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ORIGINAL ARTICLE

Metabolic effect and receptor signalling profile of a non-metabolisable insulin glargine analogue

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Abstract

Context: Insulin glargine (GLA) is rapidly metabolized *in vivo* to metabolite M1, which has *in vitro* metabolic and mitogenic profiles comparable with human insulin (HI).
Objective: To investigate the pharmacologic and signalling profiles of a non-metabolizable analogue (A21Gly,DiD-Arg) insulin (D-GLA).
Methods: Rats were injected s.c. with 1, 12.5 or 200 U/kg of GLA or D-GLA; blood glucose and phosphorylation status of the insulin receptor (IR), Akt and IGF-1 receptor (IGF1R) in tissue samples were investigated after 1 h. Plasma samples were analysed for insulin by LC-MS/MS.
Results: Blood glucose lowering was prolonged with D-GLA. D-GLA comprised ≥98% of insulin after D-GLA injection; M1 comprised 76–92% after GLA injection. IR and Akt phosphorylation were comparable with GLA and D-GLA. Neither analogue stimulated IGF1R phosphorylation.
Conclusions: Suprapharmacological doses of D-GLA did not activate IGF1R *in vivo*. Mitogenic effects of insulin and insulin analogues might be solely based on IR growth-promoting activity.

Keywords

(A21Gly,DiD-arg) insulin, IGF-1 receptor, insulin glargine, insulin receptor, receptor phosphorylation

History

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Introduction

AspB10 insulin (AspB10) is the only insulin analogue shown to promote tumour growth (Hansen *et al*., 2011). It has a higher affinity than human insulin for the insulin receptor (IR) and the IGF-1 receptor (IGF1R) *in vitro*, as well as a prolonged occupancy time at the IR and a higher proliferation rate in mammalian cell lines (Berti *et al*., 1998; Kurtzhals *et al*., 2000; Sommerfeld *et al*., 2010). Insulin glargine (A21Gly, B31Arg,B32Arg human insulin) is a long-acting insulin analogue that has an IR profile similar to human insulin but slightly higher affinity for IGF1R *in vitro* (Berti *et al*., 1998; Kurtzhals *et al*., 2000; Sommerfeld *et al*., 2010). This has led to the general belief that insulin analogues with increased IGF1R affinity *in vitro* might *per se* exert an increased growth-promoting activity *in vivo* (Hansen *et al*., 2011).

In humans and animals, glargine undergoes rapid and significant metabolism, leading to early formation of the major metabolite M1, which has *in vitro* metabolic and mitogenic profiles comparable with human insulin (Bolli *et al*., 2012; Kuerzel *et al*., 2003; Tennagels *et al*., 2013; Werner *et al*., 2012). Recently, it was reported that neither glargine nor AspB10 stimulated IGF1R phosphorylation in various tissues of rats treated with even suprapharmacological doses (Tennagels *et al*., 2013). AspB10 treatment did result in at least two-fold higher phosphorylation levels and significantly longer duration of IR and Akt (also known as protein kinase B) phosphorylation in most tissues compared with human insulin or glargine. These results led to the hypothesis that AspB10 may promote tumourigenesis via prolonged activation of the IR. In the case of glargine, the rapid metabolism to M1 may preclude IGF1R activation. However, some have speculated that glargine could possibly promote tumour growth through IGF1R activation in patients with no or low levels of the metabolising proteases that convert glargine to M1 (Müssig *et al*., 2011). In order to mimic that situation, a non-metabolizable glargine analogue, (A21Gly,DiD-Arg) insulin, was developed. Here we report on its ability to activate IR and IGF1R *in vitro* and *in vivo* compared with glargine.

Methods

Materials

Human insulin, insulin glargine and (A21Gly,DiD-Arg) insulin were produced by recombinant DNA techniques or enzymatic semi-synthesis, purified to homogeneity and made available by Process Development Biotechnology (Sanofi, Frankfurt, Germany). (A21Gly,DiD-Arg) insulin is insulin glargine with the L-arginine residues at B31 and B32 replaced with D-arginine residues. Human A14 [^{125}I]-insulin was prepared by the radio-synthesis group at Sanofi

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(Frankfurt, Germany). [2-^{14}C]-thymidine was obtained from Perkin Elmer (Boston, MA, USA). Complete Protease Inhibitor was from Roche Diagnostics (Penzberg, Germany). Polyvinyltoluene (PVT) polyethylenimine (PEI) scintillation proximity assay (SPA)-treated wheat germ agglutinin (WGA) beads were purchased from GE Healthcare (Amersham, UK). Cell culture reagents and antibodies were obtained from the suppliers as indicated in the Methods section. All other chemicals were of reagent grade.

Receptor binding assays

The binding of the different insulins to human IR-B was analysed in a competitive binding assay using the SPA as previously described (Sommerfeld *et al*., 2010). Plasma membranes were enriched from Chinese hamster ovary (CHO) cells over-expressing either human IR-B or IGF1R by a series of differential centrifugations including a single flotation through a one-step sucrose gradient. Briefly, cells were grown to confluence and gently detached, transferred to a centrifugation tube followed by centrifugation for 10 min at $600 \times g$ at 4 °C. The pellet was re-suspended in ice-cold 2.25 STM buffer (2.25 mol/l sucrose, 5 mmol/l Tris-HCl pH 7.4, 5 mmol/l $MgCl_2$, 1 × Complete Protease Inhibitor) and disrupted using a hand-held Dounce homogenizer followed by sonication. This homogenate was transferred to a centrifugation tube, overlaid with 0.8 STM buffer (0.8 mol/l sucrose, 5 mmol/l Tris-HCl pH 7.4, 5 mmol/l $MgCl_2$, 1 × Complete Protease Inhibitor) and centrifuged for 90 min at $100\,000 \times g$ at 4 °C. The emerging pellicle at the interface was collected, transferred to a new tube and washed two times with phosphate-buffered saline (PBS) by centrifugation for 10 min at $1500 \times g$. The final pellet was resuspended in a small volume of dilution buffer (50 mmol/l Tris-HCl pH 7.4, 5 mmol/l $MgCl_2$, 1 × Complete Protease Inhibitor) and homogenized with a Dounce homogenizer. These plasma membrane preparations were stored at −80 °C until use.

Insulin receptor binding experiments were conducted in 96-well microplates. Per well, 2 mg of membranes were incubated with 0.25 mg WGA PVT PEI SPA beads, 100 pmol/l A14 [^{125}I]-insulin and various concentrations of unlabelled insulins in a binding buffer containing 0.05 mol/l Tris-HCl pH 7.8, 0.15 mol/l NaCl, 0.1% bovine serum albumin (BSA) (defatted; fraction V, Sigma, Deisenhofen, Germany), Complete Protease Inhibitor for 12 h at room temperature (23 °C). The radioactivity was measured at equilibrium in a microplate scintillation counter (Wallac Microbeta, Freiburg, Germany). IGF1R binding experiments were conducted in a similar manner.

Receptor autophosphorylation

CHO cells expressing human IR-B were used for IR autophosphorylation assays using In-Cell Western as previously described (Sommerfeld *et al*., 2010). For the analysis of IGF1R autophosphorylation, the receptor was over-expressed in a mouse embryo fibroblast 3T3 Tet off cell line (BD Bioscience, Heidelberg, Germany) that was stably transfected with IGF1R tetracycline-regulatable expression plasmid resulting in the expression of $\sim 2.6 \times 10^5$ IGF1R per cell. In order to determine the receptor tyrosine phosphorylation level, cells were seeded into 96-well plates and grown for 48 h. Cells were serum starved with serum-free medium αMEM (PAN Biotech GmbH, Aidenbach, Germany) for 3–4 h. The cells were subsequently treated with increasing concentrations of either human insulin or the indicated insulin analogue for 15 min at 37 °C. After incubation the medium was discarded and the cells fixed in 3.75% freshly prepared para-formaldehyde for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Blocking was performed with Odyssey blocking buffer (LICOR, Bad Homburg, Germany) overnight at 4 °C. Anti-pTyr 4G10 (Millipore, Schwalbach, Germany) was incubated for 2 h at room temperature. After incubation of the primary antibody, cells were washed with PBS + 0.1% Tween 20 (Sigma-Aldrich, St Louis, MO, USA). The secondary antimouse-IgG-800-CW antibody (Rockland, Gilbertsville, PA, USA) was incubated for 1 h. Results were normalized by the quantification of DNA with TO-PRO3 dye (Invitrogen, Karlsruhe, Germany).

Metabolic activity

The metabolic activity of the different insulins was compared using insulin inhibition of lipolysis in in vitro differentiated human adipocytes. Human pre-adipocytes from a subcutaneous depot were obtained in frozen aliquots from Lonza (Cologne, Germany). For cell number expansion the cells were cultured in Endothelial Cell Growth Medium supplemented with supplement mix (Promo Cell GmbH, Heidelberg, Germany) at 37 °C in a humidified atmosphere. After the third passage, the expanded cell number was high enough to start the differentiation. For differentiation into adipocytes, detached and re-suspended cells were seeded in microtitre plates. After cell attachment, the cell medium was removed and replaced by differentiation medium (DMEM/Ham's F-10 Medium (1:1, v/v) (PAN-Biotech GmbH, Aidenbach, Germany), 15 mM Hepes, pH 7.4, 33 μM biotin, 17 μM pantothenate, 1 μM dexamethasone, 0.2 mM isobutylmethylxanthine, 10 nM L-thyroxine (all from Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 3% (v/v) fetal calf serum (PAN-Biotech GmbH, Aidenbach, Germany), 100 nM human insulin, 0.625x Antibiotic-Antimycotin (Life Technologies GmbH, Darmstadt, Germany), 0.1 μM PPARγ agonist. After 3 days, the differentiation media was replaced by adipocyte media (media as described above, but without isobutylmethylxanthine and L-thyroxine) and the plates incubated for ≥10 additional days; the medium was changed on a 3-4-3 day cycle.

Fourteen to 16 days after start of the differentiation, the adipocyte medium was removed and replaced with adipocyte medium without insulin and PPARγ agonist. The plates were then incubated overnight at 37 °C. The next day, medium was removed, each well washed three times with lipolysis medium (medium199 (PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 1% (w/v) HSA (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and lipolysis medium containing the test compounds in dilution series (eight different concentrations, at least quadruple determinations for each concentration) added. The plates were incubated for 4 h at 37 °C in a humidified atmosphere, after which a defined volume of supernatant was removed from each well and analysed for free glycerol content using the free glycerol determination kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) according to the manufacturer's instructions.

Mitogenic potency

Mitogenic activity was determined as described previously (Sommerfeld *et al.*, 2010). The human osteosarcoma cell line Saos-2 was obtained in frozen aliquots from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Cells were grown in McCoy's 5a medium (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (PAN Biotech GmbH, Aidenbach, Germany) and 2 mM (final) L-glutamine (Sigma Aldrich, Irvine, UK). Subconfluent cultures ($9–15 \times 10^6$ cells per $225\,cm^2$ flask) were used to determine the mitogenic activity of the test compounds. For measuring thymidine incorporation, 40 000 cells were seeded per well of a 96-well Cytostar-T scintillation microplate (GE Healthcare, Amersham, UK) and the plates were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO_2. The serum-containing medium was removed and replaced by 200 µl serum-free McCoy's 5a medium supplemented with 0.5% (w/v) BSA (Gibco, Grand Island, NY, USA), 2 mM L-glutamine and antibiotics (penicillin 100 units, streptomycin 100 units, amphotericin B 0.25 mg/ml final, Gibco, Grand Island, NY, USA). The plates were incubated for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then, 150 µl of the medium was removed and substituted by 150 µl of serum-free medium containing the different insulins at the indicated concentrations and the plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 19 h, 10 µl of [2-^{14}C]-thymidine solution (>50 mmol/l, 3.7 MBq/ml) diluted in serum-free McCoy's 5a medium was added per well to yield a final concentration of 500 nCi/ml and the plates were incubated for 6 h at 37 °C in a humidified atmosphere containing 5% CO_2. Incorporation of ^{14}C-thymidine was measured in a Wallac 1450 Micro Beta Trilux Scintillation counter (PerkinElmer, Shelton, CT, USA). Dose-response curves were obtained by testing 10 different concentrations of the ligands with every concentration tested by octuplicate samples.

Animals

Animals were housed and treated as described previously (Tennagels *et al.*, 2013). Male Wistar rats (HsdCpb:WU) were obtained from Charles River, Sulzfeld, Germany. The animals were housed in Macrolon cages ($1400\,cm^2$; Ehret, Emmendingen, Germany) on virtually dust-free wood granulate bedding, enriched with nestling material, chow stick and hide tubes ($n = 3–4$ per cage). Animal housing conditions were standardized (22 ± 2 °C, 55 ± 10% relative humidity, light cycle from 06:00 to 18:00 h) and a standard rodent pellet diet (R/M-H 1534; ssniff Spezialdiäten, Soest, Germany) was given until study start. Studies were performed with rats at 8–10 weeks of age, after acclimatization for $\geq$1 week. Free access to tap water was maintained at all times. The animals were randomized to five to eight rats per group and deprived of food 2 h before the start of an experiment.

Injections

Study 1: In the first study, rats ($n = 8$) were injected s.c. with 1 U/kg (6 nmol/kg) of glargine, (A21Gly,DiD-Arg) insulin or 0.9% saline (control group). Blood samples for glucose and insulin analyses were taken at time 0 and at various time points up to 6 h after the injection. Blood glucose was determined enzymatically from 5 µl of tail tip whole blood haemolysed with 250 µl haemolysate (haemolysis reagent H, Glucose Hexokinase Fluid 5 + 1; Hengler Analytik, Steinbach, Germany). Quantification was with a Glucoquant Glucose/HK kit (Roche Diagnostics, Penzberg, Germany) using a Beckman Coulter AU640 (Beckman Coulter, Krefeld, Germany) or a Roche/Hitachi 912 Chemistry Analyser (Roche Diagnostics, Mannheim, Germany). The amount of insulin glargine, M1 and M2 in plasma was determined by immuno-affinity extraction followed by liquid chromatography–tandem mass spectrometry as described by Bolli *et al.* (2012).

Study 2: In a second study, rats ($n = 5$) were injected s.c. with 1, 12.5 or 200 U/kg glargine or (A21Gly,DiD-Arg) insulin. After 1 h, blood was withdrawn for insulin determinations as described above. Samples of calf muscle, liver, abdominal adipose tissue and heart were removed at the same time for analysis of IR, Akt, IGF1R and extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation.

The animal studies were approved by the local ethics committee and were conducted in accordance with the Principles of Laboratory Care.

Receptor signalling *in vivo*

The phosphorylation of receptor and signalling molecules was assessed by Western blot analysis as described previously (Tennagels *et al.*, 2013). After immunoprecipitation using antibodies directed against the beta-subunit of the IR or IGF1R (Santa Cruz Biotechnology, Santa Cruz, CA, USA), proteins were separated on SDS-PAGE gels (4–12% [wt/vol.] resolving gel; Invitrogen, Carlsbad, CA, USA), transferred to polyvinylidene difluoride membranes (Roche Applied Science, Penzberg, Germany) and blocked (Roti-Block; Carl Roth, Germany) for 1 h. Membranes were incubated overnight at 4 °C with primary antibody directed against phosphotyrosine (Millipore, Germany), IR or IGF1R. Membranes were washed in TRIS-buffered saline +0.1% (vol./vol.) Tween 20 and incubated with the appropriate secondary horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualizsed with Lumi-Light (Roche Applied Science, Penzberg, Germany) and detected with a chemiluminescence detection system (Lumi-Imager; Boehringer, Mannheim, Germany). Phospho-Akt was determined using a phospho-Akt ELISA kit (Life Technologies, Grand Island, NY, USA).

Statistical analyses

For each *in vitro* experiment, IC_{50} or EC_{50} values were obtained using the four-parameter logistic model (Ratkowsky & Reedy, 1986). The adjustment was obtained by non-linear regression using the Levenberg–Marquardt algorithm in SAS v9.1.3 software (SAS Institute Inc., Cary, NC, USA) via

Table 1. Metabolic and mitogenic profile of human insulin, insulin glargine, M1 and (A21Gly,DiD-Arg) insulin glargine *in vitro*. Data are means ± SEM. All analogues were tested at least three times on different days. Activity was determined within each experiment and then averaged to yield a single reported mean value.

Analogue	IR-B affinity IC_{50} (nmol/l)	IR-B auto-phosphorylation EC_{50} (nmol/l)	Metabolic potency IC_{50} (pmol/l)	IGF-1R affinity IC_{50} (nmol/l)	IGF-1R auto-phosphorylation EC_{50} (nmol/l)	Mitogenic potency EC_{50} (nmol/l)
Human insulin	3.5 ± 0.5^{ns}	13.1 ± 0.7#	31.6 ± 1.5^{ns}	375.0 ± 61.9##	447.0 ± 48.9###	20.7 ± 3.8#
Glargine	5.2 ± 1.1^{ns}	24.3 ± 1.6**	39.0 ± 3.0^{ns}	20.3 ± 2.4**	87.5 ± 9.5***	3.7 ± 1.1*
Glargine M1	$6.4 \pm 0.5^{ns,ns}$	$23.6 \pm 2.5^{*,ns}$	$43.5 \pm 4.1^{ns,ns}$	645 ± 21.7**,###	677.0 ± 84.6**,###	40.4 ± 8.8**,###
(A21Gly, DiD-Arg) insulin	$7.8 \pm 0.6^{ns,ns}$	$21.6 \pm 5.3^{*,ns}$	$46.8 \pm 5.7^{*,ns}$	$22.8 \pm 2.7^{**,ns}$	$111.7 \pm 8.0^{***,ns}$	$1.1 \pm 0.1^{*,ns}$
IGF-1	595.3 ± 155.5***,###	> 1000***,###	$49.9 \pm 3.4^{**,ns}$	0.68 ± 0.17**,##	2.9 ± 0.3***,###	$0.31 \pm 0.07^{***,ns}$

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. human insulin; #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ vs. insulin glargine.

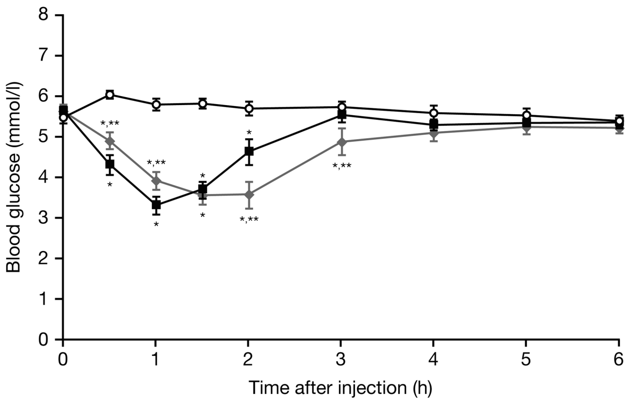

Figure 1. Time course of blood glucose following s.c. injection of 1 U/kg glargine (squares), (A21Gly,DiD-Arg) insulin (diamonds) or placebo (circles) in 8- to 10-week-old male Wistar rats. Values are mean ± SEM ($n = 8$); *$p < 0.05$ versus placebo; **$p < 0.05$ versus glargine.

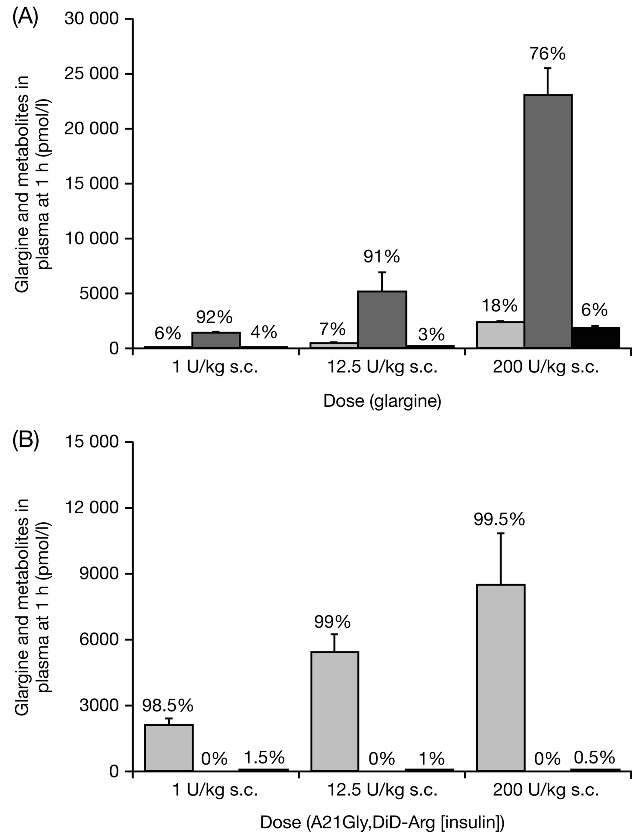

Figure 2. Plasma concentrations of parent (light grey bar), M1 (dark grey bar) and M2 (black bar) 1 h after s.c. injection of 1, 12.5 or 200 U/kg of glargine (A) or (A21Gly,DiD-Arg) insulin (B) in 8- to 10-week old male Wistar rats. Values are mean ± SEM ($n = 5$).

Biost@t-Speed V2.0-LTS internal application. If necessary, lower and upper asymptotes were set to 0 and 100, respectively. Statistical analysis was performed using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA). Data were analysed by one-way ANOVA followed by Dunnett's test. All data are presented as mean ± SEM.

Results

In vitro activity of (A21Gly,DiD-Arg) insulin

Characterization of the interaction with the insulin and IGF-1 receptor as well as the metabolic and mitogenic potencies of human insulin, insulin glargine, its main metabolite M1 (= A21Gly human insulin), (A21Gly,DiD-Arg) insulin and IGF-1 are summarized in Table 1. The binding affinity of glargine, M1 and (A21Gly,DiD-Arg) insulin for the human IR-B was 40–70% less than that of human insulin, whereas IGF-1 was 0.6%. Stimulation of IR-B autophosphorylation by insulin glargine, M1, (A21Gly,DiD-Arg) insulin and IGF-1 correlated well with their binding affinities to IR, being 54%, 56%, 61% and <1% of human insulin. Metabolic potency, as measured by anti-lipolytic action in human *in vitro* differentiated adipocytes, correlated with the ability to increase IR autophosphorylation for human insulin, glargine, M1, and (A21Gly,DiD-Arg) insulin. Interestingly a clear anti-lipolytic activity of IGF-1 with a potency similar to that of insulin glargine, M1 and (A21Gly,DiD-Arg) insulin could be observed in our cell system. The binding affinity of human insulin, glargine and M1 for IGF1R was 0.1–3% of that for IGF-1. The binding of (A21Gly,DiD-Arg) insulin was similar to that of glargine. Stimulation of IGF1R autophosphorylation by human insulin, insulin glargine and M1 correlated well with their binding affinity to IGF1R and were 0.7%, 3.3% and 0.4% of IGF-1 stimulation, respectively. Stimulation by (A21Gly,DiD-Arg) insulin was similar to that of glargine (2.6% of IGF-1). The mitogenic potency of the human insulin, glargine, M1 and (A21Gly,DiD-Arg) insulin correlated with their ability to increase autophosphorylation of human IGF1R.

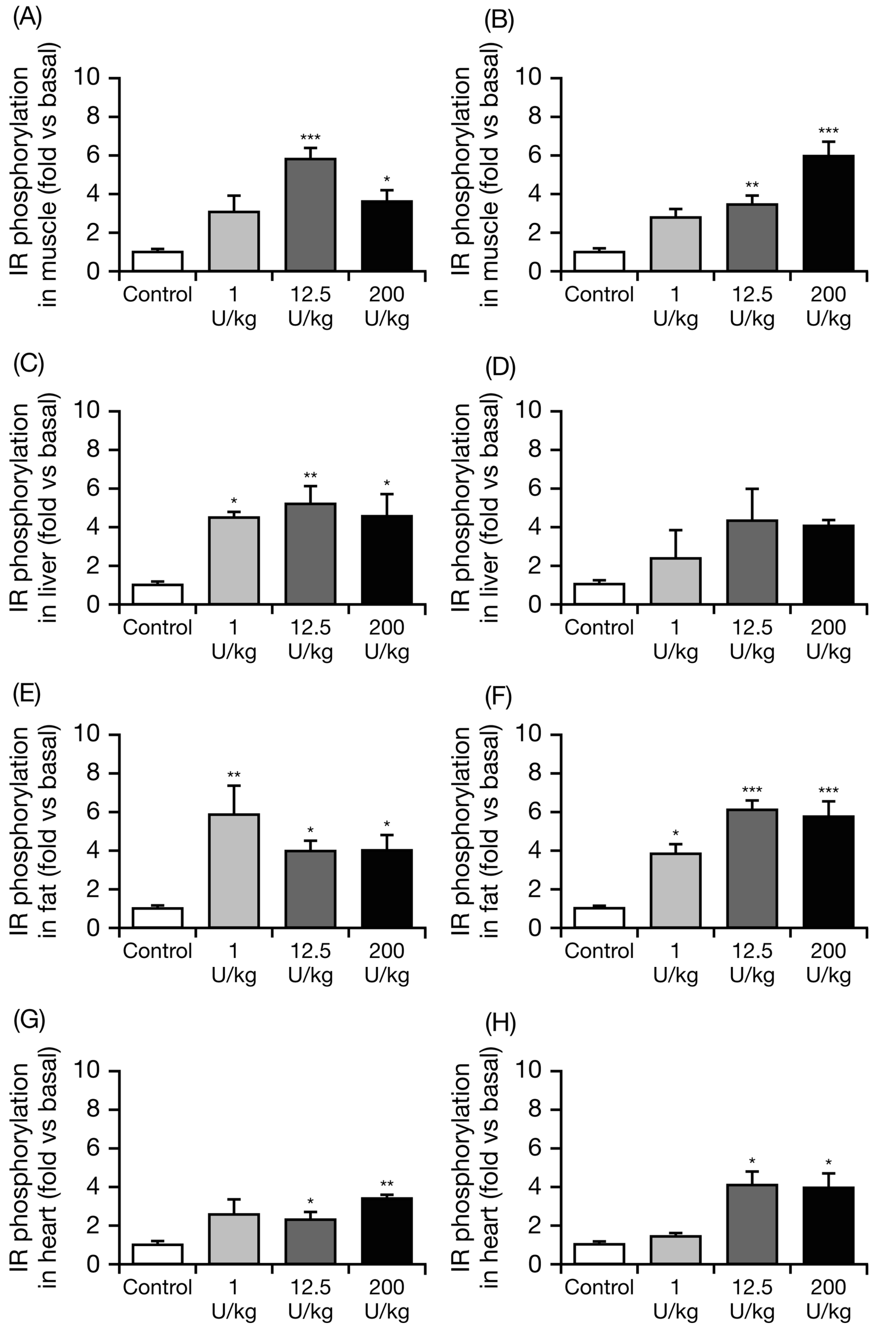

Figure 3. Insulin receptor (IR) phosphorylation in muscle (A, B), liver (C, D), fat (E, F) and heart (G, H) 1 h after s.c. injection of 1, 12.5 or 200 U/kg of (A21Gly,DiD-Arg) insulin (A, C, E, G) or glargine (B, D, F, H) in 8- to 10-week-old male Wistar rats. Values are mean ± SEM ($n = 5$); *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control.

Effects on blood glucose and plasma insulin

After s.c. injection of 1 U/kg of insulin glargine, blood glucose levels declined by 42% to 3.3 mmol/l at 1 h before returning to baseline levels at 3 h (Figure 1). A similar nadir (3.5 mmol/L) was reached after 1.5 h following injection of 1 U/kg of (A21Gly,DiD-Arg) insulin, and blood glucose remained low for another 0.5 h before returning to baseline. One hour after injection of 1, 12.5 or 200 U/kg of insulin glargine, the majority of glargine was metabolized to its main metabolite M1 accounting for 92%, 91% and 76% of the total injected insulin, respectively (Figure 2). Glargine parent only accounted for 6%, 7% and 18%, respectively. When the same doses of (A21Gly,DiD-Arg) insulin were injected, parent (A21Gly,DiD-Arg) insulin accounted for >98% of the total injected insulin, while metabolite M1 was not detected and M2 accounted for 0.5–1.5%.

Phosphorylation of receptor signalling molecules

Phosphorylation of receptor signalling molecules was examined 1 h after s.c. injection of 1, 12.5 or 200 U/kg of either glargine or (A21Gly,DiD-Arg) insulin. (A21Gly,DiD-Arg) insulin increased IR phosphorylation in muscle, heart, liver and fat tissue to a similar extent as insulin glargine at all injected doses (Figure 3). Similar results were observed with Akt phosphorylation (Figure 4). Neither (A21Gly,DiD-Arg) insulin nor glargine had any effect on IGF1R phosphorylation at any dose in muscle or heart (Figure 5; see Supplemental figures for blots).

Discussion

Glargine has a slightly greater affinity for the IGF1R *in vitro* than human insulin (Berti *et al*., 1998; Kurtzhals *et al*., 2000;

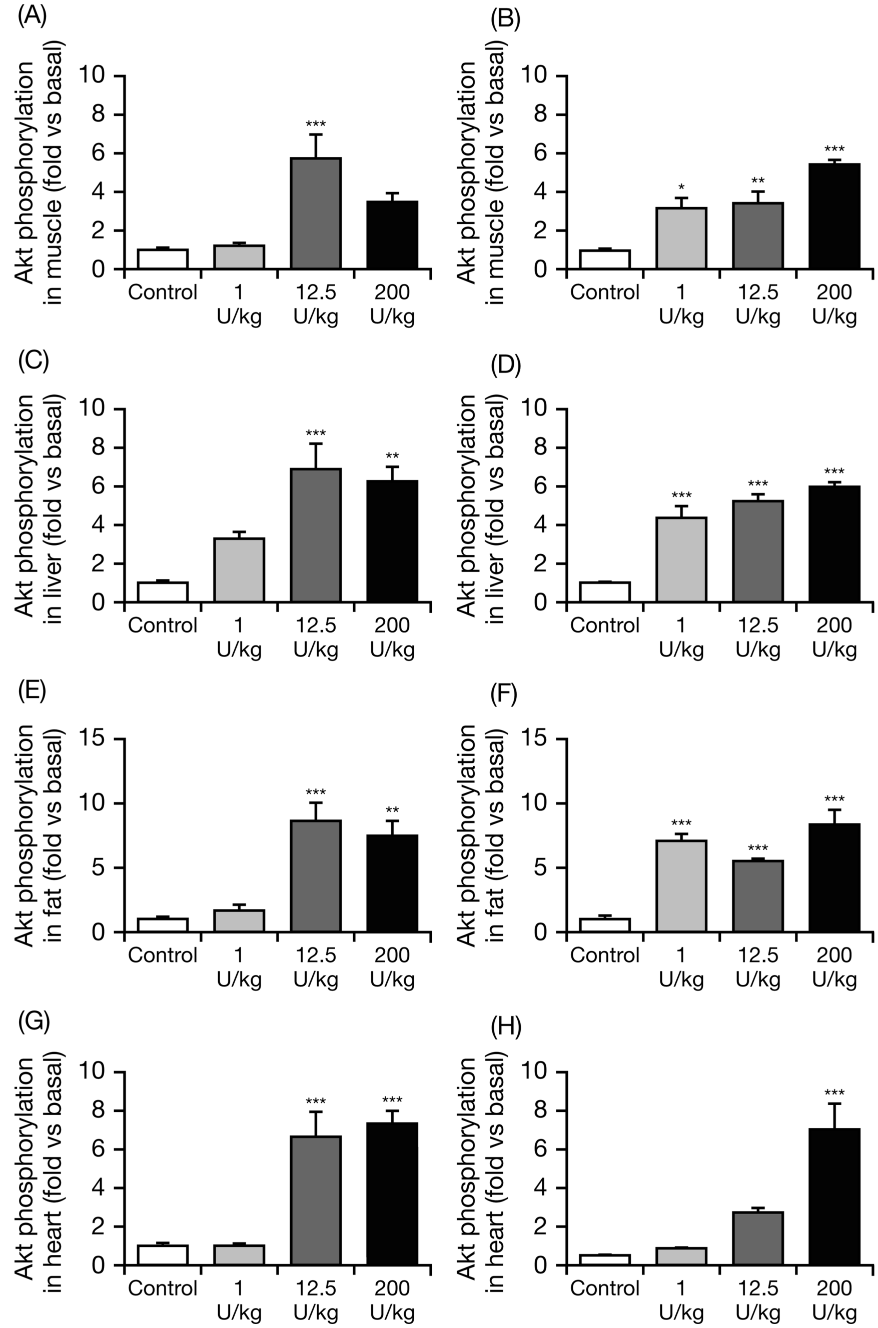

Figure 4. Akt phosphorylation in muscle (A, B), liver (C, D), fat (E, F) and heart (g, h) 1 h after s.c. injection of 1, 12.5 or 200 U/kg of (A21Gly,DiD-Arg) insulin (A, C, E, G) or glargine (B, D, F, H) in 8- to 10-week-old male Wistar rats. Values are mean ± SEM ($n = 5$); *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ versus control.

Sommerfeld *et al*., 2010), and also has greater cell proliferation effects than human insulin in some cell lines (reviewed by Tennagels & Werner, 2013). This has led some to propose a link between insulin glargine and cancer related to possible differences in stimulation of IGF1R compared with endogenous insulin (Colhoun & SDRN Epidemiology Group, 2009; Hemkens *et al*., 2009; Jonasson *et al*., 2009; Ruiter *et al*., 2012). However, little parent glargine is found in circulation after even supraphysiological doses of glargine (Tennagels *et al*., 2013), as glargine is extensively and rapidly metabolized to its M1 metabolite (Bolli *et al*., 2012; Kuerzel *et al*., 2003; Tennagels *et al*., 2013; Werner *et al*., 2012). M1 has a metabolic and mitogenic profile similar to human insulin *in vitro* (Bolli *et al*., 2012; Kuerzel *et al*., 2003; Tennagels *et al*., 2013; Werner *et al*., 2012), results consistent with the lack of an increase in cancer by glargine compared with NPH (neutral protamine Hagedorn) insulin in 2-year carcinogenicity studies in animals (Stammberger & Essermeant, 2012). Although glargine administration did not stimulate IGF1R phosphorylation *in vivo* even at supraphysiological doses (Tennagels *et al*., 2013), the fact that little parent glargine exists in circulation raises the question of whether glargine itself can stimulate IGF1R *in vivo*. To address this question, a metabolically stable analogue of glargine was developed where the di-L-arginine residues at B31 and B32 were replaced with D-arginine residues. (A21Gly,DiD-Arg) insulin was found to have similar metabolic and mitogenic activity as insulin glargine *in vitro*. *In vivo*, parent (A21Gly,DiD-Arg) insulin accounted for >98% of the injected insulin even at supraphysiological doses, with no M1 present. The extent of blood glucose lowering was similar with (A21Gly,DiD-Arg) insulin and glargine, but with

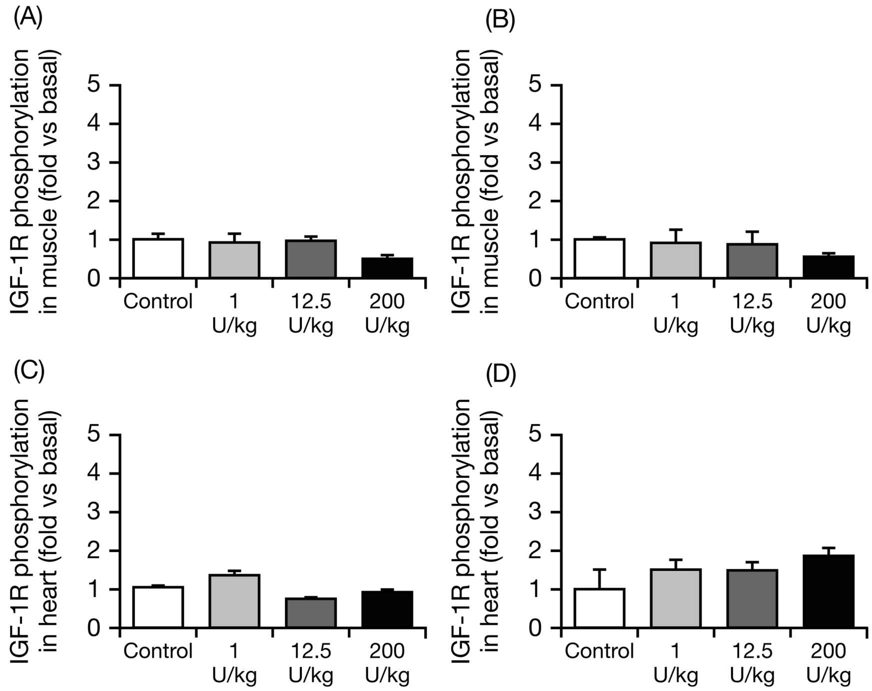

Figure 5. IGF1R phosphorylation in skeletal muscle (A, B) and heart (C, D) 1 h after s.c. injection of 1, 12.5 or 200 U/kg (A21Gly,DiD-Arg) insulin (A, D) or glargine (B, D) in 8- to 10-week-old male Wistar rats. Values are mean ± SEM ($n = 5$).

a prolonged time–action profile for (A21Gly,DiD-Arg) insulin. When receptor signalling was examined *ex vivo* 1 h after injection, no differences in IR signalling profile between glargine and (A21Gly,DiD-Arg) insulin were observed in any tissue at 1, 12.5 and 200 U/kg doses.

Activation of the IGF1R by IGF-1 has been shown to be tightly controlled. The i.v. injection of a high dose of IGF-1 (136 nmol/kg) activated IGF1R in muscle, heart and mammary tissue of rats, whereas s.c. injection of 6 nmol/kg IGF-1 was unable to generate detectable receptor autophosphorylation (Tennagels *et al*., 2013). Similarly, in mouse heart muscle, the i.v. injection of 136 nmol/kg of IGF-1 resulted in phosphorylation of IGF1R, whereas no signal could be detected after i.v. injection of a 4 nmol/kg dose (Ikeda *et al*., 2009). In addition, the s.c. injection of a supraphysiological dose (600 nmol/kg) of IGF-1 increased Akt phosphorylation in liver, colon and mammary gland of Sprague–Dawley rats (Hvid *et al*., 2011). Akt and ERK phosphorylation also occurred in mouse mammary gland tissue only after a large bolus tail vein injection of IGF-1 (Lee *et al*., 2003).

Conclusions

In the current study, neither glargine nor (A21Gly,DiD-Arg) insulin activated IGF1R in any tissue at any dose tested. The lack of IGF1R activation with glargine confirms our previous results (Tennagels *et al*., 2013), while the results with (A21Gly,DiD-Arg) insulin show that even a non-metabolized form of glargine with affinity for IGF1R *in vitro* similar to glargine itself and present in plasma at similar concentrations to glargine M1 was unable to activate the receptor. Therefore, the mitogenic effects of insulin and insulin analogues may be solely based on the growth-promoting activity of the IR itself. This would be consistent with the results of Gallagher *et al*. (2013), who showed that hyperinsulinaemia and AspB10 increase IR phosphorylation and tumour growth in MKR mice independent of any IGF1R phosphorylation. Thus, IGF1R activation by insulin analogues may be less relevant than previously realized.

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Declaration of interest

All authors are employees of Sanofi.

References

Berti L, Kellerer M, Bossenmaier B, *et al*. (1998). The long acting human insulin analog HOE 901: Characteristics of insulin signalling in comparison to Asp(B10) and regular insulin. *Horm Metab Res*, 30: 123–9.

Bolli GB, Hahn A, Schmidt R, *et al*. (2012). Plasma exposure to insulin glargine and its metabolites M1 and M2 after subcutaneous injection of therapeutic and supratherapeutic doses of glargine in subjects with type 1 diabetes mellitus. *Diabetes Care*, 35: 2626–30.

Colhoun HM, SDRN Epidemiology Group. (2009). Use of insulin glargine and cancer incidence in Scotland: A study from the Scottish Diabetes Research Network Epidemiology Group. *Diabetologia*, 52: 1755–65.

Gallagher EJ, Alikhani N, Tobin-Hess A, *et al*. (2013). Insulin receptor phosphorylation by endogenous insulin or the insulin analog AspB10 promotes mammary tumor growth independent of the IGF-1 receptor. *Diabetes*, 62: 3553–60.

Hansen BF, Kurtzhals P, Jensen AB, *et al*. (2011). Insulin X10 revisited: A super-mitogenic insulin analogue. *Diabetologia*, 54: 2226–31.

Hemkens LG, Grouven U, Bender R, *et al*. (2009). Risk of malignancies in patients with diabetes treated with human insulin or insulin analogues: a cohort study. *Diabetologia*, 52: 1732–44.

Hvid H, Fels JJ, Kirk RK, *et al*. (2011). *In situ* phosphorylation of Akt and ERK1/2 in rat mammary gland, colon, and liver following treatment with human insulin and IGF-1. *Toxicol Pathol*, 39: 623–40.

Ikeda H, Shiojima I, Ozasa Y, *et al*. (2009). Interaction of myocardial insulin receptor and IGF receptor signaling in exercise-induced cardiac hypertrophy. *J Mol Cell Cardiol*, 47: 664–75.

Jonasson JM, Ljung R, Talbäck M, *et al*. (2009). Insulin glargine use and short-term incidence of malignancies-a population-based follow-up study in Sweden. *Diabetologia*, 52: 1745–54.

Kuerzel GU, Shukla U, Scholtz HE, *et al*. (2003). Biotransformation of insulin glargine after subcutaneous injection in healthy subjects. *Curr Med Res Opin*, 19: 34–40.

Kurtzhals P, Schäffer L, Sørensen A, *et al*. (2000). Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. *Diabetes*, 49: 999–1005.

Lee AV, Taylor ST, Greenall J, *et al*. (2003). Rapid induction of IGF-IR signaling in normal and tumor tissue following intravenous injection of IGF-I in mice. *Horm Metab Res*, 35: 651–5.

Müssig K, Staiger H, Kantartzis K, *et al*. (2011). Type 2 diabetes mellitus and risk of malignancy: Is there a strategy to identify a subphenotype of patients with increased susceptibility to endogenous and exogenous hyperinsulinism? *Diabet Med*, 28: 276–86.

Ratkowsky DA, Reedy TJ. (1986). Choosing near-linear parameters in the four-parameter logistic model for radioligand and related assays. *Biometrics*, 42: 575–82.

Ruiter R, Visser LE, van Herk-Sukel MP, *et al*. (2012). Risk of cancer in patients on insulin glargine and other insulin analogues in comparison with those on human insulin: Results from a large population-based follow-up study. *Diabetologia*, 55: 51–2.

Sommerfeld MR, Müller G, Tschank G, *et al*. (2010). *In vitro* metabolic and mitogenic signaling of insulin glargine and its metabolites. *PLoS One*, 5: e9540.

Stammberger I, Essermeant L. (2012). Insulin glargine: A reevaluation of rodent carcinogenicity findings. *Int J Toxicol*, 31: 137–42.

Tennagels N, Welte S, Hofmann M, *et al*. (2013). Differences in metabolic and mitogenic signalling of insulin glargine and insulin aspart B10 in rats. *Diabetologia*, 56: 1826–34.

Tennagels N, Werner U. (2013). The metabolic and mitogenic properties of basal insulin analogues. *Arch Physiol Biochem*, 119: 1–14.

Werner U, Schmidt R, Blair E, *et al*. (2012). The molecular mechanism of insulin glargine metabolism *in vivo*. *Diabetes*, 61(Suppl 1): A425.

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