaneous assay of insulin and growth hormone. In Hormones in human blood: Detection and Assay (Antoniades, H. N., ed.), pp. 176–199, Harvard University Press
16 Wisher, M. H. and Evans, W. H. (1977) Preparation of plasma-membrane subfractions from isolated rat hepatocytes. Biochem. J. **164**, 415–422
17 Tatnell, M. A., Jones, R. H., Willey, K. P., Schüttler, A. and Brandenburg, D. (1983) Evidence concerning the mechanism of insulin-receptor interaction and the structure of the insulin receptor from biological properties of covalently linked insulin dimers. Biochem. J. **216**, 687–694
18 Cockram, C. S., Jones, R. H., Boroujerdi, M. A. and Sonksen, P. H. (1984) Evidence for separate handling *in vivo* of different regions of the insulin molecule using A14- and B1-labeled insulin tracers. Diabetes **33**, 721–727
19 Robbins, J. and Rall, J. E. (1957) The interaction of thyroid hormones and protein in biological fluids. Recent Prog. Horm. Res. **13**, 161–208
20 Mendel, C. M. and Weisiger, R. A. (1990) Thyroxine uptake by perfused rat liver. No evidence for facilitation by five different thyroxine-binding proteins. J. Clin. Invest. **86**, 1840–1847
21 Kayar, S. R., Hoppeler, H., Jones, J. H., Longworth, K., Armstrong, R. B., Laughlin, M. H., Lindstedt, S. L., Bicudo, J. E., Groebe, K. and Taylor, C. R. (1994) Capillary blood transit time in muscles in relation to body size and aerobic capacity. J. Exp. Biol. **194**, 69–81
22 Hamilton-Wessler, M., Ader, M., Dea, M., Moore, D., Jorgensen, P. N., Markussen, J. and Bergman, R. N. (1999) Mechanism of protracted metabolic effects of fatty acid acylated insulin, NN304, in dogs: retention of NN304 by albumin. Diabetologia **42**, 1254–1263
23 Owens, D. R., Zinman, B. and Bolli, G. B. (2001) Insulins today and beyond. Lancet **358**, 739–746
24 Rizza, R. A., Mandarino, L. J. and Gerich, J. E. (1981) Dose-response characteristics for effects of insulin on production and utilization of glucose in man. Am. J. Physiol. **240**, E630–E639

Received 2 February 2004; accepted 15 March 2004
Published as BJ Immediate Publication 15 March 2004, DOI 10.1042/BJ20040177