Insulin and its analogues and their affinities for the IGF1 receptor

Aimee J Varewijck and Joseph A M J L Janssen

Division of Endocrinology, Department of Internal Medicine, Erasmus Medical Center, Room D-443, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

(Correspondence should be addressed to J A M J L Janssen; Email: j.a.m.j.l.janssen@erasmusmc.nl)

Abstract

Insulin analogues have been developed in an attempt to achieve a more physiological replacement of insulin and thereby a better glycaemic control. However, structural modification of the insulin molecule may result in altered binding affinities and activities to the IGF1 receptor (IGF1R). As a consequence, insulin analogues may theoretically have an increased mitogenic action compared to human insulin. In view of the lifelong exposure and large patient populations involved, insulin analogues with an increased mitogenic effect in comparison to human insulin may potentially constitute a major health problem, since these analogues may possibly induce the growth of pre-existing neoplasms. This hypothesis has been evaluated extensively *in vitro* and also *in vivo* by using animal models. *In vitro*, all at present commercially available insulin analogues have lower affinities for the insulin receptor (IR). Although it has been suggested that especially insulin analogues with an increased affinity for the IGF1R (such as insulin glargine) are more mitogenic when tested *in vitro* in cells expressing a high proportion of IGF1R, the question remains whether this has any clinical consequences. At present, there are several uncertainties which make it very difficult to answer this question decisively. In addition, recent data suggest that insulin (or insulin analogues)-mediated stimulation of IRs may play a key role in the progression of human cancer. More detailed information is required to elucidate the exact mechanisms as to how insulin analogues may activate the IR and IGF1R and how this activation may be linked to mitogenesis.

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The introduction of insulin analogues in the treatment of diabetes

The discovery of insulin by Banting and Best in 1922 represented a milestone in clinical medicine. It has saved the lives of many who would otherwise have died, but its unforeseen effect was to transform an acute, rapidly fatal illness into a chronic disease with serious long-term complications (Tattersall 2003).

In the late-80s of the past century, methods were developed that allowed insulin to be made in the laboratory. These methods have permitted the production of limitless quantities of human insulin for therapeutic use.

The human insulin molecule is secreted by the pancreas and consists of two polypeptide chains A and B that are linked by two disulphide bridges (Fig. 1). In the body, insulin exists as monomers, dimers and as hexamers (consisting of six monomers which

self-associate in conjunction with zinc ions). An adaptation of beta-cell insulin production is the self-association of insulin molecules, at high concentrations, in conjunction with zinc ions into hexamers (Emdin *et al.* 1980). This process provides efficient spatial storage within the beta-cell vesicles, but dilution upon exocytosis ensures immediate dissociation into dimers (association of two insulin molecules) and finally into monomers. Monomers are the biologically active forms that bind to the insulin receptor (IR).

Insulin complexed to zinc ions dissociates only slowly into insulin monomers. Therefore, these preparations are used to maintain basal insulin levels (i.e. levels required in a fasting state). During a meal, more rapid-acting monomeric insulin is needed to provide meal-related increased insulin requirements.

The first available insulin preparations failed to simulate physiological insulin profiles. However,

Figure 1 Human insulin is a peptide hormone composed of 51 amino acids and has a molecular weight of 5808 Da. The primary structure of human insulin is formed by 21 amino acids in the A-chain and 30 amino acids in the B-chain. At one end of each chain (the N terminal end) is an amino group, and at the other end (the C terminal end) is a carboxylic acid group. The three-dimensional structure of insulin is further stabilised by disulphide bridges. These form between thiol groups (-SH) on cysteine residues. There are six cysteines, and so three disulphide bridges are formed: two between the A- and B-chains (between A7 and B7, and A20 and B19), and one within the A-chain (A6 and A11). Modifications made on the insulin backbone to produce short-acting insulin analogues are indicated in black, modifications made to produce long-acting insulin analogues are indicated in grey. For insulin lispro, proline at the position 28 and lysine at position 29 in the B-chain of human insulin are interchanged. For insulin aspart, the proline at position 28 in the B-chain is replaced by aspartic acid. For insulin glulisine, the asparagine at position 3 and lysine at position 29 in the B-chain are replaced by lysine and glutamic acid. For insulin glargine, aspartic acid at position 21 in the A-chain had been replaced for glycine and the B-chain contains two extra amino acids (two arginines) at positions 31 and 32. For insulin detemir, threonine at position 30 of the B-chain is removed and a 14-carbon fatty acid chain is added to position 29 of the B-chain. **Two changes have been made.

through genetic engineering of DNA, the amino acid sequence of natural insulin could be changed in such a way that alterations were made in absorption, distribution, metabolism and excretion characteristics of this molecule. Interestingly, although these modified molecules are more commonly referred to as insulin analogues, the U.S. Food and Drug Administration (FDA) refers to these also as ‘IR binding agonists’ (Guidance for industry diabetes mellitus: developing drugs and therapeutic biologics for treatment and prevention, www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071624.pdf). Two main groups of insulin analogues can be distinguished in 1) short-acting insulin analogues, genetically engineered in such a way that they dissociate more rapidly following injection and in 2) long-acting insulin analogues, which show a delayed absorption or a prolonged duration of action (see below).

Binding of insulin analogues to the IR and the IGF1 receptor

Structural modification of the insulin molecule may result in altered binding affinities and activities to the IR and/or the insulin-like growth factor 1 receptor (IGF1R). As a consequence, insulin analogues may have an

increased/decreased metabolic action and an increased/decreased mitogenic action than human insulin.

The amino acid residues in the insulin molecule that are essential for binding to the IR have been identified (Sliker *et al.* 1997). Especially, modifications at positions in the B26–B30 region, i.e. the C-terminus of the B-chain, do not seem to significantly influence insulin binding to the IR (Nakagawa & Tager 1987, Kurtzhals *et al.* 2000; Fig. 1). However, this region is important for at least two reasons. First, these amino acids are important for insulin dimerisation (Bi *et al.* 1984, Mayer *et al.* 2008). Modification of this latter region reduces the stability of monomer–monomer interactions and this effect has been used to generate monomeric insulin analogues with only slight changes in affinity for the IR.

Secondly, substitutions of amino acids in the B-chain result in insulin molecules which show increased structural homology with IGF1 and as a consequence have an increased affinity for the IGF1R (Sliker *et al.* 1997, Kurtzhals *et al.* 2000). Proline at position 28 and lysine at position 29 is the natural sequence which is present in the B-chain of human insulin (Fig. 1). The number and position of basic or acid residues in this region seem very important for IGF1R binding (Sliker *et al.* 1997). Substitution of position B28 with

basic residues increases the relative affinity to the IGF1R ~ 1.5 - to 2-fold (ArgB28ProB29 > OrnB28ProB29 = LysB28ProB29; [Sliker et al. 1997](#)). In contrast, substitution with acidic residues (CyaB28ProB29 = GluB28ProB29 > AspB28ProB29) reduces the relative affinity for the IGF1R approximately twofold. Combination of aspartic acid substitution at B10 with a modification in position B28–29 (e.g. AspB10, LysB28, ProB29) increases the affinity for the IGF1R twofold. Addition of arginine residues at position B31–32 (B31B32diArg) increases the affinity for the IGF1R tenfold compared to human insulin, while the same addition in combination with aspartic acid substitution at B10 increases the affinity to the IGF1R even 28-fold. In general, analogues with substantially increased IGF1R affinity are more potent in stimulating proliferation of cells. This could be of clinical importance since most primary tumours and malignant cells show an increased expression of IGF1Rs ([Pollak 2008](#)).

On the other hand, [Hansen et al. \(1996\)](#) have shown that an increased mitogenic potency of insulin analogues may not only be due to an increased affinity for the IGF1R, but may also result from slow ligand dissociation from the IR. A slow IR dissociation rate is associated with a sustained activation of the IR tyrosine kinase and phosphorylation of the intracellular located Shc protein.

So, there seem to be at least two mechanisms by which analogues may have an increased mitogenic potency; either through a higher affinity for the IGF1R and/or by a slower dissociation after binding to the IR (see below).

In the human body, due to alternative splicing of exon 11 of the IR gene, two IR transcripts are generated, resulting in IR isoform A (IR-A; lacking exon 11) and in IR-B (including exon 11; [Belfiore et al. 2009](#)). The IR-A is expressed ubiquitously, but is predominantly expressed in the central nervous system, not only in haematopoietic cells but can also be substantially expressed in cancer tissues. IR-A is also expressed in the foetus where it may be activated by insulin and IGF2 for growth. IR-B is expressed predominantly in the liver and also in the muscle and adipose tissue; the major target tissues for the metabolic effects of insulin ([Moller et al. 1989](#), [Mosthaf et al. 1990](#)).

The common origin of insulin, IGFs and their receptors

The structural and functional similarities between insulin and IGFs provide strong evidence that the genes encoding for these ligands are derived from a common ancestor gene ([Chan & Steiner 2000](#)).

The major structural difference between insulin and the IGFs is that the IGFs are single-chain polypeptides containing an A-, B-, C- and D-domain, whereas the insulin molecule contains a separate A- and B-chain, generated by cleavage from a single-chain proinsulin, which are bound by two disulphide bridges ([Humbel 1990](#), [Sussenbach et al. 1992](#)). In primary sequence, human insulin, IGF1 and IGF2 share 50% amino acid identity in the A- and B- domains ([Rinderknecht & Humbel 1978](#)).

As insulin and the IGFs probably arose during evolution by gene duplication, there is the hypothesis that the IR and IGF1R were also created by gene duplication of a common precursor receptor molecule ([De Meyts et al. 2007](#)). Depending on which regions are being compared, the IR and IGF1R have sequence similarities varying from 41 to 84%. Both the IR and IGF1R are composed of two monomers, each comprising an extracellular alpha-subunit and a transmembrane beta-subunit which are linked by a disulphide bridge. They belong to the family of ligand-activated receptor kinases. Unlike other tyrosine receptor kinases, these receptors exist at the cell surface as homodimers composed of two identical alpha/beta monomers, or as heterodimers composed of two different receptor monomers ([Fig. 2](#)). Binding of a ligand to the extra-cellular alpha-subunit induces the receptors to undergo a conformational change enabling autophosphorylation of the intrinsic tyrosine kinase domains within the transmembrane beta-subunits, which is the first step in the intracellular signalling cascade.

The structures of the IR and IGF1R resemble each other to such an extent that insulin and IGFs can interact with each other's receptor, although with quite different affinities. The IR demonstrates high affinity binding to insulin ($\pm 10^{-10}$ M), tenfold lower affinity for IGF2 and a 50- to 100-fold lower affinity binding for IGF1 ([Blakesley et al. 1996](#)). In this respect, it has been shown that there are differences between the IR-A and IR-B; IGF2 having a higher affinity for IR-A than for IR-B. On the other hand, the IGF1R binds the IGFs with a high affinity (10^{-10} M), but binds insulin with a 100-fold lower affinity ([Blakesley et al. 1996](#)).

The conventional view regarding the actions of insulin, the IGFs and their receptors is that insulin and the IR mainly mediate metabolic responses, whereas IGFs and the IGF1R mediate growth-promoting effects ([LeRoith et al. 1995](#), [Siddle et al. 2001](#)). Nevertheless, evidence exists showing that insulin and IGF1 can mediate very similar responses ([Froesch et al. 1993](#)). IGF1 can exert acute metabolic effects like insulin, while insulin in turn, can substitute for IGF1 inducing

Figure 2 Schematic overview of the receptors in the insulin–IGF system. Both the IR and IGF1R are composed of two monomers, each comprising an extracellular alpha-subunit and a transmembrane beta-subunit which are linked by disulphide bridges (not shown). They exist at the cell surface as homodimers composed of two identical alpha/beta monomers, or as heterodimers composed of two different receptor monomers (so called hybrid receptors). Owing to alternative splicing of exon 11 of the IR gene, two insulin receptor (IR) isoforms exist; isoform A (IR-A; lacking exon 11) and in IR isoform B (IR-B; including exon 11). Reproduced, with permission, from [Brugts \(2009\)](#).

growth-promoting and differentiation enhancing activities ([Froesch *et al.* 1993](#)). In addition, also the IR and IGF1R share very similar intracellular signalling pathways ([Vigneri *et al.* 2009](#); [Fig. 3](#)). Moreover, studies have suggested that differences between IR-A and IR-B in terms of receptor activation and signalling may result in different functions of each IR isoform ([Belfiore *et al.* 2009](#)). Hence, the general consensus is that the IR and IGF1R besides their distinct functions, also have overlapping functions.

What determines growth-promoting and metabolic effect actions of Insulin and IGF1?

Structural differences of the beta-subunit and kinase domains of the IR and the IGF1R leading to differences in substrate interactions have been suggested to be partly responsible for insulin-IGF1 specificity ([Dupont & LeRoith 2001](#)). Moreover, the signal transduction by the receptors may not be limited to its activation at the cell surface. It has been suggested that the activated ligand–receptor complex, initially at the cell surface, is internalised into endosomes and that the lifetime of this complex within the endosomes might be an important factor in influencing the types of response produced by a particular receptor ([Bevan 2001](#)). However, the role of ligand internalisation and endosomal residence time

as mechanism of selective signalling has yet never been confirmed.

Although the IR and IGF1R have distinct and overlapping functions, it was recently suggested that *in vivo* specificity of insulin and IGF1 reflects at least in part the levels and timing of the expression of IRs and IGF1Rs in target tissues in combination with ligand concentration and availability ([Boucher *et al.* 2010](#)). As Boucher stated: IR and IGF1R act as identical portals for the regulation of gene expression, with differences between insulin and IGF1 effects due to a modulation of the amplitude of the signal created by the specific ligand–receptor interaction ([Boucher *et al.* 2010](#)).

Many details of insulin/IGF1 signalling remain to be clarified including the specific roles of hybrids ([Fig. 2](#)). In some tissues and cells where significant levels of both IRs and IGF1Rs are present, hybrids may be formed. These hybrids are heterodimeric receptors consisting of an IR-alpha/beta monomer and an IGF1R-alpha/beta monomer linked by disulphide bonds. Such hybrids are probably formed during normal post-translational processing of both receptors ([LeRoith *et al.* 1995](#)) and are widely expressed on normal tissues and often aberrantly expressed in cancer cells ([Pandini *et al.* 2002](#)). Although the precise biological role of these hybrids is still unclear, it has been suggested that hybrid receptors may play a role in the overlapping functions of insulin and IGF1 ([LeRoith](#)

Figure 3 Schematic overview of the IR and IGF1R signalling pathways. After activation of the receptors by insulin, insulin analogues or IGF1, autophosphorylation of kinase subunits leads to phosphorylation of adaptor proteins. The two main intracellular pathways, activated by both receptors, are the RAS-ERK-MAPK pathway and the PI3K/AKT pathway. Reproduced, with permission, from Brugts (2009).

et al. 1995). Binding of insulin to a hybrid receptor would result in autophosphorylation of its own beta-subunit which, through subsequent transphosphorylation, activates the beta-subunit of the IGF1R monomer, resulting in a growth-promoting signal (LeRoith *et al.* 1995). In the other way around, IGF1 binding to a hybrid receptor would result in autophosphorylation of its own beta-subunit which could activate the beta-subunit of the IR monomer by the same mechanism, thereby promoting metabolic actions (LeRoith *et al.* 1995). Although this could explain why insulin under certain circumstances may induce cellular proliferation and IGF1 may stimulate metabolic functions, functional studies have demonstrated that hybrid receptors behave more like IGF1Rs than IRs (Pandini *et al.* 2002).

The story of the first rapid-acting analogue insulin X10

The first rapid-acting insulin analogue was developed by replacing a histidine residue for the negatively charged aspartic acid at position B10 (insulin X10 or B10Asp; Hansen *et al.* 2011). The applied genetic modification led to disrupting the ability of insulin molecules to self-associate as hexamers. Therefore, after s.c. injection of insulin X10, a much higher and

earlier insulin peak was reached. Although clinical results were quite promising (Nielsen *et al.* 1995), further development of this analogue was discontinued when a dose-dependent increase in the occurrence of mammary tumours was observed in female Sprague-Dawley rats that were treated with supraphysiological doses of insulin X10 (Drejer 1992).

Insulin X10 has been shown to induce enhanced mitogenic effects due to the activation of both the IRs and the IGF1Rs (Milazzo *et al.* 1997). In most studies, its binding affinity for the IR has been found to be 200–400% higher than that of human insulin (Hansen *et al.* 2011). Although it has an identical ‘on-rate’, it has a much slower ‘off-rate’ from the IR than human insulin (Drejer *et al.* 1991). In addition, its affinity for the IGF1R has been found to be increased compared to human insulin, although fairly lower compared to IGF1 (Drejer *et al.* 1991). Both effects may have resulted in an increased mitogenicity of this insulin analogue.

Currently available insulin analogues

Currently, there are three rapid-acting insulin analogues (insulin lispro, insulin aspart and insulin glulisine) and two long-acting insulin analogues (insulin glargine and insulin detemir) commercially available.

Rapid-acting insulin analogues

Insulin lispro (LysB28, ProB290 human insulin) was the first clinically available insulin analogue (Fig. 1). In insulin lispro, the natural amino acid sequence of the B-chain is reversed at positions 28 and 29. As a consequence, there is a proline at position 28 and a lysine at position 29, like in IGF1 (Fig. 1). This amino acid sequence reduces the ability of insulin to self-associate, leading to a significantly higher absorption and elimination rate than that of human insulin after s.c. injection. For insulin aspart (AspB28), another strategy is used to reduce self-association (Fig. 1). Insulin aspart is obtained by changing proline at position B28 by the negatively charged amino acid aspartic acid. The pharmacokinetic and pharmacodynamic characteristics of insulin aspart resemble and are very similar to that of insulin lispro. For both insulin analogues, the affinities for the IR and the IGF1R have been reported to be similar to that of human insulin (LeRoith *et al.* 1995, Owens 2000, Vigneri *et al.* 2010).

Insulin glulisine has been developed by substituting aspartic acid at position B3 with lysine and lysine at position B29 with glutamine (Fig. 1). These changes also reduce the self-association when injected s.c. and thereby provide a quick biological availability after injection. Insulin glulisine has similar or slightly less binding affinity for the IR than human insulin. In addition, it has been suggested that IGF1R binding affinity is significantly lower than that of human insulin (Stammberger *et al.* 2006).

Long-acting insulin analogues

Two strategies to protract absorption by genetically modifying the insulin molecule have been tested clinically (Havelund *et al.* 2004).

The first principle was to shift the isoelectric point of the molecule towards neutrality to provide reduced solubility at physiological pH values. This principle has been used for insulin glargine (GlyA21, ArgB31, ArgB32 human insulin); so it is injected as an acid solution (pH 4.0) and forms a slowly absorbed precipitate in the neutral environment of the subcutis. This property means that it cannot be mixed with neutral formulations of other insulins. Insulin glargine has been produced by substituting asparagine with glycine in the A-chain at position 21 and by adding two arginine residues to the B-chain at position 30 (Fig. 1).

In vitro studies using rat fibroblast cells showed similar binding characteristics for insulin glargine and human insulin. The IGF1R binding affinity *in vitro* on cardiac myocytes has been reported to be stronger than that for human insulin (Owens 2000). However, native

insulin glargine and its metabolites M1 and M2 are released from the subcutaneous depot and M1 has been found to be the most abundant in the circulation and is therefore most likely to be the biologically active form (Kuerzel *et al.* 2003). This ultimately makes it even more difficult to interpret the *in vitro* results of insulin glargine.

Another strategy to protract absorption has been to acylate fatty acid species to the insulin molecule to allow reversible albumin–insulin binding in an attempt to protract the time action profile while retaining the practical advantages of a neutral liquid preparation. This strategy has been applied to insulin detemir (LysB29 (*N*-tetradecanoyl) des (B30) human insulin). In insulin detemir, the ϵ -amino group on the side-chain of lysine at position B29 is acylated, while threonine at position B30 is removed (Fig. 1). After s.c. injection, insulin detemir binds to albumin through this fatty acid chain (Owens 2000, Vigneri *et al.* 2010). This binding prolonged half-life in pigs to 14.3 h as compared to 10.5 h with NPH insulin and reduces the biological availability of free insulin detemir, making it more predictable in terms of the risk for hypoglycaemic episodes (Markussen *et al.* 1996, Vague *et al.* 2003, Havelund *et al.* 2004, Heise *et al.* 2004).

The affinity of insulin detemir for the IR has been found to be reduced, both *in vitro* and *in vivo* (Markussen *et al.* 1996). *In vivo*, fourfold higher doses were required to obtain a similar blood glucose-lowering effect as observed after regular insulin injections or injections with other insulin analogues. Therefore, the commercially available insulin detemir has a fourfold concentrated formulation in order to match the biological potency of human insulin (Owens & Bolli 2008).

The binding affinity of insulin detemir to IGF1R is as low as its affinity to the IR. The ratio between IR affinity and IGF1R affinity is therefore approximately the same as that for human insulin (Hastings KL (2005) US FDA Pharmacology and Toxicology Review of Insulin Detemir, 2003, www.accessdata.fda.gov/drugsatfda_docs/nda/2005/021-536_Levemir_pharmr.PDF).

Methods to assess the metabolic activity and mitogenic activity of insulin and insulin analogues

As previously discussed, insulin and insulin analogues may have different metabolic and mitogenic activities. In view of the lifelong exposure and large patient populations involved, insulin analogues with an increased mitogenic effect in comparison to human insulin may potentially constitute a major health

problem, since these analogues may possibly induce the growth of pre-existing neoplasms. This has been extensively evaluated *in vitro* and *in vivo* using animal models.

In vitro, the metabolic activity of insulin and insulin analogues is often assessed by glucose uptake of cells or by inhibition of lipolysis. *In vitro*, the mitogenic activity of insulin and insulin analogues is assessed by measuring cell proliferation and more recently also by methods assessing apoptosis and colony formation. The metabolic and mitogenic activities are assessed both in benign and malignant cells.

The biological response of a target cell to insulin analogues is not only determined by the concentration of an insulin analogue and the number of receptors, but also by the affinity of the IR and IGF1R for a particular insulin analogue. Both the IR and IGF1R – as previously discussed – may be involved in metabolism, proliferation and apoptosis. Besides the above-mentioned factors, the mitogenic response of a cell to insulin analogues may also be dependent on the dissociation rate of insulin analogues from the IR and IGF1R and also related to the duration of action of an insulin or an insulin analogue at the IR and IGF1R (see below).

All these cellular responses are downstream effects of processes that are, in fact, initiated by the stimulation of the IR or IGF1R. Therefore, receptor-mediated signalling is now widely used to characterise the post-receptor events in specific pathways known to play a role in cell growth and metabolism. However, the post-receptor intracellular signalling pathways for the IR and IGF1R are very complex, overlap, and are still an area of active research. In view of the high complexity of these signalling pathways, the evaluation of the total activity of an insulin analogue *in vitro* and *in vivo* is relevant. As discussed above, published literature has shown that a number of specific domains in the insulin molecule are important for binding to the IR and IGF1R. However, receptor binding is only a first step in the activation of specific pathways. At present, it is not possible to predict with any degree of certainty whether certain modifications in the structure of the insulin molecule may induce mitogenic effects *in vivo*.

The discussion about the enhanced proliferative activity of certain insulin analogues has mainly focused on increased signalling via the IGF1R, since prospective studies showed that individuals with circulating IGF1 levels in the high range of normal were at an increased risk of several common cancers (Juul 2003). However, as scientific knowledge evolves, it seems that not only effects on the IGF1R should be studied, but that interactions with the IR should also be taken into account.

In the following section, we will give a summary of the key findings of the *in vitro* and *in vivo* effects of insulin analogues that have been reported in the literature.

***In vitro/ex vivo* cell culture systems**

Insulin analogues have been tested for their affinity to the IR and IGF1R, their metabolic potency, their mitogenic potency and their dissociation rate from the IR.

In vitro, all at-present commercially available insulin analogues have lower affinities for the IR (Table 1). In contrast, insulin X10 has significantly higher affinity for the IR than human insulin.

All *in vitro* studies have documented that insulin X10 and the long-acting insulin analogue insulin glargine have a higher affinity for the IGF1R than human insulin (Table 2). Affinity for the IGF1R for all available insulin analogues, except insulin lispro, is significantly lower than that for IGF1 (Table 2).

Until now, insulin X10 is the only insulin analogue which has been shown to be metabolically more potent *in vitro* than human insulin (Table 3). All other currently commercially available insulin analogues show equal or less metabolic potency than human insulin (Table 3).

Both insulin X10, B31B32diArg and insulin glargine have higher mitogenic potencies *in vitro* than human insulin, while most commercially available insulins show less mitogenic activity *in vitro* than human insulin (Table 3). However, in all circumstances, the mitogenic potency of insulin analogues was significantly lower than that for IGF1. A recent paper compared the insulin analogues for mitogenic effects (cell proliferation and colony formation) in engineered cells expressing only one receptor type (IR-A, IR-B or IGF1R) in order to analyse the individual contribution of each receptor type (Sciaccia *et al.* 2010). They found that the long-acting insulin analogues insulin glargine and insulin detemir, through all three receptors, induced significantly more cellular proliferation than human insulin and short-acting insulin analogues. Interestingly, although only a significant effect was reported for insulin X10, all insulin analogues (except insulin glargine) induced more anchorage-independent cell growth (a transformation marker) through the IR-A than human insulin (Table 4).

As previously discussed, occupancy time at the IR has been correlated with mitogenic potential. Insulin X10, insulin aspart and B31B32diArg have a lower insulin off-rate than human insulin, while the other insulin analogues (like insulin glargine) show a higher IR off-rate (Table 1). Whether the same phenomenon

Table 1 Affinity and off-rate of commercially available insulin analogues for the insulin receptor

Analogue	Insulin receptor affinity (%)					Insulin receptor off-rate (%)
Human insulin	100 ^a	100 ^b	100 ^c	100 ^d	100 ^e	100 ^a
X10	205 ^a	817 ^b	271 ^c	148 ^d	245 ^e	14 ^f
Aspart	92 ^a	ND	ND	67 ^d	70 ^e	81 ^f
Lispro	84 ^g	ND	ND	93 ^d	91 ^e	100 ^g
Glargine	86 ^a	59 ^b	53 ^c	43 ^d	84 ^e	152 ^a
Glargine M1	ND	48 ^b	42 ^c	ND	ND	ND
Glargine M2	ND	53 ^b	52 ^c	ND	ND	ND
A21Gly	78 ^a	ND	ND	ND	ND	162 ^a
B31B32diArg	120 ^a	ND	ND	ND	ND	75 ^a
Detemir	46 ^h	ND	ND	44 ^d	3.5 ^e	204 ^a
Glulisine	ND	ND	68 ⁱ	66 ^d	94 ^e	Comparable to human insulin ^j
IGF1	ND	0.8 ^b	0.3 ^c	0.6 ^d	<0.5 ^e	ND

All values relative to human insulin. ND, not determined.

^aData from Kurtzhals *et al.* (2000).

^bData from Sommerfeld *et al.* (2010) (IR-A).

^cData from Sommerfeld *et al.* (2010) (IR-B).

^dData from Sciacca *et al.* (2010) (IR-A).

^eData from Sciacca *et al.* (2010) (IR-B).

^fData from Hansen (2008).

^gData from Slieker *et al.* (1997).

^hData from Markussen *et al.* (1996).

ⁱData from Stammberger *et al.* (2006).

^jData from Hennige *et al.* (2005).

plays a role for the IGF1R has not yet been studied and is therefore still unknown.

There is conflicting evidence as to whether or not the mitogenic effects of insulin and insulin analogues at high doses are mediated via the IR and/or the IGF1R. The ratio of IGF1Rs:IRs may play a role in the sensitivity of cells to insulin and insulin analogues *in vitro* (Zelobowska *et al.* 2009). Differences in this ratio between different cell lines may explain at least partly the observed differences in mitogenic potencies of insulin analogues; for example, an increased potency of insulin glargine is only seen in cells with a relatively high proportion of IGF1R (Table 5; Hansen 2008). However, also this is not a consistent finding. Staiger *et al.* (2007) failed to detect any increased mitogenicity of insulin glargine in MC7-cells, despite the fact that these cells express fourfold more IGF1Rs compared to IRs. They suggested that a certain ratio of IGF1Rs:IRs is needed before insulin glargine induces a mitogenic response (Hansen 2008). On the other hand, as mentioned before, Sciacca *et al.* (2010) compared the analogues for mitogenic effects in engineered cells expressing only one receptor type (IR-A, IR-B or IGF1R) and found that the mitogenic effects were also induced in cells not expressing the IGF1R.

Furthermore, IR and IGF1R expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF1R

system may function as a critical determinant of the mitogenic potency of insulin analogues (Eckardt *et al.* 2007).

Thus, the question remains whether all these *in vitro* observations may have any clinical implications. There are several uncertainties which make it very difficult to answer the question decisively (Hansen 2008). There seems to be a consensus that insulin and insulin

Table 2 Affinity of insulin, insulin analogues and insulin-like growth factor 1 (IGF1) for the IGF1 receptor

Analogue	IGF1 receptor affinity (%)			
Human insulin	100 ^a	100 ^b	100 ^c	100 ^d
X10	587 ^a	277 ^b	364 ^c	≈ 250 ^d
Aspart	81 ^a	ND	ND	≈ 100 ^d
Lispro	156 ^a	–	144 ^c	≈ 100 ^d
Glargine	641 ^a	457 ^b	ND	≈ 333 ^d
Glargine M1	ND	45 ^b	ND	ND
Glargine M2	–	68 ^b	ND	ND
A21Gly	42 ^a	ND	ND	ND
B31B32diArg	2049 ^a	ND	2275 ^c	ND
Detemir	16 ^a	ND	ND	≈ 333 ^d
Glulisine	ND	68 ^e	ND	≈ 100 ^d
IGF1	ND	32 472 ^b	75 428 ^c	> 44 843 ^d

All values relative to human insulin. ND, not determined.

^aData from Kurtzhals *et al.* (2000).

^bData from Sommerfeld *et al.* (2010).

^cData from Slieker *et al.* (1997).

^dData from Sciacca *et al.* (2010).

^eData from Stammberger *et al.* (2006).

Table 3 *In vitro* metabolic potency and mitogenic potency of commercially available insulin analogues

Analogue	Metabolic potency (%)		Mitogenic potency (%)		
Human insulin	100 ^a	100 ^b	100 ^a	100 ^b	100 ^c
X10	207 ^a	145 ^b	975 ^a	806 ^b	340 ^c
Aspart	101 ^a	ND	58 ^a	ND	ND
Lispro	82 ^a	ND	66 ^a	ND	89 ^c
Glargine	60 ^a	68 ^b	783 ^a	760 ^b	ND
Glargine M1	ND	32 ^b	ND	75 ^b	ND
Glargine M2	ND	52 ^b	ND	68 ^b	ND
A21Gly	88 ^a	ND	34 ^a	ND	ND
B31B32diArg	75 ^a	ND	2180 ^a	ND	ND
Detemir	27 ^a	ND	11 ^a	ND	ND
Glulisine	ND	ND	ND	ND	^d
IGF1	ND	0.02 ^b	ND	5568 ^b	5700 ^c

All values relative to human insulin. ND, not determined.

^aData from Kurtzhals *et al.* (2000).

^bData from Sommerfeld *et al.* (2010).

^cData from Slieker *et al.* (1997).

^dComparable to human insulin. Data from Stammberger *et al.* (2006).

analogues have growth-promoting activity (Sandow 2009). Moreover, insulin and insulin analogues have no carcinogenic activity (cell transformation) and are not a co-carcinogen when evaluated in special toxicology (Sandow 2009). Although Giordano *et al.* (1991) found that supraphysiological overexpression of IRs does favour ligand-dependent cell transformation *in vitro*, which underlines the potency of insulin to do so, it certainly does not mean that this occurs *in vivo*. On the other hand, one should keep in mind that increased mitogenic activity *per se* might increase the chances of mutations, thereby initiating tumour formation. However, circulating concentrations of injected insulin analogues are normally quite low compared to the levels needed to elicit a mitogenic response (Hansen 2008).

As previously discussed, it has been suggested that the IR and IGF1R act at identical portals to the regulation of gene expression, with differences between insulin and IGF1 effects due to a modulation of the signal created by the specific ligand–receptor interactions (Boucher *et al.* 2010). As a consequence, it is almost impossible in most *in vitro* cell lines to disentangle the individual contribution of each type of receptor to the final downstream event. In this respect, Sciacca *et al.* (2010) have made a great effort when they compared analogues for binding, post-receptor signalling and mitogenic effects in mouse embryonic fibroblasts expressing only the human IR-A, IR-B or the human IGF1R.

In our laboratory, we have developed three bioassays, specific for the human IR-A, the human IR-B and the human IGF1R, which quantify ligand-stimulated

phosphorylation of tyrosine residues within the specific receptors (Brugts *et al.* 2008, Varewijck *et al.* 2010). These assays use human embryonal kidney (HEK) cells which are stably transfected with cDNA of either the human IR-A, the human IR-B or the human IGF1R. The endpoint signal of the assays is 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 the IGF1R-specific bioassay, we previously confirmed that *in vitro*, at high concentrations, insulin glargine is more potent than human insulin, insulin detemir and insulin NPH in activating the IGF1R (Varewijck *et al.* 2010, 2012). However, since insulin glargine is metabolised after s.c. injection, we recently studied whether differences in IGF1R activation *in vitro*

Table 4 *In vitro* anchorage-independent cell growth (in terms of colony formation) in engineered cells expressing only one receptor type

Analogue	Mitogenic potency (%)		
	IR-A	IR-B	IGF1R
Human insulin	100	100	100
X10	173	100	118
Aspart	134	100	64
Lispro	112	89	91
Glargine	97	75	91
Detemir	127	94	91
Glulisine	103	94	82
IGF1	89	8.3	509

All values relative to human insulin. Data from Sciacca *et al.* (2010).

Table 5 Receptor expression in experimental cell lines and observed response after stimulation with insulin glargine (modified from Hansen (2008))

Cell lines	IGF1 receptor/ insulin receptor ratio	IGF1 receptor affinity	mitogenicity
SaosB10 ^a	Predominantly IGF1 receptor	↑	↑
HMEC ^b	Predominantly IGF1 receptor	↑	↑
Rat-1 over expressing IR ^c	Predominantly IGF1 receptor	ND	←
MCF-7 cells ^d	4:1	ND	← →
MCF-10 ^d	1:1	ND	← →
MCF-7-cells ^e	7:1	ND	← →
SKBR-3 cells ^e	1:1	ND	← →

ND, not determined.

^aData from Kurtzhals *et al.* (2000).

^bData from Kohn *et al.* (2007).

^cData from Berti *et al.* (1998).

^dData from Staiger *et al.* (2007).

^eData from Liefvendahl & Arnqvist (2008).

translate into different effects on IGF1 bioactivity in patients *in vivo* (Varewijck *et al.* 2012). We compared serum IGF1 bioactivity in participants of the Lanmet Study (Yki-Jarvinen *et al.* 2006), who were insulin-naïve type 2 diabetic patients treated with either high doses of insulin glargine or NPH insulin for 36 weeks. At baseline and after 36 weeks of insulin therapy, there was no difference in IGF1 bioactivity between the treatment groups. Moreover, circulating IGF1 bioactivity decreased significantly during insulin therapy, whereas (immunoreactive) serum total IGF1 concentrations remained unchanged. The data in our study do not support the idea that treatment with insulin glargine in type 2 diabetes leads to a stronger stimulation of the IGF1R than NPH insulin. Our findings are in line with a recently published paper in which, in an animal model of type 2 diabetes, no differences were demonstrated in the degree of colonic epithelial proliferation between animals treated with insulin glargine or NPH insulin (Nagel *et al.* 2010). Nevertheless, in that study, insulin treatment did result in a higher degree of colonic epithelial proliferation, thereby pointing towards the potential mitogenic properties of insulins, irrespective of the type of insulin (Nagel *et al.* 2010). However, it is important to underline once again that increased mitogenicity does not necessarily lead to increased carcinogenicity.

Given our findings and the affinity of insulin and insulin analogues to the IGF1R, it is unlikely that mitogenic responses to insulin and insulin analogues *in vivo* are exclusively mediated by the IGF1R. Currently, the possibility is discussed whether insulin and insulin analogues may increase the proliferation rate of already pre-existing subclinical tumours. These already transformed cells could be more sensitive to insulin and insulin analogues due to changes in receptor expression and/or distribution (i.e. the proportion between IRs and IGF1R). The higher responses after stimulation with insulin glargine in cells *in vitro* with an increased IGF1R expression, as shown in Table 5, are also in accordance with this latter possibility.

Moreover, recent data have elucidated some other molecular mechanisms by which insulin (or insulin analogues)-mediated stimulation of IRs may play a key role in the progression of human cancer (Frasca *et al.* 2008). IRs, like IGF1Rs, are overexpressed in many human malignancies (Frasca *et al.* 2008). Interestingly, especially the IR-A isoform is overexpressed in cancer and has the peculiar characteristic to bind not only insulin but also IGF2; and IGF1 as well, but to a lesser extent (Denley *et al.* 2004, Belfiore *et al.* 2009). In addition, as previously discussed, IRs contribute to the formation of hybrid receptors together with IGF1Rs. By binding to these hybrid receptors, insulin and insulin analogues may stimulate specific IGF1R signalling pathways (Belfiore *et al.* 2009). Overexpression of IR-A may play a key role in the development and progression of human cancers after starting treatment with insulin or insulin analogues.

When IRs are activated, they activate at least two different downstream signalling pathways, one involving MAP kinases and the other involving PI3 kinase (Belfiore *et al.* 2009; Fig. 3). Another pathway is the direct translocation of insulin (and insulin analogues?) and IRs to the cellular nucleus (Podlecki *et al.* 1987, Harada *et al.* 1999). Recently, it was reported that after stimulation, the IRs together with components of the MAP kinase signalling cascade were translocated into the nucleus, finally leading to the direct induction of transcription of two important insulin target genes (early growth response 1 and glucokinase; Nelson *et al.* 2011). Further studies will be required to reveal which signalling pathways are actually involved in the different effects of insulin and insulin analogues and whether in this respect (some) insulin analogues differ from human insulin.

In conclusion, the introduction of insulin and insulin analogues has rendered diabetes as a chronic disease with potentially serious long-term complications. Beside metabolic actions, insulin and insulin analogues

may induce mitogenic effects and thereby change the risk on malignancies. Although it has been suggested that especially insulin analogues with an increased affinity for the IGF1R (such as insulin glargine) are more mitogenic when tested *in vitro* in cells expressing a high proportion of IGF1R, the question remains whether this has any clinical implications. In addition, recent data suggest that insulin (or insulin analogues)-mediated stimulation of IRs may play a key role in the progression of human cancer.

More detailed information is required to elucidate the exact mechanisms as to how insulin and insulin analogues may activate the IR and IGF1R and how this activation is linked to mitogenesis and metabolism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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