Differences in metabolic and mitogenic signalling of insulin glargine and insulin aspart B10 in rats

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Abstract
Aims/hypothesis In vitro, insulin glargine (A21Gly,B31Arg, B32Arg human insulin) has an insulin receptor (IR) profile similar to that of human insulin, but a slightly higher affinity for the IGF-1 receptor (IGF1R). Insulin aspart B10 (B10Asp human insulin) (AspB10), the only insulin analogue with proven carcinogenic activity, has a greater affinity for IGF1R and IR, and a prolonged IR occupancy time. The pharmacological and signalling profile of therapeutic and suprapharmacological doses of glargine were analysed in different tissues of rats, and compared with human insulin and AspB10.

Methods Male Wistar rats were injected s.c. with human insulin or insulin analogue at doses of 1 to 200 U/kg, and the effects on blood glucose and the phosphorylation status of IR, IGF1R, Akt and extracellular signal-regulated protein kinase 1/2 in muscle, fat, liver and heart samples were investigated.

Results Glargine, AspB10 and human insulin lowered blood glucose, with the onset of action delayed with glargine. Glargine treatment resulted in phosphorylation levels of IR and Akt that were comparable with those achieved with human insulin, although delayed in time in some tissues. AspB10 treatment resulted in at least twofold higher phosphorylation levels and significantly longer duration of IR and Akt phosphorylation in most tissues. None of the insulin treatments resulted in detectable IGF1R phosphorylation in muscle or heart tissue, whereas intravenous injection of IGF-1 increased IGF1R phosphorylation.

Conclusions/interpretation The IR signalling pattern of AspB10 in vivo is distinctly different from that of human insulin and insulin glargine, and might challenge the notion that activation of IGF1R plays a role in the observed carcinogenic effect of AspB10.

Keywords Akt · AspB10 insulin · Human insulin · IGF-1 receptor · Insulin glargine · Insulin receptor · Receptor phosphorylation

Introduction
Insulin analogues in the treatment of patients with type 1 or type 2 diabetes have been shown to be more efficient, reproducible and convenient than regular insulin [1]. Due to either sequence or secondary structural modifications, analogues may differ from insulin with respect to metabolic potency, stability, or onset and duration of action. Although these changes were introduced to alter the time-action profile of the respective insulin analogues, they may also lead to an altered activation profile of the insulin receptor (IR) and (or) IGF-1 receptor (IGF1R) signalling pathways, and may change metabolic or mitogenic responses [2]. A careful investigation of acute and long-term effects of insulin analogues has been a major research focus.

The insulin analogue insulin aspart B10 (B10Asp human insulin) (AspB10) was withdrawn from clinical development
due to a higher incidence of breast cancer in rats [3]. AspB10 differs from human insulin by the substitution of histidine by aspartate in position 10 of the B chain. In vitro, AspB10 displays higher affinity towards the IR and IGF1R, a prolonged occupancy time at the IR and a higher proliferation rate in mammalian cell lines. Although the mechanism by which AspB10 exerts its mitogenic effect is not clear, it is still contended that the analogue’s greater affinity to the IGF1R might be at least in part responsible [4–7]. 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.

However, in vitro studies cannot be directly applied to the in vivo situation. First, the affinity of the endogenous ligand IGF-1 for IGF1R is at least 100-fold greater than that of insulin or insulin analogues. Thus, IGF-1 competes with insulin for IGF1R occupation. Second, in vitro studies use supraphysiological (nanomolar) concentrations vs picomolar concentrations in vivo. Third, the plasma and tissue concentration and distribution of insulin analogues are different in vivo than in vitro. Fourth, insulin analogues undergo biotransformation in vivo, necessitating analysis of the active metabolites.

Insulin glargine (A21Gly,B31Arg,B32Arg human insulin) is a long-acting human insulin analogue with an activity profile very closely mimicking the natural physiological profile of basal endogenous insulin secretion. Glargine differs from human insulin by substitution of asparagine by glycine in position 21 of the A chain and by carboxy-terminal extension of the B chain by two arginine residues. These changes cause a shift in the isoelectric point from pH 5.4 to 6.7. Following s.c. administration as a clear solution of pH 4, insulin glargine precipitates at the injection site because of its low solubility at physiological pH levels. The prolonged blood glucose-lowering activity of insulin glargine may result from the subsequent slow dissolution of the microprecipitate on the basis of a low dissociation rate. The dissolution process is followed by rapid proteolytic degradation of parent glargine, leading to soluble metabolites as demonstrated in metabolism studies in humans, rats and dogs [8–10]. The two main metabolites of insulin glargine, M1 ([GlyA21]insulin) and M2 ([GlyA21,des-ThrB30]insulin) are formed by the sequential removal of the two arginines from the carboxy-terminus of the B chain and additional deamination of threonine in position B30. In plasma, the principal circulating compound is the metabolite M1, the exposure of which increases as the dose of administered insulin glargine increases [11].

Insulin glargine has an in vitro IR signalling and metabolic profile comparable to that of human insulin, but displays a slightly greater IGF1R affinity in vitro [4, 5, 7]. However, in 2 year carcinogenicity studies, no difference was observed in the incidence of mammary tumours in mice and rats compared with controls or animals treated with NPH insulin [12], a finding that can be attributed to the pharmacodynamic effect of M1, which has in vitro metabolic and mitogenic profiles comparable with human insulin [7].

The aim of this study was to analyse the time–action profile of glargine in responsive tissues of rats with respect to pharmacological and signalling variables and to compare that profile to those of human insulin and AspB10, using therapeutic as well as suprapharmacological doses. We also investigated the effect of human insulin, glargine and AspB10 on the phosphorylation of IGF1R and compared it with the effect of IGF-1.

Methods

Animals

Male Wistar rats (HsdCpb:WU) were obtained from Charles River, Sulzfeld, Germany. The animals were housed in Macrolon cages (1,400 cm²; 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 standardised (22±2°C, 55%±10% relative humidity, light cycle from 06:00 to 18:00 hours) 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 to 10 weeks of age, after acclimatisation for at least 1 week. Free access to tap water was maintained at all times. The animals were randomised to five to six rats per group and deprived of food 2 h before the start of an experiment.

Injections

All injection solutions were freshly prepared. To achieve final doses, regular human insulin and AspB10 were dissolved in 0.9% (wt/vol.) saline, while glargine was dissolved in a solution matching the glargine placebo at pH 4. All test drugs and placebo were administered as a single subcutaneous injection.

Study 1 In the first study, rats (n=5–6) were injected s.c. with 1 U/kg (6 nmol/kg) of human insulin, glargine, AspB10 or 0.9% saline (control group). Blood samples for glucose and insulin analyses were taken at time point 0 and at various time points up to 120 min 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

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was with a Gluco-quant Glucose/HK kit (Roche Diagnostics, Penzberg, Germany) using a Beckman Coulter AU640 (Beckman Coulter, Krefeld, Germany) or a Roche/Hitachi 912 Chemistry Analyzer (Roche Diagnostics, Mannheim, Germany). Serum insulin was determined by human insulin immunnoassay (ELISA 10-1113-01; Mercodia, Uppsala, Sweden) unless otherwise stated. The amount of insulin glargine, M1 and M2 in plasma was determined by immunoaffinity extraction followed by liquid chromatography–tandem mass spectrometry as described by Bolli et al [11]. Samples of skeletal (calf) muscle, liver, abdominal adipose tissue and heart were removed at the same time points for analysis of IR, Akt (also known as protein kinase B) and extracellular signal-regulated protein kinase (ERK)1/2 phosphorylation.

Study 2 In a second study, rats (n=5) were injected s.c. with 12.5, 50 or 200 U/kg human insulin, glargine or AspB10 to determine whether these high doses led to an increase in IGF1R phosphorylation. Samples of calf muscle, liver, abdominal adipose tissue and heart were removed after 60 min. As a control intended to demonstrate an IGF-1 effect on IGF1R downstream signalling, rats were injected s.c. with 6 nmol/kg or i.v. with 6 or 136 nmol/kg des[1-3] IGF-1, and calf muscle and heart samples were taken 60 min after the s.c. injection or 5 min after the i.v. injection. All tissue samples were subsequently analysed for IR, IGF1R and Akt phosphorylation. Des[1-3]IGF-1 is a truncated variant, which is more potent than human IGF-1 due to reduced binding to IGF-binding proteins [13].

Study 3 In a third study, the effects on IR and IGF1R phosphorylation in mammary tissue were examined in 74-week-old female Sprague–Dawley rats (Charles River). The rats (n=3–4) were injected s.c. with 12.5 U/kg human insulin, glargine, AspB10 or saline, and mammary tissue was removed at time 0 and 60 min. Other rats (n=3–4) were injected intravenously with 1 mg/kg des[1-3]IGF, with skeletal muscle and heart samples being removed after 5 min.

Analyses

The phosphorylation of receptor and signalling molecules was assessed by western blot analysis as described by Baus et al [14]. 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, Germany) and blocked (Roti-Block; Carl Roth, Germany) for 1 h. Membranes were incubated overnight at 4°C with primary antibody directed against phosphorytosine (Millipore, Germany), IR or IGF1R. Membranes were washed in TRIS-buffered saline + 0.1% (vol./vol.) Tween 20 and incubated with the appropriate secondary horseaterish peroxidase-conjugated antibody (Santa Cruz). Immunoreactive bands were visualised with LumiLight (Roche) 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).

Study approval and statistical analysis

The animal studies were approved by the local Ethics Committee and were conducted in accordance with the Principles of Laboratory Care. All data are presented as means±SEM. Statistical analysis was by one-way ANOVA followed by Dunnett’s test, and was performed with a statistics analysis software (Prism; Graph Pad Software, San Diego, CA USA).

Results

Effects on blood glucose and plasma insulin

After injection of 1 U/kg human insulin or AspB10, blood glucose levels started to decline immediately, reaching minimum values of 4.3±0.1 mmol/l and 3.6±0.2 mmol/l after 30 and 60 min, respectively. Starting baseline values were similar at 5.9 and 6.1 mmol/l (Fig. 1a). The glucose-lowering action of 1 U/kg glargine was initially delayed by 30 min but glucose levels reached a similar level of 4.1 ±0.2 mmol/l after 90 min. However, the AUC was smaller with glargine than with human insulin or AspB10, indicating a prolonged glucose-lowering action of this insulin analogue. With higher doses, blood glucose was lowered to the same extent by human insulin and insulin analogues, reaching 28 to 41% of baseline without significant hypoglycaemia (data not shown).

As in humans, glargine was effectively and rapidly metabolised in rats. At 1 h after the injection of 1 U/kg glargine, 90% of total insulin was identified as the M1 metabolite (1,407 pmol/l), whereas the parent compound was barely detectable and the M2 metabolite was below the level of quantification (Fig. 1b). The M1 metabolite also accounted for 91% (5,122 pmol/l) and 76% (22, 969 pmol/l) of glargine in plasma after injection of 12.5 and 200 U/kg glargine, where the total insulin glargine concentration including metabolites was 5,600 and 30,100 pmol/l, respectively (electronic supplementary material [ESM] Fig. 1). However, even with a suprapharmacological dose of 200 U/kg, the relative proportion of glargine parent compound (18%, 5,288 pmol/l) and M2 (6%, 1,826 pmol/l) remained low.
Phosphorylation of the IR and signalling molecules

The time course of IR phosphorylation in skeletal muscle, liver and fat tissue was examined following s.c. injection of 1 U/kg human insulin, glargine or AspB10 (Fig. 2a). In muscle, the time to peak phosphorylation with human insulin and AspB10 was reached after 15 min, whereas with glargine it was delayed until 30 min (Fig. 2a). AspB10 treatment resulted in at least twofold higher peak phosphorylation levels and a significantly longer duration of IR phosphorylation than treatment with human insulin. Peak phosphorylation with glargine was lower than with AspB10, but greater than with human insulin; the duration was also longer than with human insulin. In liver, the time to peak phosphorylation was the same for all three insulins (Fig. 2b). Only AspB10 showed greater phosphorylation and a longer duration than human insulin. In fat tissue, the time to peak phosphorylation was later and peak phosphorylation was greater with human insulin than with glargine or AspB10, while the duration of phosphorylation was longer with glargine (Fig. 2c).

Subcutaneous injection of 1 U/kg glargine resulted in peak Akt phosphorylation comparable with that of 1 U/kg of human insulin in skeletal muscle, liver and fat tissue (Fig. 3). Time to peak phosphorylation was delayed in muscle and fat tissue, but not in liver. AspB10 induced significantly greater peak phosphorylation in muscle and liver, and also prolonged the duration of Akt phosphorylation in skeletal muscle. None of the insulins significantly increased ERK1/2 phosphorylation in any tissue examined (data not shown).

The effect of increasing suprapharmacological doses of human insulin, glargine and AspB10 on IR and Akt phosphorylation in muscle was examined 60 min after s.c. injection, at a time when similar glucose-lowering effects were observed with each insulin. Each insulin increased IR and Akt phosphorylation, with no significant difference between them detectable (Fig. 4). Comparable results were observed in heart (data not shown).

Phosphorylation of IGF1R It was of interest to determine whether the injection of high doses of human insulin, AspB10 or glargine led to an increase in IGF1R phosphorylation in skeletal muscle. As a control to demonstrate an IGF-1 effect on IGF1R and downstream signalling, des[1-3] IGF-1 was injected and the phosphorylation of IGF1R, IR and Akt was determined (Fig. 5). Subcutaneous injection of...
6 nmol/kg had no effect on any phosphorylation, whereas i.v. injection of 6 or 136 nmol/kg increased IGF1R and Akt phosphorylation in a dose-dependent manner, but had no effect on IR phosphorylation. In contrast, s.c. injection of increasing doses of human insulin, glargine or AspB10 had no effect on muscle IGF1R phosphorylation after 60 min at any dose (Fig. 6). The slight increase in the tyrosine phosphorylation signal observed at very high doses may have been due to contaminating phospho-IR in the immunoprecipitate. Similar results were observed in heart (data not shown). To show whether i.v. delivery of insulin analogues resulted in IGF1R phosphorylation in muscle, 1 U/kg of each analogue was injected, and IR and IGF1R phosphorylation was investigated after 5 min (ESM Fig. 2). Whereas significant phosphorylation of the IR was detected, no IGF1R phosphorylation was measurable with any insulin. Total insulin levels after i.v. injection reached 14,844±1,101 pmol/l. The majority of the insulin present in plasma was the M1 metabolite (84%, 9,350 pmol/l), followed by M2 (10%, 1,140 pmol/l) and parent glargine (6%, 652 pmol/l), indicating rapid metabolism in plasma.

Phosphorylation of IR and IGF1R in mammary tissue IGF1R phosphorylation in mammary tissue was not significantly increased when 74-week-old female rats were injected s.c. with 12.5 U/kg human insulin, glargine or AspB10 (Fig. 7). The increased phosphorylation detected after AspB10 injection mainly resulted from the presence of phospho-IR in the IGF1R immunoprecipitate. Only AspB10 significantly increased IR phosphorylation. Intravenous injection of 1 mg/kg des[1-3]IGF-1 increased IGF1R phosphorylation 16-fold but had no significant effect on IR phosphorylation.

Discussion

Given that patients with diabetes often require life-long insulin treatment, it is essential to examine all steps in the action of an insulin analogue in vitro and in vivo, to exclude unwanted effects like growth-promoting activities [15]. Malignant cell growth is often associated with aberrant signalling of both IR isoforms (IR-A and IR-B) and IGF1R. The insulin and IGF receptors trigger a complex

![Fig. 3](a) Time course of Akt phosphorylation in skeletal muscle, (b) liver and (c) fat following s.c. injection of 1 U/kg human insulin (triangles), glargine (circles) and AspB10 (squares) 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 vs human insulin.

![Fig. 4](a–c) Muscle IR and (d–f) Akt phosphorylation 60 min after s.c. injection of (a, d) 12.5, (b, e) 50 or (c, f) 200 U/kg human insulin (HI), glargine or AspB10 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 vs control.
range of intracellular signals for metabolism, cell growth and proliferation [16]. Their relative abundance affects intracellular signalling and has important consequences for tissue-specific responses to insulin, IGFs and insulin analogues [17, 18]. In addition, it has been demonstrated that overexpression of IR and IGF1R in human breast carcinomas allows insulin and IGF-1 hybrid receptors to form. These hybrid receptors become tyrosine autophosphorylated when breast cancer cells are exposed to IGF-1, but not to insulin, and also mediate growth in response to IGF-1 [19–22].

As cancer cells have aberrant IR and IGF1R signalling patterns, it is important to understand how insulin analogues affect normal and cancerous cells, as this has implications for diabetes, cancer and cancer treatment. It is generally believed that insulin analogues with a higher affinity than human insulin for IGF1R in vitro have greater mitogenic activity in vivo. This belief is based on AspB10, the only insulin analogue with proven carcinogenicity in rats [3]. Insulin glargine is also thought to have greater mitogenic activity in vivo, based on its slightly higher affinity for IGF1R in vitro, but unlike AspB10, glargine does not have greater affinity for the IR or a prolonged occupancy time at the IR [4, 5, 7].

It is, however, difficult to predict results in vivo on the basis of in vitro data [23], and in vitro studies do not conclusively support IGFR activation as the mechanism of increased mitogenic activity. The mitogenic action of insulin glargine is increased in certain cell lines with high IGFR:IR ratios [6, 7, 24–28], but other cell lines with high IGFR:IR ratios do not respond to insulin glargine treatment with...
increased proliferation [4, 5, 7, 24, 25, 29–34]. Moreover, some cell lines respond to AspB10, but not to other analogues with increased proliferation [35], while cell lines that do not have a greater affinity for IGFR have also been reported to show increased proliferation upon exposure to other insulin analogues currently used in therapy [27, 28, 36].

One way of examining mitogenic activity in vivo is to directly determine tumour formation after chronic treatment with insulin and (or) insulin analogues. AspB10 was withdrawn from clinical development due to a higher incidence of breast cancer in rats after 12 months [3]. In contrast, insulin glargine did not induce a higher incidence of mammary tumours in lifetime carcinogenic studies in female rats and mice [37], confirming that this basal insulin analogue is unlikely to pose a cancer risk in humans. Moreover, in a mouse model prone to tumour formation, Nagel et al. [38] demonstrated that tumour formation did not increase with insulin glargine vs NPH insulin treatment after 18 months. The balance of evidence indicates that no commercially available insulin analogue has carcinogenic effects in the human clinical setting [10]. The approach presented here was to examine the time course of phosphorylation of the IR and IGF1R, and the effects on downstream signalling molecules of insulin and insulin analogues in different tissues in rats. Previous studies have been performed in mice [39, 40]. The one rat study that has been reported to date used suprapharmacological doses and only investigated downstream signalling [17].

Blood glucose levels dropped immediately after the injection of human insulin or AspB10, with no difference between the two insulins. In contrast, glucose lowering was delayed with insulin glargine, as expected for a long-acting insulin analogue, where the mode of action involves precipitation and subsequent slow release from the tissue depot [41]. As in humans [11, 42], the lowering of blood glucose could be correlated to the biortransformation of insulin glargine into the M1 metabolite, which lacks the di-arginine residues [8]. Glargine parent and M2 were not detectable. Consequently M1, and not glargine itself, is the primary driver of the pharmacodynamic effect and the long-acting time–action profile observed with insulin glargine treatment [11, 42]. The proteases responsible for this activity appear to be independent of the species investigated [43]. M1 is the major active metabolite even at a dose of 200 U/kg, suggesting that the protease system involved has a high capacity. However, at this high dose, glargine can be found in the circulation, indicating that saturation of the system can occur.

Peak IR and Akt phosphorylation levels induced by insulin glargine were generally comparable with those achieved with human insulin, although in some tissues the effects of insulin glargine were delayed and (or) prolonged in time. Similar differences have been described by Agouni et al. [39], possibly reflecting differences in pharmacokinetic and/or pharmacodynamic properties across tissues. The comparable peak phosphorylation of insulin glargine vs human insulin reflects the comparable activity of M1 vs human insulin and supports the conclusion that insulin glargine behaves like human insulin in terms of signalling. In contrast, IR and Akt phosphorylation was increased and prolonged with AspB10, most strikingly in muscle and liver. These results are compatible with the greater affinity of the IR for AspB10 and with the prolonged signalling of the IR when exposed to AspB10 in vitro [6, 7]. Interestingly, at higher doses (12.5 and 200 U/kg), the differences in peak phosphorylation of the IR and Akt observed between AspB10, glargine and insulin were no longer detectable, apparently demonstrating the saturation of peak phosphorylation under these high-dose conditions. Hvid et al. have reported that 100 U/kg s.c. resulted in comparable peak phosphorylation of Akt over 150 min [17, 18]. Consequently, a therapeutic dose of 1 U/kg seems to reflect differences in the affinity of AspB10, insulin and insulin glargine to the IR in vitro [7].

The presence and activation of the IGF1R in muscle, heart and mammary tissue was demonstrated by intravenous injection of a high dose of IGF-1 (136 nmol/kg), whereas 6 nmol/kg IGF-1 injected s.c. was unable to generate detectable receptor autophosphorylation. A similar result was reported in mouse heart muscle, where the injection of 136 nmol/kg IGF-1 i.v. resulted in strong phosphorylation of the receptor, whereas no signal could be detected after i.v. injection of a therapeutic 4 nmol/kg dose [44]. The tight control of IGF1R phosphorylation was also demonstrated by Hvid et al. [17], who reported that s.c. injection of a supraphysiological dose (600 nmol/kg) of IGF-1 in rats increased Akt phosphorylation in liver, colon and mammary gland of Sprague–Dawley rats. In agreement with a relatively poor response to IGF-1, Lee et al reported that Akt and ERK phosphorylation occurred in mouse mammary gland tissue only after a large bolus tail vein injection [45]. Although it has been demonstrated that the large bolus can result in the majority of the IGF-1 being in circulation [46], it should be noted that part of the dose could be bound to IGF-binding proteins, which would limit the free concentration and explain a reduced response [45]. In any case, we used des[1-3]IGF-1, which lacks the N-terminal tripeptide Gly-Pro-Glu and has increased potency due to reduced binding to most of the IGF-binding proteins [13]. Thus i.v. injection of des[1-3]IGF-1 should directly allow characterisation of the tissue IGF1R response.

Neither human insulin nor insulin analogues generated significant IGF1R signals in these tissues at s.c. doses up to 200 U/kg. The same holds true when the analogues were administered i.v. and the phosphorylation pattern was studied after 5 min. Importantly, animals that received an insulin glargine injection of 200 U/kg showed a free serum insulin
M1 level of 22 nmol/l, which is 30-fold below the reported affinity of M1 to the IGF1R [7]. In the same study, the measured concentrations of parent glargine and M2 were 5 nmol/l and 2 nmol/l, respectively, with the total insulin concentration reaching about 30 nmol/l. Assuming similar concentrations were achieved with AspB10 at a dose of 200 U/kg, the lack of IGF1R phosphorylation would be remarkable, since this concentration should be able to increase IGF-1 phosphorylation in vitro [7]. The tendency for a high dose of AspB10 to show an increase in the tyrosine phosphorylation signal of the immunoprecipitate may be a reflection of phosphorylated IR contamination due to coprecipitation of IR by the antibody used in these studies. It may also reflect IR subunits precipitated as IR and/or IGFRI hybrids. Hybrid receptors have been detected in a number of tissues including human skeletal and heart muscle, and adipose tissue [6, 47]. Glargine itself might have a higher affinity for hybrid receptors in vitro [6, 47], but M1 is like human insulin and thus no signal was observed in vivo.

In summary, AspB10 treatment resulted in a blood glucose profile comparable to that of insulin. The phosphorylation of signalling molecules was increased and (or) prolonged in most tissues, which resembles in vitro findings. The glycaemic action of insulin glargine was (slightly) retarded compared with insulin. Insulin glargine is rapidly and effectively metabolised to M1 under therapeutic and high-dose conditions, and the phosphorylation of signalling molecules in tissues was generally comparable to that of insulin, but retarded in time in some tissues. IGF1R phosphorylation could not be detected in several tissues upon exposure to insulin glargine or AspB10, even at high dose and different routes of administration. We conclude that in rats AspB10 has a different IR signalling profile to that of insulin and insulin glargine, and that the slightly elevated IGF1R activity of AspB10 in vitro did not translate into IGF1R phosphorylation in vivo. Consequently, we hypothesise that the greater mitogenic effect of AspB10 is most likely to be based on its altered IR profile in vivo. It is therefore tempting to speculate that the greater mitogenic effects of insulin and insulin analogues are solely based on the growth-promoting activity of the IR itself, and that IGF1R activation by insulin analogues may be less relevant under therapeutic conditions than previously discussed.

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Contribution statement NT and UW made substantial contributions to the conception and design of the study, to the analysis and interpretation of the data, to the drafting and revising of the manuscript, and also approved the version to be published. SW, MH, PB and RS contributed substantially to the acquisition of data, critically reviewed the manuscript and approved the version to be published.

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