Insulin and its analogues and their affinities for the IGF-I receptor

Aimee J. Varewijck & Joseph A.M.J.L. Janssen

Department of Internal Medicine, Division of Endocrinology, Erasmus MC, Rotterdam, The Netherlands

Corresponding author:
J.A.M.J.L. Janssen, MD PhD
Department of Internal Medicine,
Erasmus MC, Room D-443
's-Gravendijkwal 230, 3015 CE Rotterdam,
The Netherlands
Tel: +31-(0)10-7033975
Fax: +31-(0)10-7033268
E-mail: j.a.m.j.l.janssen@erasmusmc.nl

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Abstract

Insulin analogues have been developed in an attempt to achieve a more physiological replacement of insulin and thereby a better glycemic control. However, structural modification of the insulin molecule may result in altered binding affinities and activities to the IGF-I receptor (IGF-IR). As a consequence insulin analogues may theoretically have an increased mitogenic action compared to human insulin. In view of the life long 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 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 IGF-IR (such as insulin glargine) are more mitogenic when tested in vitro in cells expressing a high proportion of IGF-IR, 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 how insulin analogues may activate the IR and IGF-IR and how this activation may be linked to mitogenesis.
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 eighties 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 (Figure 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 biological 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, 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 "insulin receptor binding agonists" (FDA 2008). 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 insulin receptor and the IGF-I receptor

Structural modification of the insulin molecule may result in altered binding affinities and activities to the IR and/or the IGF-I receptor (IGF-IR). 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 (Slieker, et al. 1997). Especially modifications at positions the B26-B30 region i.e. the C-terminus of the B-chain, do not seem to significantly influence insulin binding to the IR (Kurtzhals, et al. 2000; Nakagawa and Tager 1987) (Figure 1). However, this region is important for at least 2 reasons. First, these aminoacids 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 IGF-I and as a consequence have an increased affinity for the IGF-IR ((Kurtzhals et al. 2000; Slieker et al. 1997). Proline at position 28 and lysine at position 29 is the natural sequence which is present in the B- chain of human insulin (Figure
1) The number and position of basic or acid residues in this region seems very important for IGF-IR binding (Sliker et al. 1997). Substitution of position B28 with basic residues increases the relative affinity to the IGF-IR approximately 1.5-2-fold (ArgB28ProB29> OrnB28ProB29=LysB28ProB29) (Sliker et al. 1997). In contrast, substitution with acidic residues (CyaB28ProB29=GluB28ProB29>AspB28ProB29) reduces relative affinity for the IGF-IR approximately 2-fold. Combination of aspartic acid substitution at B10 with a modification in position B28-29 (e.g. AspB10, LysB28, ProB29) increases the affinity for the IGF-IR 2-fold. Addition of arginine residues at position B31-32 (B31B32diArg) increases the affinity for the IGF-IR 10-fold compared to human insulin, while the same addition in combination with aspartic acid substitution at B10 increases the affinity to the IGF-IR even 28-fold. In general, analogues with substantially increased IGF-IR 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 IGF-IRs (Pollak 2008).

On the other hand, Hansen et al. have shown that an increased mitogenic potency of insulin analogues may not only be due to an increased affinity for the IGF-IR but may also result from slow ligand dissociation from the IR (Hansen, et al. 1996). 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 IGF-IR 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 isoform B (IR-B) (including exon 11) (Belfiore, et al. 2009). The IR-A is expressed ubiquitously, but is
predominantly expressed in the central nervous system, hematopoietic cells but can also be
substantially expressed in cancer tissues. IR-A is also expressed in the foetus where it maybe
activated by insulin and IGF-II for growth. IR-B is expressed predominantly in the liver and
also in muscle and adipose tissue; the major target tissues for the metabolic effects of insulin

The common origin of insulin, insulin-like growth factors 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 SJ
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 disulfide bridges (Humbel 1990; Sussenbach, et al. 1992). In primary sequence,
human insulin, IGF-I and IGF-II share 50% amino acid identity in the A- and B- domains
(Rinderknecht and Humbel 1978).

As insulin and the IGFs probably arose during evolution by gene duplication, there is the
hypothesis that the IR and IGF-IR were also created by gene duplication of a common
precursor receptor molecule (De Meyts 2007). Depending on which regions are being
compared, the IR and IGF-IR have sequence similarities varying from 41-84%. Both the IR
and IGF-IR are composed of two monomers, each comprising an extracellular alpha-subunit
and a transmembranic beta-subunit which are linked by a disulfide 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 (Figure 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 transmembranic beta-subunits which is the first step in the intracellular signalling cascade.

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

The conventional view regarding actions of insulin, the IGFs and their receptors is that insulin and the IR mainly mediate metabolic responses, whereas IGFs and the IGF-IR mediate growth promoting effects (Siddle, et al. 2001) (LeRoith, et al. 1995). Nevertheless, evidence exists showing that insulin and IGF-I can mediate very similar responses (Froesh, et al. 1993). IGF-I can exert acute metabolic effects like insulin, while insulin in turn, can substitute for IGF-I inducing growth-promoting and differentiation enhancing activities (Froesh et al. 1993). In addition, also the IR and IGF-IR share very similar intracellular signalling pathways (Vigneri, et al. 2009) (Figure 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). So the general consensus is that the IR and IGF-IR besides their distinct functions, also have overlapping functions.
What determines growth-promoting and metabolic effects actions of Insulin and IGF-I?

Structural differences of the beta-subunit and kinase domains of the IR and the IGF-IR leading to differences in substrate interactions have been suggested to be partly responsible for insulin-IGF-I specificity (Dupont and 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 internalization and endosomal residence time as mechanism of selective signalling has yet never been confirmed.

Although the IR and IGF-IR have distinct and overlapping functions, it was recently suggested that in vivo specificity of insulin and IGF-I reflects at least in part the levels and timing of the expression of IRs and IGF-IRs in target tissues in combination with ligand concentration and availability (Boucher, et al. 2010). As Boucher stated: IR and IGF-IR act as identical portals for the regulation of gene expression, with differences between insulin and IGF-I 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/IGF-I signalling remain to be clarified including the specific roles of hybrids (Figure 2). In some tissues and cells where significant levels of both IRs and IGF-IRs are present, hybrids may be formed. These hybrids are heterodimeric receptors consisting of an IR alpha/beta monomer and an IGF-IR alpha/beta monomer linked by disulfide 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 IGF-I (LeRoith 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 IGF-IR monomer resulting in a growth-promoting signal (LeRoith et al. 1995). In the other way around, IGF-I 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 IGF-I may stimulate metabolic functions, functional studies have demonstrated that hybrid receptors behave more like IGF-IRs 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 disrupt the ability of insulin molecules to self-associate as hexamers. Therefore, after subcutaneous 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 supraphysiologic 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 IGF-IRs (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 IGF-IR has been found to be increased compared to human insulin although fairly lower compared to IGF-I (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, insulin glulisine) and two long-acting insulin analogues (insulin glargine and insulin detemir) commercially available.

A] Rapid-acting insulin analogues

Insulin lispro (LysB28, ProB290 human insulin) was the first clinically available insulin analogue (Figure 1). In insulin lispro the natural amino acid sequence of the B chain is reversed at position 28 and 29. As a consequence there is a proline at position 28 and a lysine at position 29, like in IGF-I (Figure 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 subcutaneous injection. For insulin aspart (AspB28) another strategy is used to reduce self-association (Figure 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 IGF-IR have been reported to be similar to that of human insulin. (LeRoith et al. 1995; Owens D 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 (Figure 1). These changes also reduce the self-
association when injected subcutaneously 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 IGF-IR binding affinity is significantly
lower than of human insulin (Stammberger, et al. 2006)

B] 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 (Figure 1).

In vitro studies using rat fibroblasts cells showed similar binding characteristics for insulin
glargine and human insulin. The IGF-IR binding affinity in vitro on cardiac myocytes has
been reported to be stronger than that for human insulin (Owens D 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 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 (Figure 1). After subcutaneous injection, insulin detemir binds to albumin through this fatty acid chain (Owens D 2000; Vigneri et al. 2010). This binding prolonged half-life in pigs to 14.3 hrs compared with 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 (Havelund et al. 2004; Heise, et al. 2004; Markussen, et al. 1996; Vague, et al. 2003).

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, 4-fold 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 4-fold concentrated formulation in order to match biological potency of human insulin (Owens and Bolli 2008).

Binding affinity of insulin detemir to IGF-IR is as low as its affinity to the IR. The ratio between IR affinity/IGF-IR affinity is therefore approximately the same as for human insulin (Hastings 2005).

Methods to assess 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 life long 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
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. Metabolic and mitogenic activity 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 the IGF-IR for a particular insulin analogue. Both the IR and IGF-IR -as previously discussed- may be involved in metabolism, proliferation and apoptosis. Besides the above mentioned factors, mitogenic response of a cell to insulin analogues may also be dependent on the dissociation rate of insulin analogues from the IR and IGF-IR and also related to the duration of action of an insulin or an insulin analogue at the IR and the IGF-IR (see below).

All these cellular responses are downstream effects of processes that are, in fact, initiated by the stimulation of the IR or IGF-IR. Therefore, receptor-mediated signalling is now widely used to characterize 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 the IGF-IR 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 the IGF-IR. However, receptor binding is only a first step in the activation of specific pathways. At present, it 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 enhanced proliferative activity of certain insulin analogues has mainly focused on increased signalling via the IGF-IR, since prospective studies showed that individuals with circulating IGF-I levels in the high range of normal were at an increased risk for several common cancers (Juul 2003). However, as scientific knowledge evolves, it seems that not only effects on the IGF-IR 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 the IGF-IR, 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 IGF-IR than human insulin (Table 2). Affinity for the IGF-IR for all available insulin analogues is significantly lower than that for IGF-I (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 mitogenic potency of insulin analogues was significantly lower than that for IGF-I. A recent paper compared insulin analogues for mitogenic effects (cell proliferation and colony formation) in engineered cells expressing only one receptor type (IR-A, IR-B or IGF-IR) in order to analyze the individual contribution of each receptor type (Sciacca, 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 plays a role for the IGF-IR 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 IGF-IR. The ratio of IGF-IRs/ 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 which a relatively high proportion of IGF-IR (Table 5) (Hansen 2008). However, also this is not a consistent finding. Staiger et al. failed to detect any increased mitogenicity of insulin glargine in MC7-cells,
despite the fact that these cells express 4-fold more IGF-IRs compared to IRs (Staiger, et al. 2007). They suggested that a certain ratio of IGF-IRs/IRs is needed before insulin glargine induces a mitogenic response (Hansen 2008). On the other hand, as mentioned before, Sciacca et al. compared analogues for mitogenic effects in engineered cells expressing only one receptor type (IR-A, IR-B or IGF-IR) and found that mitogenic effects were also induced in cells not expressing the IGF-IR (Sciacca et al. 2010).

Furthermore, IR and IGF-IR expression may vary in a tissue-specific manner and inter-individual differences in the levels of proteins of the IGF-IR system may function as a critical determinant of the mitogenic potency of insulin analogues (Eckardt, et al. 2007).

Thus the question remains whether all these in vitro observations may have any clinical implications. There are several uncertainties which makes it very difficult to answer the question decisively (Hansen 2008). There seems consensus that insulin and insulin 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 Giorgino et al. found that supraphysiological overexpression of IRs does favour ligand-dependent cell transformation in vitro, which underlines the potency of insulin to do so (Giorgino, et al. 1991), 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 may increase the chances of mutations thereby initiating tumor 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 IGF-IR act at identical portals to the regulation of gene expression, with differences between insulin and IGF-1 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 receptor to the final downstream event. In this respect, Sciacca et al. 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 IGF-IR (Sciacca et al. 2010).

In our laboratory we have developed three bioassays, specific for the human IR-A, the human IR-B and the human IGF-IR, 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 IGF-IR. 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 IGF-IR 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 IGF-IR (Varewijck et al. 2010; Varewijck, et al. 2012). However, since insulin glargine is metabolized after subcutaneous injection, we recently studied whether differences in IGF-IR activation in vitro translate into different effects on IGF-I bioactivity in patients in vivo (Varewijck et al. 2012). We compared serum IGF-I bioactivity in participants of the Lanmet Study (Yki-Jarvinen, et al. 2006), who were insulin naive 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 IGF-I bioactivity between the treatment groups. Moreover, circulating IGF-I bioactivity decreased significantly during insulin therapy, whereas (immunoreactive) serum total IGF-I 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 IGF-IR 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 more that increased mitogenicity does not necessarily lead to increased carcinogenicity.

Given our findings and the affinity of insulin and insulin analogues to the IGF-IR it is unlikely that mitogenic responses to insulin and insulin analogues in vivo are exclusively mediated by the IGF-IR. 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 IGF-IR). The higher responses after stimulation with insulin glargine in cells in vitro with an increased IGF-IR 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 IGF-IRs, 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 IGF-II and also IGF-I but to a lesser extend (Belfiore et al. 2009; Denley, et al. 2004). In addition, as previously discussed, IRs contribute to the formation of hybrid receptors together with IGF-IRs. By binding to these hybrid receptors insulin and insulin analogues may stimulate specific IGF-IR signalling pathways (Belfiore et al. 2009). Overexpression of IR-A may play a key
role in the formation 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) (Figure
3). Another pathway is the direct translocation of insulin (and insulin analogues?) and IRs to
the cellular nucleus (Harada, et al. 1999; Podlecki, et al. 1987). Recently, it was reported that
after stimulation, the IR together with components of the MAP kinase signalling cascade were
translocated into the nucleus finally leading to 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 into 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 IGF-IR (such as insulin glargine) are more mitogenic when tested in
vitro in cells expressing a high proportion of IGF-IR, 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 how insulin and
insulin analogues may activate the IR and IGF-IR and how this activation is linked to
mitogesis and metabolism.
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Duality of interest statement

The authors declare that there is no duality of interest associated with this manuscript.
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Legends

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 6 cysteines, so 3 disulphide bridges are formed: 2 between the A and B chains (between A7 & B7, and A20 & B19), and one within the A chain (A6 & 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 is 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 position 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.

Figure 2

Schematic overview of the receptors in the insulin-IGF system. Both the IR and IGF-IR are composed of two monomers, each comprising an extracellular alpha-subunit and a transmembranic beta-subunit which are linked by disulfide 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). Due 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). With permission retrieved and modified from the thesis entitled ‘IGF-I bioactivity in Aging, Health and Disease’ by M.P. Brugts (ISBN 9789085599159).

Figure 3

Schematic overview of the IR and IGF-IR signalling pathways. After activation of the receptors by insulin, insulin analogues or IGF-I, 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 PI3/AKT pathway. With permission retrieved and modified from the thesis entitled ‘IGF-I bioactivity in Aging, Health and Disease’ by M.P. Brugts (ISBN 9789085599159).
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Figure 2
Figure 3
Table 1. Affinity and off rate of commercially available insulin analogues for the insulin receptor

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Insulin Receptor affinity (%)</th>
<th>Insulin Receptor off rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin</td>
<td>100&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>X10</td>
<td>205&lt;sup&gt;1&lt;/sup&gt;</td>
<td>817&lt;sup&gt;4a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspart</td>
<td>92&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Lispro</td>
<td>84&lt;sup&gt;2&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Glargine</td>
<td>86&lt;sup&gt;1&lt;/sup&gt;</td>
<td>59&lt;sup&gt;4a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glargine M1</td>
<td>ND</td>
<td>48&lt;sup&gt;4a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glargine M2</td>
<td>ND</td>
<td>53&lt;sup&gt;4a&lt;/sup&gt;</td>
</tr>
<tr>
<td>A21Gly</td>
<td>78&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>B31B32diArg</td>
<td>120&lt;sup&gt;1&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Detemir</td>
<td>46&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Glulisine</td>
<td>ND</td>
<td>68&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-I</td>
<td>ND</td>
<td>0.8&lt;sup&gt;4a&lt;/sup&gt;</td>
</tr>
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</table>

<sup>1</sup> Data from Kurtzhals et al.
<sup>2</sup> Data from Slieker et al.
<sup>3</sup> Data from Markussen et al.
<sup>4a</sup> Data from Sommerfeld et al. (Sommerfeld, et al. 2010) (IR-A)
<sup>4b</sup> Data from Sommerfeld et al. (IR-B)
<sup>5</sup> Data from Hansen et al. (2008)
<sup>6</sup> Data from Hennige et al. (Hennige, et al. 2005)
<sup>7</sup> Data from Stammberger et al.
<sup>8a</sup> Data from Sciacca et al. (IR-A)
<sup>8b</sup> Data from Sciacca et al. (IR-B)
<sup>9</sup> ND= not determined

All values relative to human insulin.
Table 2. Affinity of insulin, insulin analogues and IGF-I for the IGF-I receptor

<table>
<thead>
<tr>
<th>Analogue</th>
<th>IGF-I Receptor affinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human insulin</td>
<td>100&lt;sup&gt;1&lt;/sup&gt; 100&lt;sup&gt;2&lt;/sup&gt; 100&lt;sup&gt;4&lt;/sup&gt; 100&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>X10</td>
<td>587&lt;sup&gt;1&lt;/sup&gt; 277&lt;sup&gt;2&lt;/sup&gt; 364&lt;sup&gt;4&lt;/sup&gt; ≈250&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aspart</td>
<td>81&lt;sup&gt;1&lt;/sup&gt; ND ND ≈100&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lispro</td>
<td>156&lt;sup&gt;1&lt;/sup&gt; - 144&lt;sup&gt;4&lt;/sup&gt; ≈100&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glargine</td>
<td>641&lt;sup&gt;1&lt;/sup&gt; 457&lt;sup&gt;2&lt;/sup&gt; ND ≈333&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glargine M1</td>
<td>ND 45&lt;sup&gt;2&lt;/sup&gt; ND ND</td>
</tr>
<tr>
<td>Glargine M2</td>
<td>- 68&lt;sup&gt;2&lt;/sup&gt; ND ND</td>
</tr>
<tr>
<td>A21Gly</td>
<td>42&lt;sup&gt;1&lt;/sup&gt; ND ND ND</td>
</tr>
<tr>
<td>B31B32diArg</td>
<td>2049&lt;sup&gt;1&lt;/sup&gt; ND 2275&lt;sup&gt;4&lt;/sup&gt; ND</td>
</tr>
<tr>
<td>Detemir</td>
<td>16&lt;sup&gt;1&lt;/sup&gt; ND ND ≈333&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glulisine</td>
<td>ND 68&lt;sup&gt;3&lt;/sup&gt; ND ≈100&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>IGF-I</td>
<td>ND 32472&lt;sup&gt;2&lt;/sup&gt; 75428&lt;sup&gt;4&lt;/sup&gt; &gt;44843&lt;sup&gt;5&lt;/sup&gt;</td>
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All values relative to human insulin
1. Data from Kurtzhals et al.
2. Data from Sommerfeld et al.
3. Data from Stammberger et al.
4. Data from Slicker et al.
5. Data from Sciacca et al. (IGF-IR)
6. ND= not determined
Table 3. In vitro metabolic potency and mitogenic potency of commercially available insulin analogues

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Metabolic Potency (%)</th>
<th>Mitogenic Potency (%)</th>
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<tbody>
<tr>
<td>Human insulin</td>
<td>100(^1) 100(^2)</td>
<td>100(^1) 100(^2)</td>
</tr>
<tr>
<td>X10</td>
<td>207(^1) 145(^2)</td>
<td>ND</td>
</tr>
<tr>
<td>Aspart</td>
<td>101(^1) ND</td>
<td>58(^1) 806(^2)</td>
</tr>
<tr>
<td>Lispro</td>
<td>82(^1) ND</td>
<td>66(^1) ND</td>
</tr>
<tr>
<td>Glargine</td>
<td>60(^1) 68(^2)</td>
<td>783(^1) 760(^2)</td>
</tr>
<tr>
<td>Glargine M1</td>
<td>ND 32(^2)</td>
<td>ND 75(^2) ND</td>
</tr>
<tr>
<td>Glargine M2</td>
<td>ND 52(^2)</td>
<td>ND 68(^2) ND</td>
</tr>
<tr>
<td>A21Gly</td>
<td>88(^1) ND</td>
<td>34(^1) ND</td>
</tr>
<tr>
<td>B31B32diArg</td>
<td>75(^1) ND</td>
<td>2180(^1) ND</td>
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<tr>
<td>Detemir</td>
<td>27(^1) ND</td>
<td>11(^1) ND</td>
</tr>
<tr>
<td>Glulisine</td>
<td>ND ND ND</td>
<td>ND ND Comparable to human insulin(^4)</td>
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<tr>
<td>IGF-I</td>
<td>ND 0.02(^2)</td>
<td>ND 5568(^2) 5700(^3)</td>
</tr>
</tbody>
</table>

3 All values relative to human insulin
1 Data from Kurtzhals et al.
2 Data from Sommerfeld et al.
3 Data from Slieker et al.
4 Data from Stammberger et al.
5 ND = not determined
Table 4. In vitro anchorage-independent cell growth (in terms of colony formation) in engineered cells expressing only one receptor type

<table>
<thead>
<tr>
<th>Analogue</th>
<th>IR-A</th>
<th>IR-B</th>
<th>IGF-IR</th>
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<tr>
<td>Human insulin</td>
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<td>100</td>
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<td>Aspart</td>
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<td>89</td>
<td>91</td>
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<tr>
<td>Glargine</td>
<td>97</td>
<td>75</td>
<td>91</td>
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<tr>
<td>Detemir</td>
<td>127</td>
<td>94</td>
<td>91</td>
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<tr>
<td>Glulisine</td>
<td>103</td>
<td>94</td>
<td>82</td>
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<tr>
<td>IGF-I</td>
<td>89</td>
<td>8.3</td>
<td>509</td>
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All values relative to human insulin
Data from Sciacca et al.
Table 5. Receptor expression in experimental cell lines and observed response after stimulation with insulin glargine (modified from Hansen 2008)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IGF-I receptor/insulin receptor ratio</th>
<th>IGF-I receptor affinity</th>
<th>mitogenicity</th>
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<tbody>
<tr>
<td>SaosB10</td>
<td>Predominantly IGF-I receptor</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>HMEC</td>
<td>Predominantly IGF-I receptor</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Rat-1 over expressing IR</td>
<td>Predominantly IGF-I receptor</td>
<td>ND</td>
<td>←</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>4:1</td>
<td>ND</td>
<td>←</td>
</tr>
<tr>
<td>MCF-10</td>
<td>1:1</td>
<td>ND</td>
<td>←</td>
</tr>
<tr>
<td>MCF-7-cells</td>
<td>7:1</td>
<td>ND</td>
<td>←</td>
</tr>
<tr>
<td>SKBR-3 cells</td>
<td>1:1</td>
<td>ND</td>
<td>←</td>
</tr>
</tbody>
</table>

1. Data from Kurzhals et al.
2. Data from Kohn et al.
3. Data from Berti et al.
4. Data from Staiger et al.
5. Data from Liefvendahl et al.
6. ND= not determined