Insulin glargine is more potent in activating the human IGF-I receptor than human insulin and insulin detemir

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Abstract

Objective: To investigate whether human insulin (HI) and insulin analogues differ in their ability to activate the human IGF-I receptor (IGF-IR), the human insulin receptor A (IR-A) and the human insulin receptor B (IR-B) in vitro.

Methods: HI, short-acting insulin analogues (insulin aspart; insulin lispro) and long-acting insulin analogues (insulin glargine; insulin detemir) were compared by using kinase receptor activation (KIRA) bioassays specific for IGF-IR, IR-A or IR-B, respectively. These assays quantify ligand activity by measuring receptor auto-phosphorylation upon ligand binding. HI and insulin analogues were tested in a range from 0.1 to 100 nM.

Results: Short-acting analogues: Overall, short-acting insulin analogues did not differ substantially from HI, nor from each other. Insulin lispro was slightly more potent than HI and insulin aspart in activating the IGF-IR, only reaching statistical significance at 100 nM (p<0.01).

Long-acting analogues: At <10 nM insulin glargine was as potent as HI in activating the IRs and IGF-IR. At 10–100 nM insulin glargine was significantly more potent than HI in activating the IR-B (p<0.05) and IGF-IR (p<0.001). Insulin glargine was more potent than insulin detemir in activating all three receptors (p<0.001). Insulin detemir was less potent than HI in activating the IRs at 1–10 nM (p<0.05) and IGF-IR at >1 nM (p<0.05).

Conclusions: Insulin glargine was more potent in activating the IGF-IR than HI and insulin detemir. Since KIRA bioassays do not mimic the exact in vivo situation, further research is needed to find out whether our data have implications for clinical use of insulin glargine.

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1. Introduction

The insulin receptor (IR) is a transmembrane receptor tyrosine kinase and exists in two isoforms: isoform A (IR-A) and isoform B (IR-B). The IR-A is expressed ubiquitously but is predominantly expressed in central nervous system, hematopoietic cells and in cancer tissues [1–4]. The IR-B is expressed predominantly in the liver, but is also substantially expressed in muscle and adipose tissue, the major target tissues for the metabolic effects of insulin [1–4]. Both IR isoforms show great homology with the IGF-I Receptor (IGF-IR) [4–6]. The IGF-IR is found in most tissues and brings about mitogenic, pro-invasive and anti-apoptotic effects [7].

Insulin cannot only stimulate the IR-B, but also the IR-A and IGF-IR [4]. Subtle modifications of the insulin structure made to engineer insulin analogues, may affect receptor specificity signalling and thereby result in abnormal metabolic: mitogenic ratios, e.g. an increased activation of the IGF-IR may inhibit apoptosis and promote cancer by increasing cell proliferation [4]. Recently a possible relationship between insulin analogues, in particular insulin glargine, and risk of cancer was raised by epidemiological studies [8–10]. However, the results of these studies were found to be inconsistent [11,12]. Although, based on the current evidence, short-acting insulin analogues do not seem to bring additive risks in this respect, further evaluation of especially insulin glargine is required [12].

Kinase Receptor Activation (KIRA) bioassays make use of cell lines stably transfected with receptors and quantify ligand bioactivity by measuring ligand-induced receptor tyrosine kinase activation in terms of receptor-phosphorylation [13]. In our laboratory we have running KIRA bioassays specific for the human IGF-IR [14], human IR-A or human IR-B, respectively. This gives us the possibility to compare bioactivity of insulin analogues with respect to their ability to activate the IGF-IR, IR-A and IR-B.

In the present study we investigated whether (short- and long-acting) insulin analogues differ from HI in their bioactivity.

2. Materials and methods

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The human embryonic kidney cell-line (HEK) Flip-in™_293 was obtained from Invitrogen life technologies (Breda, The Netherlands). Plasmids (pNTK-2) containing a cDNA insert of the human IR-B

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(pNTK2-IR-B) or IR-A (pNTK2-IR-A) were kindly provided by Axel Ullrich (Martinsried, Germany). The HEK IGF-IR cell-line was a kind gift from P. de Meyts (Gentofte, Denmark).

Dulbecco’s Modified Eagles Medium (DMEM: gluc+, L-Glutamin +, Ppy+), Penicillin/ Streptomycin, Hygromycine, Geneticin, Fugene® transfection reagents and FBS were obtained from Invitrogen life technologies (Breda, The Netherlands). Human Serum Albumin (HSA), (Octalbine®) was obtained from Octopharma (Lachen, Switzerland). Culture plates (flat-bottomed 48 wells) were obtained from Corning Costar (Schiphol, The Netherlands). Microtiter 96-wells plates were purchased from Biozym (Landgraaf, The Netherlands).

Antibody coating buffer (ABC) contained 15 mM sodium carbonate and 35 mM sodium hydrogen carbonate (pH 9.6). Blocking solution contained 40 mM phosphate, 0.05% (wt/vol) Na2O, 0.6% (wt/vol) NaCl, 0.2% (wt/vol) Tiritrex V (EDTA), and 1% (wt/vol) HSA (pH 8.0). Krebs Ringer bicarbonate (KRB) buffer was adjusted to pH 7.4 by CO2 and supplemented with 0.1% (wt/vol) HSA. Lysis buffer contained 50 mM sodium phosphate, 0.05% (wt/vol) Na2O, 0.6% (wt/vol) NaCl, 0.2% (wt/vol) Tiritrex V (EDTA, and 1% (wt/vol) HSA (pH 7.4). Edetate disodium (EDTA) was added to 50 mL of lysis buffer.

Culture plates (flat-bottomed 48 wells) were obtained from Corning Costar (Schiphol, The Netherlands). Microtiter 96-wells plates were purchased from Biozym (Landgraaf, The Netherlands).

2. Methods

Insulin analogues were compared to HI by using in-house KIRA bioassays specific for IGF-IR, IR-A or the IR-B. The IGF-IR KIRA assays has been previously described [15] and the IR-A and IR-B specific bioassays have recently been developed based on this same principle (article in preparation). All three assays use human embryonic renal cells stably transfected with either cDNA of the human IR-A or human IR-B gene (HEK IR-A or IR-B) or with cDNA of the human IGF-IR gene (HEK IGF-IR).

After 48 h of culture, HEK IR-A and HEK IR-B cells were stimulated for 10 min at 37 °C with an equimolar dose titration ranging from 0.1 to 100 nM of insulin analogues or HI, respectively. HEK IGF-IR cells were stimulated for 15 min at 37 °C with an equimolar dose titration ranging from 0.1 to 100 nM of insulin analogues, HI or human recombinant IGF-I.

After stimulation cells were lysed. Crude lysates were transferred to a sandwich assay. Capture antibodies MA1 and MAD1 were used in a concentration of 2.5 μg/mL and 5.0 μg/mL respectively. Eu-PY20 was used as tracer antibody in a concentration of 1.25 μg/mL. Contents were read in a time-resolved fluorometer. Assays were performed in 48 well plates.

A control dose-titration-curve of HI or human recombinant IGF-I were obtained from Invitrogen (Breda, The Netherlands). DELFIA assay reagents (assay buffer, wash buffer and label counter) were also purchased from Perkin Elmer-life sciences (Groningen, The Netherlands). Europium-labelled PY20 (Eu-PY20); a monoclonal anti-phosphotyrosine antibody was obtained from Novozymes-Gropep (Adelaide, Australia). Europium-orthovanadate 14H20 was added to 50 mL of lysis buffer.

MA1 and MAD1, monoclonal antibodies directed against the extracellular domain of the human IR and human IGF-IR respectively, were obtained from Novozymes-Gropep (Adelaide, Australia). Europium-labelled PY20 (Eu-PY20); a monoclonal anti-phosphotyrosine antibody was obtained from Perkin Elmer-life sciences (Groningen, The Netherlands). DELFIA assay reagents (assay buffer, wash buffer and enhancement solution) and a time-resolved fluorometer (Victor® multi-label counter) were also purchased from Perkin Elmer-life sciences.

HI (Actrapid®, insulin aspart (Novorapid®) and insulin detemir (Levemir®) were obtained from Novo Nordisk (Bagsvaerd, Denmark). HI (Actrapid®, insulin aspart (Novorapid®) and insulin detemir (Levemir®) were obtained from Novo Nordisk (Bagsvaerd, Denmark).

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values of insulin detemir for both IR isoforms and for the IGF-IR were higher relative to HI (440% for IR-A and 690% for IR-B and 170% for the IGF-IR, respectively).

5. Discussion

Our main finding was that insulin glargine was significantly more potent than HI in activating the IGF-IR. This result is in agreement with a study of Kurtzhals et al., where a 3–8 fold increased mitogenicity of insulin glargine compared to HI was found [16]. They used downstream events, such as glucose uptake (of mouse adipocytes) and proliferation (of human osteosarcoma cells), to compare metabolic and mitogenic potencies of insulin analogues with human insulin and correlated these effects to IGF-IR and IR-A affinity. However, there is a considerable crosstalk between IRA, IR-B and IGF-IR-mediated functions at receptor- and at post-receptor level and the final effects are due to a combination of IGF-IR- and IRS-mediated processes. Recently it was even suggested that the IR and IGF-IR act as identical portals to the regulation of gene expression, with differences between insulin and IGF-I effects due to a modulation of the signal created by the specific ligand-receptor interactions [17]. As a consequence it is almost impossible in most in vitro cell lines to disentangle the individual contribution of each type receptor to the final downstream event.

Recently, Sciacca et al. found an increased mitogenic potency of insulin glargine and insulin detemir compared to HI [18]. However, in contrast to our findings and previous reports [16,19], they found that

![Activation of IR-B](image1.png)

**A. Activation of insulin receptor isoform B (IR-B).** A: comparing human IGF-I (dashed black line), human insulin (solid black line) and short-acting insulin analogues insulin aspart (red line) and insulin lispro (green line). B: comparing human IGF-I (dashed black line), human insulin (solid black line) and long-acting insulin analogues insulin glargine (red line) and insulin detemir (green line). Dose-response profiles ranged from 0.1–100 nM. Stimulation with 100 nM of human IGF-I was arbitrarily set at 100% (thin horizontal dashed line). Each point represents the mean value ± SD of four independent experiments. ** p < 0.01 insulin aspart vs. human insulin. ●● p < 0.05 insulin glargine vs. human insulin. *** p < 0.001 insulin detemir vs. human insulin.

![Activation of IR-A](image2.png)

**B. Activation of insulin receptor isoform A (IR-A).** A: comparing human insulin (black line) and short-acting insulin analogues insulin aspart (red line) and insulin lispro (green line). B: comparing human insulin (black line) and long-acting insulin analogues insulin glargine (red line) and insulin detemir (green line). Dose-response profiles ranged from 0.1 to 100 nM. Stimulation with 100 nM of human insulin was arbitrarily set at 100% (thin horizontal dashed line). Each point represents the mean value ± SD of four independent experiments. ** p < 0.01. *** p < 0.001 insulin detemir vs. human insulin.

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insulin detemir induced significantly more IGF-IR phosphorylation than HI, while IGF-IR phosphorylation by insulin glargine was similar to HI. Sciacca et al. suggested that different findings on the effects of insulin detemir in vitro may be attributed to different albumin concentrations used in different studies.

In our study we used three KIRA assays; one developed for the human IGF-IR, one for the human IR-A and one for the human IR-B. These bioassays are very specific in that only the initial activation step of a particular receptor (i.e. tyrosine-phosphorylation) after stimulation with an insulin analogue is used to quantify receptor-mediated signalling. By using these three KIRA bioassays, we were thus able to assess the specific activation of each type of receptor after stimulation with an insulin analogue.

As for all in vitro systems, KIRA bioassays do not mimic the exact in vivo situation. KIRA bioassays only provide a crude, albeit convenient, measure of kinase activation. Although the endpoint signal is readily direct and specific, the IR and IGF-IR have up to 6 key tyrosine residues of which some the role in vivo is not yet fully clear. In addition, the antibody used to capture the tyrosine residues may well not recognize all residues equally because of dependence of affinity on flanking sequence and proximity of other sites. Furthermore, IR and IGF-IR expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may function as a critical determinant of the mitogenic potency of insulin analogues [20]. Moreover, IR/IGF-IR hybrids (Hybrid-Rs) are formed in many tissues and have been shown to be the most represented subtype [21]. However, although the precise biological role of these Hybrid-Rs is still unclear, functional studies have demonstrated that Hybrid-Rs behave more like IGF-IRs than IRS [21]. So, in this context the effects we found on IGF-IR phosphorylation could be important in vivo.

Increased IGF-IR activation by insulin glargine may result in an abnormal metabolic/mitogenic ratio in vivo. However, differences in IGF-IR activation between insulin glargine and HI only reached statistical significance when stimulating cells with relatively high concentrations. On the other hand, in contrast to in vitro conditions, cells in vivo are continuously exposed to insulin glargine 24 h a day during many years. In vivo, insulin glargine is partially degraded into two bioactive products (M1 and M2, respectively) after subcutaneous injection [22]. It has been found that one of these degradation products (M1) is less mitogenic than insulin glargine [16], which could implicate that insulin glargine is less mitogenic in vivo than it is in vitro. To make relationships even more complex, markedly increased circulating IGF-I levels have been reported during treatment with insulin glargine [23–25].

Comparing HI and long-acting insulin analogues in their ability to activate the IR-B, we found that insulin glargine was at least as potent as HI. Insulin detemir was less potent than HI in IR-B activation; only at 100 nM insulin detemir induced similar IR-B activation levels compared to HI. This could be attributed to lower receptor affinity and to binding of insulin detemir to HSA [16] although in our experiments the HSA concentration was only 0.1%. In vivo, a reduced potency to activate IR-B will result in decreased glucose lowering effects. Indeed, insulin detemir has a 4-fold concentrated formulation in order to match biological potency of HI [26]. Therefore, administration of insulin detemir has been shown to result in higher plasma insulin levels than HI and insulin glargine [27,28]. Sciacca et al. tested insulin and insulin analogues at bioequivalent (i.e. glucose lowering) concentrations [18]. They found that insulin glargine, insulin detemir and HI had a similar effect on IR-A and IR-B phosphorylation but that insulin detemir was more potent than HI in activating the IGF-IR. In accordance with these latter results we found that insulin detemir at highest concentrations (100 nM) was as potent as HI in phosphorylating both IR isoforms. However, we found that, even at 100 nM, insulin detemir was not able to activate the IGF-IR to any extent.

Sacco et al. have shown that equivalent IR-A activation can elicit different downstream signalling pathways and biological effects depending on the ligand activating the receptor [29]. So, even at equivalent IR-A activation, HI, insulin detemir and insulin glargine
may result in different downstream signalling effects, being either more metabolic or more mitogenic.

Overall, short-acting insulin analogues did not differ substantially from Hl, nor from each other. In agreement with previous studies [16], insulin lispro was slightly more potent than Hl and insulin aspart in activating the IGF-IR, only reaching statistical significance at 100 nM.

In conclusion, insulin glargine was more potent than Hl especially in IGF-IR activation, whereas insulin detemir did not activate the IGF-IR to any extent. This result clearly underlines the importance of further research to elucidate whether increased IGF-IR activation by insulin glargine in vitro translates into clinical effects on cancer risk.

**Conflict of interest statement**

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**References**