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Effects of new insulin analogues HMR1964 (insulin glulisine) and HMR1423 on insulin receptors

Received: 1 March 2005 / Accepted: 10 March 2005 / Published online: 29 July 2005
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Abstract *Aims/hypothesis:* New insulin analogues have been created by amino-acid exchange to provide an improved pharmacokinetic profile. However, safety issues have been raised regarding their use, as amino-acid exchange of insulin may induce altered metabolic and mitogenic effects. For example, the insulin analogue Asp(B10) causes breast cancer in rodents. The aim of this study was to compare two new insulin analogues HMR1964 (Lys[B3],Glu[B29]) (insulin glulisine) and HMR1423 (Gly[A21],His[B31],His[B32]) with regular insulin and the mitogenic analogue Asp(B10). *Materials and methods:* We analysed insulin receptor binding characteristics and dissociation kinetics, as well as insulin-induced receptor auto- and dephosphorylation kinetics, in rat-1 fibroblasts overexpressing the human insulin receptor isoform B. Mitogenic activity was tested in the non-malignant cell line MCF10. *Results:* Regular insulin, HMR1964 and HMR1423 showed no significant differences in receptor association, dissociation and receptor binding affinity, while Asp(B10) displayed markedly increased insulin receptor affinity. All of the analogues induced rapid insulin receptor autophosphorylation, reaching a maximum 10 min after stimulation (10^{-9} mmol/l insulin). In contrast, Asp(B10) induced a prolonged phosphorylation and dephosphorylation state of the 95 kDa insulin receptor β -subunit. With respect

to [3 H]thymidine incorporation, the new analogues had similar (HMR1423) or even lower (HMR1964) effects than regular insulin in the mammary epithelial cell line MCF10, while Asp(B10) showed increased [3 H]thymidine incorporation. *Conclusions/interpretation:* HMR1964 and HMR1423 displayed the same association, dissociation and insulin receptor affinity kinetics as regular insulin, and might therefore be useful for the treatment of diabetes.

Keywords Autophosphorylation · Dephosphorylation · Insulin analogues · Insulin glulisine · Insulin receptor binding · Mitogenicity

Abbreviations HIR-B: human insulin receptor isoform B · PTYR: phosphotyrosine

Introduction

In the past few years insulin analogues have been introduced for the treatment of diabetes [1]. There are now two rapid-acting insulin analogues and two long-acting insulin analogues on the market [2–5]. When modifying the pharmacokinetic profile by introducing mutations into the insulin gene, it is crucial that these insulin analogues still behave like regular insulin with respect to insulin receptor binding and activation. Moreover, they should trigger similar intracellular signalling pathways. Keeping in mind that the initiation of insulin therapy is most often followed by life-long treatment, the issue of mitogenic effects is very important when testing new insulin analogues. Previous studies have shown that the insulin analogue Asp(B10) behaves differently from regular insulin with respect to dissociation from the insulin receptor, phosphorylation and dephosphorylation of substrates, and mitogenic activity in different cell lines [6–9].

Although the exact mechanisms for the enhanced mitogenic potential of Asp(B10) have not yet been elucidated, it seems to be related to a delayed insulin receptor dissociation and dephosphorylation rate and due to phos-

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phorylation of different substrates [10]. It has, moreover, been shown that Asp(B10) displays increased binding to the IGF-I receptor, which can also contribute to mitogenic activity [11, 12].

By introducing amino-acid exchange, the physicochemical properties of regular human insulin were changed, creating the new rapid-acting insulin analogue, insulin glulisine (HMR1964, Lys[B3],Glu[B29]). The mutations of HMR1423 (Gly[A21],His[B31],His[B32]) introduced two additional histidines serving as additional zinc-binding sites. This makes HMR1423 a potential tool for an insulin analogue with a prolonged, or even variable, duration of insulin action (depending on the proportion of HMR1423 in a mixed preparation with non-mutated insulin). The aim of this study was to investigate the insulin receptor binding and autophosphorylation characteristics of the above-mentioned insulin analogues in rat-1 fibroblasts, and their mitogenicity in the non-malignant human breast cell line MCF10 [13] compared with regular human insulin and Asp(B10).

Materials and methods

Materials

Cell culture reagents and fetal calf serum were purchased from Gibco (Karlsruhe, Germany). Aprotinin, phenylmethylsulphonyl fluoride, Na_3VO_4 and Triton X-100 were from Sigma (Munich, Germany). Nitrocellulose was from Sartorius (Göttingen, Germany). Visualisation of immunocomplexes after western blotting was performed with the non-radioactive enhanced chemiluminescence system from Amersham Biosciences Europe (Freiburg, Germany). [^3H]Thymidine was purchased from Amersham Biosciences Europe. The monoclonal anti-phosphotyrosine (PTYR) antibody (PY20) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All insulins used in this study were supplied by Aventis Pharmaceuticals (Bad Soden, Germany).

Cell culture and western blot analysis

Rat-1 fibroblasts stably overexpressing human insulin receptor isoform B (HIR-B) were grown in DMEM/Ham's F12 medium mix supplemented with 10% FCS. Cells were grown to confluence, and subsequently starved for 18 h, in medium with 0.5% FCS, and then incubated with regular human insulin, or the analogues, as indicated in the figures.

Receptor autophosphorylation was measured as previously described [10]. For dephosphorylation kinetics, HIR-B-overexpressing cells were incubated with regular human insulin, or analogues, for 3 min. Dephosphorylation of the insulin receptor was initiated by washing off the insulin. Cells were lysed, on ice, with 50 mmol/l HEPES buffer containing 150 mmol/l NaCl, 1.5 mmol/l MgCl_2 , 1 mmol/l EGTA, 10% glycerol, 1% Triton X-100, 1 mmol/l

l phenylmethylsulphonyl fluoride, 10 mg/l aprotinin, 0.4 mmol/l orthovanadate, 10 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$, and 100 mmol/l NaF after various time intervals (0–60 min). After centrifugation, supernatants were boiled in Laemmli buffer, and proteins were separated by 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with anti-PTYR antibody or anti-insulin-receptor antibody (CT 104), overnight at 4°C. The membranes were washed four times before incubating with horseradish peroxidase-conjugated anti-mouse IgG, or anti-rabbit IgG, for 1 h at room temperature. Visualisation of immunocomplexes was performed by enhanced chemiluminescence.

Association kinetics of ^{125}I -insulin (human) and ^{125}I -analogues to the insulin receptor in intact cells

Binding studies were performed on intact cells grown to approximately 80% confluence. Cells were scraped from the plates and kept in binding buffer (50 mmol/l Tris-HCl, 1% BSA, 10 mmol/l MgSO_4 , pH 7.6). The reaction was started with the addition of 0.0035 nmol/l of ^{125}I -labelled regular human insulin HMR1799 (1.34×10^{10} Bq/mg), ^{125}I -HMR1423 (1.30×10^{10} Bq/mg), ^{125}I -HMR1964 (1.34×10^{10} Bq/mg) or ^{125}I -Asp(B10) (1.35×10^{10} Bq/mg) and incubated at 21°C for the times indicated. Radioactivity was measured in a gamma counter. Non-specific binding was determined in the presence of excess unlabelled insulin.

Inhibition-competition curves of ^{125}I -insulin binding obtained with labelled Asp(B10) insulin in HIR-B cells

Cells were serum starved for 16 h and then incubated for 45 min with ^{125}I -Asp(B10) insulin in the presence of increasing concentrations of unlabelled analogues or regular insulin (10^{-6} – 10^{-10} mol/l, final concentration). At the end of the incubation period the supernatants were aspirated and the pellets counted in a gamma counter. The specific binding was calculated by total minus non-displaceable binding. Specific binding is plotted as a function of free ligand concentration.

Determination of the dissociation rate of cell-bound ^{125}I -insulin

The kinetics of dissociation from HIR-B cells were analysed by incubating cells with ^{125}I -insulin or the analogues for 3 h at 4°C, washing them quickly and then allowing ^{125}I -insulin to dissociate into 2 ml of fresh buffer. At each time-point, cell-bound ^{125}I -insulin was withdrawn and measured. Cell-associated radioactivity was measured as a function of time. All the results are expressed as a percentage of the amount of ^{125}I -insulin bound to cells at time 0.

Determination of [^3H]thymidine incorporation in MCF10 cells

To measure [^3H]thymidine incorporation, cells were grown in DMEM/Ham's F12 medium (1:1, with 5% horse serum, 10 $\mu\text{g/ml}$ insulin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 0.1 $\mu\text{g/ml}$ cholera toxin and 20 ng/ml epidermal growth factor) to confluence in six-well culture plates, and subsequently starved for 48 h in serum-free medium. After stimulation with insulin for 16 h, [^3H]thymidine (1.8×10^4 Bq/ml) was added for 4 h. The dishes were then rinsed twice with ice-cold PBS, and once with 10% trichloroacetic acid. After 20 min, dishes were washed once with ice-cold 10% trichloroacetic acid, cells were lysed with 500 μl 0.2 mol/l NaOH/1% SDS, and neutralised with 0.5 ml 0.2 mol/l HCl. Radioactivity was determined by liquid scintillation counting.

Statistics

Statistical analyses were carried out by Student's *t*-test. Data in figures are expressed as means $\pm$ SEM. *p* values less than 0.05 were considered significant.

Results

Insulin receptor association kinetics

It has been shown that insulin analogues can exhibit altered receptor-binding characteristics. We examined association kinetics of HMR1964 (insulin glulisine) and HMR1423 in rat-1 fibroblasts overexpressing approximately 1,250,000 insulin receptors per cell (HIR-B) to estimate specific binding to the human insulin receptor. For HMR1964, HMR1423 and regular insulin, similar receptor association kinetics were observed (Fig. 1). In contrast, Asp(B10) showed markedly increased insulin receptor binding affinity (bound insulin $10.96 \pm 2.61\%$ of total) compared with regular insulin ($5.28 \pm 1.78\%$). Therefore, only Asp(B10) insulin behaved differently with respect to affinity for the insulin receptor.

Insulin competition curves

The inhibition of ^{125}I -Asp(B10) binding by unlabelled regular insulin or the analogues was studied. HIR-B cells were incubated for 45 min with ^{125}I -Asp(B10) insulin in the presence of increasing concentrations of unlabelled analogues or regular insulin (10^{-6} – 10^{-10} mol/l, final concentration). In HIR-B cells, ^{125}I -Asp(B10) binding was inhibited by unlabelled regular insulin with an EC_{50} effect at ~ 60 nmol/l, for HMR1964 the EC_{50} effect was at ~ 70 nmol/l and at ~ 80 nmol/l for HMR1423, whereas Asp(B10) was approximately 10-fold more potent in competing for insulin receptor binding compared with regular human insulin ($\text{EC}_{50} \sim 6$ nmo/l) (Fig. 2).

Fig. 1 Association kinetics for ^{125}I -human insulin and its analogues. Rat-1 fibroblasts overexpressing HIR-B were incubated with regular insulin or the analogues HMR1423, HMR1964 or Asp(B10). Ligand association was determined for 0–30 min. Cell pellets were washed three times and radioactivity was determined in a gamma counter. $n=6$, means $\pm$ SEM. ** $p < 0.01$, Asp(B10) insulin vs regular insulin; *** $p < 0.001$, Asp(B10) insulin vs regular insulin. *Diamonds*, regular insulin; *squares*, HMR1423; *crosses*, HMR1964; *circles*, Asp(B10)

Insulin receptor dissociation kinetics

The dissociation rate was measured by incubating HIR-B cells with labelled insulin or the analogues over a 45-min period. To minimise internalisation, the experiment was carried out at 4°C and the results are expressed as a percentage of the initial binding. Asp(B10) dissociated significantly more slowly from the receptor, whereas the

Fig. 2 Inhibition-competition curves of ^{125}I -insulin binding obtained with labelled Asp(B10) insulin in HIR-B cells. Cells were incubated for 45 min with ^{125}I -Asp(B10) insulin and increasing concentrations (10^{-6} – 10^{-10} mol/l, final concentration) of the analogues HMR1423, HMR1964 or Asp(B10) or regular insulin. Binding is expressed as per cent of maximal specific ^{125}I -insulin binding. Each point represents four independent experiments $\pm$ SEM. ** $p < 0.01$, analogue vs regular insulin; *** $p < 0.001$, Asp(B10) insulin vs regular insulin. *Diamonds*, regular insulin; *squares*, HMR1423; *crosses*, HMR1964; *circles*, Asp(B10)

Fig. 3 Dissociation of cell-bound ^{125}I -insulin. Rat-1 fibroblasts overexpressing HIR-B were incubated with labelled ^{125}I -insulin or analogues for 3 h at 4°C , washed quickly and ^{125}I -insulin was allowed to dissociate into 2 ml fresh buffer. At each time-point, cell-bound ^{125}I -insulin was withdrawn and measured. All the results are expressed as a percentage of the amount of ^{125}I -insulin bound to cells at time 0. Each time-point represents four independent experiments $\pm$ SEM. *** $p < 0.001$, Asp(B10) insulin vs regular insulin. Diamonds, regular insulin; squares, HMR1423; crosses, HMR1964; circles, Asp(B10)

analogues HMR1964 and HMR1423 dissociated more rapidly and to the same extent as regular insulin (Fig. 3).

Insulin receptor autophosphorylation kinetics

To determine the effect on insulin receptor autophosphorylation, we stimulated rat-1 fibroblasts overexpressing HIR-B with either regular insulin or the insulin analogues

for 3–120 min. Insulin receptor activation by autophosphorylation was detected by western blot analysis of whole-cell lysates, using an anti-PTYR antibody (Fig. 4a). Protein levels of insulin receptor were assessed by western blot analysis with an anti-receptor β -subunit antibody (Fig. 4b).

In HIR-B cells, regular insulin and the new analogues showed no significant differences in receptor autophosphorylation, while different kinetics were observed with Asp(B10). Each of the new analogues induced rapid autophosphorylation of the insulin receptor, reaching a maximum after 10 min of stimulation with 10^{-9} mmol/l insulin. Despite rapid autophosphorylation, Asp(B10) insulin induced a prolonged phosphorylation state of the insulin receptor β -subunit, which could still be observed at 120 min, a time-point where autophosphorylation by the other analogues was already reduced (quantification of six different experiments is depicted in Fig. 4c).

Dephosphorylation kinetics of the insulin receptor

Dephosphorylation kinetics for regular insulin and the analogues HMR1964, HMR1423 and Asp(B10) were assessed by incubating HIR-B cells with the respective ligand. After a 3-min incubation, insulin was washed off and dephosphorylation was measured between 3 and 60 min. Representative western blots are shown in Fig. 5a,b. Delayed dephosphorylation of the insulin receptor was observed in rat-1 cells stimulated with Asp(B10), while HMR1964 and HMR1423 behaved like regular human insulin (Fig. 5a). Quantification of five different experi-

Fig. 4 Autophosphorylation kinetics of human insulin receptor. Rat-1 fibroblasts overexpressing HIR-B were incubated for various times with regular insulin or the analogues (final concentration 10^{-9} mmol/l). Cells were lysed, proteins were separated by SDS gel electrophoresis and western blots were probed with anti (α)-PTYR antibody (a) or anti-insulin receptor β -subunit antibody (b). Representative western blots are shown.

c Quantification of the receptor autophosphorylation kinetics by scanning densitometry. Scanning data obtained from six independent experiments are expressed as a percentage of maximal autophosphorylation (100%) $\pm$ SEM. * $p < 0.05$, Asp(B10) insulin vs regular insulin; *** $p < 0.001$, Asp(B10) insulin vs regular insulin. Diamonds, regular insulin; squares, HMR1423; crosses, HMR1964; circles, Asp(B10). IR, insulin receptor

Fig. 5 Dephosphorylation kinetics of human insulin receptor. Rat-1 fibroblasts were incubated for 3 min with regular insulin or the analogues (final concentration 10^{-9} mmol/l). Subsequently, the incubation medium was replaced with medium not containing insulin, and cells were incubated at 37°C for an additional 0–60 min. Immunoblots were performed with anti (α)-PTYR antibody (a) or anti-insulin receptor β -subunit antibody (b). Representative western blots

are shown. **c** Quantification of the receptor dephosphorylation kinetics by scanning densitometry. Scanning data obtained from five independent experiments are expressed as a percentage of maximal receptor autophosphorylation ($100\% \pm \text{SEM}$). * $p < 0.05$, Asp(B10) insulin vs regular insulin; ** $p < 0.01$, Asp(B10) insulin vs regular insulin. Diamonds, regular insulin; squares, HMR1423; crosses, HMR1964; circles, Asp(B10). IR, insulin receptor

ments is shown in Fig. 5c, which indicates that Asp(B10) induced prolonged dephosphorylation kinetics of the insulin receptor, compared with the other insulins tested.

Measurement of mitogenic activity in the human epithelial breast cell line MCF10

Asp(B10) insulin has been shown to increase mitogenic activity and induce mammary tumours in rodents. Therefore, we measured stimulation of [^3H]thymidine incorporation in the human breast cell line MCF10 with the respective new insulin analogues. The results of [^3H]thymidine incorporation in MCF10 cells confirmed earlier data showing a significantly increased mitogenic activity for Asp(B10) insulin [10, 14]. In contrast, the insulin analogue HMR1423 behaved like regular insulin, while HMR1964 was even less effective in stimulation of [^3H]thymidine incorporation (Fig. 6).

Fig. 6 [^3H]Thymidine incorporation. MCF10 cells were incubated with regular insulin, or the analogues HMR1423, HMR1964 and Asp(B10) at the indicated concentrations. Results are means of four experiments expressed as a percentage of the maximal response seen in the presence of 10% FCS ($100\% \pm \text{SEM}$). * $p < 0.05$, Asp(B10) insulin vs regular insulin; ** $p < 0.01$, HMR1964 vs regular insulin. Diamonds, regular insulin; squares, HMR1423; crosses, HMR1964; circles, Asp(B10)

Discussion

Insulin analogues have been created to improve therapy of diabetic patients. When introducing point mutations in the insulin gene, metabolic as well as mitogenic effects must be tested carefully in a preclinical and clinical setting. Since the concentrations of insulin or insulin analogues which might cause mitogenesis through the IGF-I receptor are unlikely to occur in vivo, it is a major concern that an insulin analogue might cause mitogenic effects, not via the

IGF-I receptor, but via the insulin receptor. Therefore, we examined insulin receptor association and dissociation kinetics as well as auto- and dephosphorylation of the insulin receptor after stimulation with the new rapid-acting insulin analogues insulin glulisine (HMR1964) and the potentially long-acting analogue HMR1423.

Compared with regular human insulin, we found similar receptor binding characteristics and insulin receptor autophosphorylation and dephosphorylation kinetics for these two new analogues. These findings are in contrast to Asp(B10) insulin, which shows markedly increased affinity for the insulin receptor and prolonged insulin receptor auto- and dephosphorylation rates. This altered behaviour of Asp(B10) on the insulin receptor goes along with increased mitogenic activity shown in rat-1 fibroblasts [10] as well as in MCF10 cells [14]. Moreover Asp(B10) induced tumour formation in mammary glands of rats [14]. While this suggests that the mitogenic effects of Asp(B10) are mediated by the insulin receptor itself, Kurtzhals et al. [15] reported that increased IGF-I binding of insulin analogues may contribute to mitogenicity. However, increased affinity to the IGF-I receptor appears not to be the only mechanism for increased mitogenicity, given that some insulin analogues, like lispro, do not show increased mitogenicity, although their IGF-I receptor binding is somewhat increased. Therefore, under physiological concentrations of insulin, mitogenic properties are much more likely to be determined by the effect of the insulin analogues on the insulin receptor itself. This is in agreement with a report from Hansen et al. [16] showing that the mitogenic effects of different insulin analogues can be predicted by a reduced dissociation rate from the insulin receptor. Indeed, this might be an important mechanism for the increased mitogenic activity of Asp(B10) insulin, since this insulin shows prolonged occupancy on the insulin receptor, which may allow coupling to mitogenic signalling pathways.

Mitogenic activity of the insulin analogues was also tested in the non-malignant human epithelial breast cell line MCF10. This cell line has several advantages over other frequently used malignant cell lines. First, increased mitogenic activity has already been demonstrated with Asp(B10) in this cell model [10]. Secondly, Asp(B10) induced increased mammary tumours in rodents [14], suggesting that mammary cells represent a relevant tissue to study mitogenic effects. Thirdly, MCF10 cells express both insulin receptor isoforms (A and B) to the same extent, as there is increasing evidence that the HIR-A is of prime importance for mitogenic signalling [17].

In this non-malignant cell line, Asp(B10) was more potent in stimulating [³H]thymidine incorporation, while HMR1423 and regular insulin showed no differences, and HMR1964 was even less mitogenic than regular insulin. A reasonable mechanism for the decreased mitogenic activity of HMR1964 was recently suggested by Rakatzi et al. [18], who demonstrated in an in vitro model that HMR1964 shows lower IRS-1 phosphorylation than regular insulin.

In summary, our data show that the new insulin analogues insulin glulisine (HMR1964) and HMR1423 behave like regular human insulin in terms of insulin receptor binding and phosphorylation characteristics. Moreover, HMR1964 was even less mitogenic than regular human insulin in MCF10 cells. Therefore, these new insulin analogues may be safe in terms of mitogenicity. However, as with all new insulin analogues, its side-effects and mitogenic potential must be fully evaluated in vivo.

Duality of Interest

H.-U. Häring received grant support and consulting fees for participating on scientific boards of Aventis Pharma Deutschland. M. Kellerer has received honoraria for speaking engagements and was supported by a grant from Aventis Pharma Deutschland. G. Seipke is employed by Aventis Pharma Deutschland and received fees.

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