Analysis of signaling pathways related to cell proliferation stimulated by insulin analogs in human mammary epithelial cell lines

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Abstract

Insulin and insulin analogs stimulate proliferation of human mammary epithelial cells. We identified and analyzed the signaling pathways related to cell proliferation induced by regular insulin and by four insulin analogs presently approved for therapeutical use. Benign and malignant mammary cell lines showing different insulin receptor (IR) and IGF-I receptor (IGF-IR) expression patterns were studied. Cell proliferation was studied by crystal violet staining (BrdU-FACS analysis). Activation of insulin and IGF signaling pathways was studied by analysis of the phosphorylation status of IGF-IR and of key signaling proteins of the phosphoinositide 3-kinase (PI3K)/Akt and MAP kinase pathways, by the use of specific PI3K and MAP kinase inhibitors, and by silencing of IR and IGF-IR. Lantus stimulated the growth of MCF7 cells, which show high IGF-IR/IR ratio, significantly at 0.3 nmol/l, while regular insulin (Actrapid and bovine insulin) and other insulin analogs (Novorapid, Humalog, and Levemir) stimulated cell growth at 1.5–15 nmol/l concentrations. No difference between Lantus and the other insulin analogs was observed regarding growth stimulation of MCF10A cells showing low IGF-IR/IR ratio. Growth stimulation of MCF7 cells by Lantus was mainly due to strong activation of the IGF-IR and the MAP kinase pathway. Regular insulin and other insulin analogs tested activated mainly the IR and the PI3K/Akt pathway. We conclude that unlike regular insulin and other insulin analogs, Lantus strongly activates the IGF-IR and the MAP kinase pathway in MCF7 cells and is a strong mitogen for cells characterized by a high-IGF-IR/IR ratio.

Introduction

Insulin and IGFs bind to and stimulate their cognate receptors, the insulin receptor (IR) and the IGF-I receptor (IGF-IR). IR is predominantly related to metabolic control but may also transduce mitogenic stimuli (Sliker et al. 1997, Shymko et al. 1999), and IGF-IR is mainly related to stimulation of proliferation and inhibition of apoptosis (Pollak et al. 2004). Artificial insulin molecules or insulin analogs, aimed to replace regular insulin for better control of blood glucose in diabetes have been developed by modifying the backbone of the insulin molecule (Sliker et al. 1997, Eckardt & Eckel 2008). Insulin modified in the B-chain (Sliker et al. 1997) may possess altered binding properties to the IR or acquire higher affinity to the IGF-IR and thus can exert a stronger proliferative effect than regular insulin on cells expressing these receptors. The increased proliferative potency of insulin analogs with enhanced time of occupancy of the IR was explained by the mitogenic signaling properties of the IR (Hansen et al. 1996). Insulin analogs with increased affinity toward IGF-IR elicited

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strong proliferative response in various tumor cell lines (Kurtzhals et al. 2000) as well as in normal human mammary epithelial cells (HMEC; Slieker et al. 1997). These observations suggest potentially increased cancer risk and raised safety concerns about the long-term clinical use of insulin analogs.

Several reports confirmed the high-mitogenic potency of some insulin analogs. B10Asp, generated by replacing glutamic acid at position B10 by aspartic acid, caused increased focus formation in MCF10A cells, a non-malignant human breast cell line, and was more potent than insulin in stimulating colony formation in MCF7 cells, a human breast cancer cell line (Milazzo et al. 1997). Furthermore, administration of B10Asp, to female Sprague–Dawley rats led to strong increase in the incidence of mammary tumors (Dideriksen et al. 1992). B31ArgB32Arg insulin stimulated HMEC proliferation tenfold over regular insulin (Slieker et al. 1997). Insulin glargine (A21Gly-B31ArgB32Arg), an insulin analog with high affinity for the IGF-IR, had up to eightfold higher mitogenic potency on Saos/B10 osteosarcoma cells compared with regular insulin (Kurtzhals et al. 2000). Furthermore, Eckardt et al. (2007) reported that insulin glargine was mitogenic in primary cultures of normal fibroblasts and smooth muscle cells expressing high levels of IGF-IR and insulin receptor substrate-1 (IRS-1) proteins.

IR and IGF-IR show high expression levels in epithelial cells of the normal human mammary gland and particularly IGF-IR has been shown to be significantly overexpressed in breast cancer and in malignant mammary cell lines (Papa et al. 1990, Milazzo et al. 1992, Schnarr et al. 2000, Sachdev & Yee 2001, Frasca et al. 2008). Thus, the mammary gland may represent a sensitive target for growth stimulation by insulin analogs. Reports on the proliferative effect of insulin analogs on mammary epithelial cells in vitro (Slieker et al. 1997, Gammeltoft et al. 1999, Staiger et al. 2007, Lievendahl & Arnqvist 2008) showed varying results regarding the mitogenic potency of the compounds. However, results from different studies are difficult to compare because data have been obtained under different experimental conditions and evaluated by different procedures. Moreover, all these studies are limited in terms of the number of cell lines and insulin analogs tested.

The aim of the present study was to perform a comparative detailed analysis of the proliferative potency of regular insulin and four insulin analogs that are presently approved for therapeutic use and to characterize the signaling pathways activated by them in human mammary epithelial cell lines. We were especially interested in studying the contribution of IGF-IR and IR signaling into the mitogenic potency of insulin and insulin analogs and in the potential correlation of the proliferative effect with the IGF-IR/IR ratio expressed in HMECs. Seven-cell lines expressing different levels of IR, IGF-IR, and the downstream effector IRS-1 (Mayer et al. 2008) were studied. We show that only one insulin analog (Lantus) in comparison with regular insulin had significantly stronger mitogenic properties on MCF7 breast cancer cells characterized by a high IGF-IR/IR ratio and this effect was mainly due to the activation of the IGF-IR and mitogen-activated protein kinase (MAPK) signaling pathway. In MCF10A cells characterized by a low IGF-IR/IR ratio, Lantus induced proliferation was not different from that induced by regular insulin.

### Materials and methods

#### Materials

Crystalline bovine insulin was from Sigma. Recombinant regular human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), insulin aspart (Novorapid, Novo Nordisk), insulin detemir (Levemir, Novo Nordisk), insulin glargine (Lantus, Sanofi Aventis, Paris, France), insulin lispro (Humalog, Lilly, Indianapolis, IN, USA) were obtained from the pharmacy. Recombinant human IGF-I was from R&D Systems (Wiesbaden, Germany), Wortmannin and U0126 from Calbiochem (EMD Biosciences, Darmstadt, Germany). Rabbit anti-IGF-IR, rabbit anti-IRβ, rabbit anti-IRβ, and rabbit anti-Akt-1/2 were from Santa Cruz (Heidelberg, Germany). The following antibodies were from Cell Signaling Technologies (NEB, Frankfurt, Germany): rabbit monoclonal anti-IR-β, mouse anti-phospho-Akt (Ser-473), rabbit anti-phospho-glycogen synthase kinase 3α/β (GSK3α/β) (Ser-21/-9), mouse anti-phospho-extracellular-signal regulated kinase 1/2 (Erk1/2), rabbit anti-Erk1/2, mouse anti-phospho-p38, and rabbit anti-p38. Mouse anti-GSK3α/β was from BioSource (Solingen, Germany). Mouse anti-phospho Tyr 4G10, mouse anti-IGF-IRβ, and rabbit normal IgG were from Upstate (Millipore, Eschwege, Germany). The FITC BrdU Flow Kit was from Becton Dickinson (Heidelberg, Germany).

#### Cell culture

MCF7 (from DSMZ, Braunschweig, Germany), MDA-MB231, and HCC1937 cells were maintained in phenol red-free DMEM (4.5 g/l glucose; Invitrogen) containing 10% FCS (Biochrom, Berlin, Germany) and penicillin/streptomycin (100 U/ml per 100 μg/ml respectively). BT474, T47D, and ZR75-1 (from ATCC/LGC, Wesel, Germany) were maintained in...
RPMI-1640 (PAA; Cölbe, Germany). MCF10A cells (ATCC) were maintained in mammary epithelial growth medium with supplements (MEGM; Provitro, Berlin, Germany). Three days before the experiment, cells were grown in medium containing 10% dextran-coated charcoal-treated FCS (DCC-FCS; Medunjanin et al. 2005).

**Proliferation assay**

Cells were plated at $10^4$ cells/well in 96-well plates. After 24 h, cells were starved for additional 24 h in medium containing 2% DCC-FCS except MCF10A cells which were starved in MEGM without insulin. Thereafter, MCF7 cells were stimulated with insulin or insulin analogs every 24 h for total of 72 h, MCF10A cells were stimulated for 48 h. It should be mentioned that Levemir is not an insulin analog in the classical sense but regular human insulin modified by myristylation at B29 position which increases its affinity for albumin and thus prevents rapid elimination (Morales 2007). Levemir will be listed in the following as insulin analog for easier description. Controls remained untreated or were treated with the carrier solution of the various preparations obtained by the inactivation of insulin and insulin analogs by boiling for 30 min under reductive conditions, followed by centrifugation. At the end of incubation time, cells were washed with PBS, fixed with 3% paraformaldehyde in PBS, and stained with 1% crystal violet dissolved in 10% ethanol. Optical density was read at 595 nm using a plate reader (Multiscan MX, Thermo, Dreieich, Germany).

**Immunoblot analysis**

Cell lysates were prepared as described (Medunjanin et al. 2005) and subjected to SDS-PAGE and immunoblotting (Grisouard et al. 2007). Phosphorylated proteins and total proteins were detected with appropriate primary antibodies. Protein bands were visualized with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies using the ECLplus system (GE Healthcare, Freiburg, Germany). Stripping of the membrane was performed as described (Grisouard et al. 2007).

**Immunoprecipitation**

Seven hundred and fifty micrograms of cellular protein in 750 µl lysis buffer (Medunjanin et al. 2005) was incubated overnight with 2 µg polyclonal anti-IGF-IRβ antibody and 25 µl protein-A-agarose beads (Roche Diagnostics). The specificity of immunoprecipitation was determined by incubation with non-immune IgG instead of anti-IGF-IRβ antibody. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting procedures.

**siRNA transfection**

MCF7 cells were transfected with 25 nmol/l control siRNA (Dharmacon, Boulder, CO, USA, cat# D-001210-02-20), 25 nmol/l siRNA targeting IGF-IR (cat# L-003012-00) or 25 nmol/l siRNA targeting IR (cat# L-003014-00) using Oligofectamine (Invitrogen). To study activation of signaling pathways, cells were starved in serum-free medium for 24 h after 48 h of transfection. Thereafter, cells were stimulated with 15 nmol/l Actrapid, 15 nmol/l Lantus or 15 nmol/l IGF-I for 10 min. Subsequently, cell lysate was prepared and subjected to immunoblotting. To study proliferation, cells were starved in medium containing 2% DCC-FCS for 24 h after 48 h of transfection and then stimulated with 15 nmol/l Actrapid, 15 nmol/l Lantus or 15 nmol/l IGF-I for 72 h. Cells were fixed and processed as described above.

**Quantitative real-time RT-PCR**

Total RNA was isolated using the RNeasy kit (Qiagen). Two hundred and fifty nanograms of RNA was reverse transcribed using ‘RevertAid™ H Minus First Strand cDNA Synthesis’ kit (Fermentas, St Leon-Rot, Germany). cDNA was purified with the QIAquick PCR purification kit (Qiagen). Quantitative real-time PCR was performed using the iQ SYBR Green supermix (Bio-Rad) on PTC-200 Peltier Thermal Cycler (MJ Research, Miami, FL, USA) using 50 ng of cDNA and 0.5 µmol/l of following primers: GAPDHfwd: 5’-AGCCACATCGCTCAGACA-3’ GAPDHref: 5’-GCCCAATACGACCAAATCC-3’ CCND1fwd: 5’-CCTGTCCTACTACCGCCTCA-3’ CCND1rev: 5’-TGGGGTCCATGTTCTGCT-3’

**Statistical analysis**

Immunoblots were quantitatively evaluated using ImageJ software (NIH, Bethesda, MD, USA). Signal intensities of phospho-proteins were normalized to the corresponding protein signals. Data presented are mean ± s.d. of at least three independent experiments; data were analyzed by t-test. Proliferation assays (dose–response curves) were evaluated using a four-parametric log-logistic model (Ritz & Streibig 2005) and t-tests in a multiple contrast testing approach (Hothorn et al. 2008). P values below 0.05 were considered statistically significant.
Results

MCF10A and MCF7 exhibit the highest insulin-induced proliferation among the panel of cell lines tested

We screened a panel of benign (MCF10A; Soule et al. 1990) and malignant (MCF7 (Brooks et al. 1973; BT474 (Lasfargues et al. 1978); T47D (Keydar et al. 1979); ZR75-1 (Engel et al. 1978); MDA-MB231 (Cailleau et al. 1978); HCC1937 (Tomlinson et al. 1998)) mammary epithelial cell lines showing different expression patterns of IR and IGF-IR (Mayer et al. 2008) for proliferative response to regular insulin. We treated the cells with 1.5 μmol/l of regular insulin (Actrapid or bovine insulin) and performed proliferation assays. Control cells remained untreated. Most of the cell lines analyzed showed only low proliferative response toward insulin treatment (Table 1). Only MCF10A (benign mammary epithelial cell line) and MCF7 (malignant mammary epithelial cell line) responded strongly to insulin treatment and showed maximum growth response of 1.7- and 2.2-fold respectively, of the control. These two cell lines were utilized for testing the mitogenic potency of insulin analogs.

Lantus shows strong proliferative potency in MCF7 cells

The insulin analogs tested are dissolved or suspended in carrier solutions whose composition is not known. None of the carrier solutions of the different insulin preparations gained as described in ‘Materials and methods’, showed any proliferative effect on MCF10A and MCF7 cells. MCF10A and MCF7 cells were treated with concentrations ranging from 1.5 nmol/l to 1.5 μmol/l of insulin or insulin analog and proliferative response was measured. In MCF10A cells, a significant proliferative response to regular insulin and insulin analogs was observed at 15 nmol/l (Fig. 1A). Treatment with higher concentrations did not lead to further significant increase in cell proliferation.

MCF7 cells showed a slight positive growth response to 1.5 nmol/l Actrapid. Treatment with increasing Actrapid concentrations resulted in gradual increase in proliferation which reached maximum of 2.2-fold of control with 1.5 μmol/l Actrapid (Fig. 1B). The dose–response curve achieved with Humalog was similar to that obtained with regular insulin. Novorapid tended to induce stronger proliferation than regular insulin at all concentrations studied (Fig. 1B), however, growth stimulation was not statistically significantly different from regular insulin. Levmir was found to be a weaker mitogen than regular insulin at concentrations from 15 nmol/l to 1.5 μmol/l. Treatment of MCF7 cells with 1.5 μmol/l–1.5 μmol/l Lantus resulted in 1.6- to 3.1-fold higher proliferation compared with untreated cells respectively. Statistical analysis showed that growth stimulation by ≥1.5 Lantus was significantly stronger in comparison with Actrapid and bovine insulin. The strong proliferative effect of 1.5 nmol/l Lantus prompted us to test Lantus at concentrations from 1.5 to 150 pmol/l. MCF7 cells treated with 150 pmol/l Lantus showed a significantly stronger growth response compared with 150 pmol/l Actrapid (Fig. 1C). The dose–response model showed initial statistically significant growth response (given by the ED10 estimation) of MCF7 cells to Actrapid at 5.2 nmol/l (95% confidence intervals, lower: $-1.0571 \times 10^{-9}$ mol/l, upper: $1.143 \times 10^{-8}$ mol/l) and to Lantus at 0.31 nmol/l (95% confidence intervals: lower: $-7.8890 \times 10^{-11}$ mol/l, upper: $7.059 \times 10^{-10}$ mol/l) concentrations.

The results obtained with the colorimetric proliferation assay were confirmed by BrdU-FACS analysis. Lantus treatment resulted in strong increase in the percentage of S-phase cells (Supplementary Fig. 1, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/).

Lantus strongly activates PI3K/Akt pathway in MCF10A and MCF7 cells

To understand the molecular basis of the high-proliferative ability of Lantus observed in MCF7 but not in MCF10A cells, we examined the activation of phosphoinositide 3-kinase (PI3K)/Akt and MAPK signaling pathways. In all experiments, IGF-I was

Table 1 Screening of mammary epithelial cell lines for proliferative response to insulin

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Proliferation (fold of control)</th>
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<tbody>
<tr>
<td>MCF10A</td>
<td>1.7 ± 0.20</td>
</tr>
<tr>
<td>BT474</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td>MCF7</td>
<td>2.2 ± 0.28</td>
</tr>
<tr>
<td>T47D</td>
<td>1.1 ± 0.04</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>1.4 ± 0.20</td>
</tr>
<tr>
<td>MDA-MB231</td>
<td>1.3 ± 0.12</td>
</tr>
<tr>
<td>HCC1937</td>
<td>1.1 ± 0.05</td>
</tr>
</tbody>
</table>

Cells were starved for 24 h in medium containing 2% charcoal-stripped serum and then were treated with 1.5 μmol/l regular insulin for 48 h (MCF10A) or for 72 h (all other cell lines). Thereafter, cells were fixed with 3% paraformaldehyde in PBS, stained with crystal violet and absorbance was read at 595 nm. Fold of control was calculated by comparing the proliferation achieved in the absence or presence of insulin. Data show mean ± s.d., n=20. The two cell lines that show the strongest proliferative response are given in bold.
included as positive control. As PI3K pathway activation leads to phosphorylation of Akt and its downstream target, GSK3α/β (Torres-Arzayus et al. 2004), the phosphorylation status of these two proteins was considered as an indicator for the strength of PI3K/Akt pathway activation. Cells were treated with insulin, insulin analogs or IGF-I for 10 min and phosphorylation levels of Akt and GSK3α/β were compared with the levels observed in untreated cells. In MCF10A cells, treatment with regular insulin or IGF-I resulted in strong phosphorylation of Akt and GSK3α/β, IGF-I showing an even stronger effect than insulin (Fig. 2A and C). Treatment with Humalog, Novorapid or Levemir resulted in Akt and GSK3α/β phosphorylation levels similar to insulin induced phosphorylation levels. By contrast, Lantus treatment

**Figure 1** Proliferative ability of insulin and insulin analogs in MCF10A and MCF7 cells. MCF10A cells (A) were starved for 24 h and then were treated with increasing concentrations of insulin and insulin analogs for 48 h. Thereafter, cells were fixed and stained as described in 'Materials and methods' section. Proliferation is shown as fold of control (untreated cells). Values are the mean ± S.D. of n=16. Asterisks show statistically significant increase in proliferation compared with untreated controls. t-test, P<0.05 was considered significant. MCF7 cells (B and C) were starved for 24 h and then were treated with increasing concentrations of insulin and insulin analogs for 72 h. Values are the mean ± S.D. of n=20. Asterisks show statistically significant increase in proliferation compared with regular human insulin (*) or bovine insulin (**). t-test, P<0.05 was considered significant.
in comparison with Actrapid resulted in significantly stronger phosphorylation of Akt but not of GSK3α/β.

In MCF7 cells, short-term treatment with regular insulin or IGF-I also led to strong phosphorylation of Akt and GSK3α/β, IGF-I being a stronger activator than insulin (Fig. 2B and D). Humalog and Novorapid were equipotent to insulin in stimulating Akt and GSK3α/β phosphorylation. Treatment of MCF7 cells with Levemir resulted in significantly weaker phosphorylation of Akt and GSK3α/β in comparison with Actrapid. MCF7 cells treated with Lantus showed strong phosphorylation of Akt and GSK3α/β which was significantly higher than that obtained with Actrapid. Interestingly, the ratio of phosphorylated to total proteins of Akt and GSK3α/β was higher in MCF10A cells than in MCF7 cells.

**Lantus strongly activates MAPK pathway in MCF7 cells**

Activation of the MAPK pathway implies the strong phosphorylation of Erk1 and Erk2 proteins (Ahmad et al. 2004). Therefore, activation of the MAPK pathway by insulin analogs was assessed by studying the phosphorylation status of Erk1/2. In MCF10A cells, treatment with regular insulin and particularly IGF-I led to strong increase in phosphorylation of Erk1/2 compared with untreated cells (Fig. 3A). Treatment with Humalog, Novorapid, Levemir, and Lantus, resulted in similar levels of Erk1/2 phosphorylation as observed with regular insulin.

In MCF7 cells, insulin or IGF-I treatment resulted in an increase in the phosphorylation of Erk1/2 compared with untreated cells (Fig. 3B). As in MCF10A cells, IGF-I treatment of MCF7 cells led to very strong Erk1/2 phosphorylation. Humalog and Novorapid induced similar effects as regular insulin. Levemir was a weak inducer of Erk1/2 phosphorylation in MCF7 cells. Treatment of MCF7 cells with Lantus resulted in a very strong induction of Erk1/2 phosphorylation which was significantly stronger compared with regular insulin. Surprisingly, the ratio of phosphorylated to total Erk1/2 proteins was higher in MCF10A cells compared with MCF7 cells.

As insulin analogs induced phosphorylation of Erk1/2 in MCF7 cells, it was interesting to study also their effect on p38, another MAPK pathway effector (Zarubin & Han 2005). Treatment with insulin or insulin analogs did not result in alterations of the phosphorylation level of p38 (data not shown).
Lantus induced proliferation in MCF7 cells is mainly due to MAPK pathway activation

Activation of both PI3K and MAPK pathways can induce proliferation in MCF7 cells (Lu & Campisi 1992, Jhun et al. 1994, Dufourny et al. 1997). As Lantus strongly phosphorylated and thus activated key signaling proteins of both pathways (Figs 2 and 3), we were interested in studying the role of either of the pathways in the proliferative ability of Lantus. To study this, we chose pathway-specific inhibitors, Wortmannin for PI3K and U0126 for MAPK pathway. We treated MCF7 cells with 150 nmol/l Actrapid or 150 nmol/l Lantus in combination with 200 nmol/l Wortmannin or 10 μmol/l U0126 and performed proliferation assays. Figure 4A and B shows complete inhibition of Akt phosphorylation by Wortmannin and complete inhibition of Erk1/2 phosphorylation by U0126, detected by western blotting. In agreement with results shown in Fig. 1B, Lantus-induced MCF7 cell proliferation was higher than that induced by Actrapid in the absence of inhibitors. In the presence of Wortmannin, we observed only slight inhibition of Actrapid or Lantus induced MCF7 cell proliferation. By contrast, MAPK pathway inhibition by U0126 led to complete inhibition of MCF7 cell proliferation induced by Actrapid or Lantus (Fig. 4C). It may be concluded that MAPK pathway is the major pathway involved in Actrapid or Lantus induced proliferation.

IGF-IR knockdown decreases Lantus-induced PI3K and MAPK activation in MCF7 cells

To understand whether strong activation of PI3K and MAPK pathway by Lantus in MCF7 cells is a result of the strong activation of IR or rather of activation of IGF-IR, we studied the effect of Lantus on phosphorylation of signaling proteins of PI3K and MAPK pathways under IR or IGF-IR knockdown conditions. Figure 5A demonstrates significant downregulation of IGF-IR and IR after transfection of IGF-IR siRNA and IR siRNA respectively. Cells transfected with control siRNA, 10 min treatment with 15 nmol/l Actrapid, Lantus or IGF-I resulted in weaker phosphorylation of Akt and Erk1/2 compared with that observed for non-transfected cells (Figs 2 and 3), which may be a consequence of the transfection procedure. IGF-IR knockdown significantly decreased IGF-I ability to phosphorylate Akt (Fig. 5B) and Erk1/2 (Fig. 5C). Furthermore, IGF-IR knockdown significantly compromised Lantus ability to induce Akt (Fig. 5B) and Erk1/2 (Fig. 5C) phosphorylation, underlining the importance of IGF-IR in Lantus mediated PI3K and MAPK pathway activation.

Cells transfected with IR siRNA became less responsive to Actrapid as reflected in decreased Akt and Erk1/2 phosphorylation compared with that observed for non-targeting conditions (Fig. 5B and C). However, IR knockdown did not diminish the ability of Lantus to stimulate PI3K and MAPK pathway activation. These data show that IGF-IR plays an important role in the strong effect of Lantus on Akt and Erk1/2 phosphorylation. Interestingly, treatment of IR siRNA transfected cells with IGF-I, and to a lower extent with Lantus, resulted in Akt and Erk1/2 phosphorylation which was higher compared to non-targeted control.
higher than the level observed after transfection with control siRNA. These data suggest a ‘quenching’ of IR function by IGF-IR in cells characterized by high IGF-IR/IR ratios.

IGF-IR knockdown results in similar proliferative potency of Lantus and Actrapid in MCF7 cells

To determine the role of IGF-IR in the proliferative ability of Lantus, we carried out proliferation assays in MCF7 cells after IGF-IR knockdown. IGF-IR silencing markedly decreased the ability of both Lantus and IGF-I to stimulate proliferation of MCF7 cells (Fig. 5D). We observed that under IGF-IR knockdown conditions, the proliferative abilities of Lantus and Actrapid were similar leading to the suggestion that in cells with low IGF-IR/IR ratio Lantus is equi-potent to Actrapid.

Lantus stimulates phosphorylation of IGF-IR in MCF7 cells

To corroborate the involvement of IGF-IR in the mitogenic ability of Lantus, we studied the phosphorylation status of IGF-IR after immunoprecipitation from lysates of MCF7 cells treated with 15 nmol/l Actrapid, Lantus or IGF-I (Fig. 6). IGF-I strongly stimulated phosphorylation of its cognate receptor, IGF-IR. Treatment with Actrapid did not increase IGF-IR phosphorylation over the basal phosphorylation level observed in untreated cells. However, treatment with Lantus led to marked increase in IGF-IR phosphorylation which demonstrates the activation of IGF-IR by Lantus.

Cyclin D1, often used as proliferation marker, is an IGF-responsive gene (Furlanetto et al. 1994, Sachdev & Yee 2001). Cyclin D1 was strongly induced by Lantus (Supplementary Fig. 2, which can be viewed online at http://erc.endocrinology-journals.org/supplemental/) which further confirms that Lantus activates IGF-IR and the IGF signaling pathway. The weaker induction of cyclin D1 by Actrapid may be due to the low-mitogenic effect of the IR.

Discussion

This study, in agreement with others, shows that insulin is a mitogen for mammary epithelial cells. However, out of seven mammary epithelial cell lines tested only MCF7 and MCF10A cells showed a clear proliferative response to insulin treatment (Table 1). ZR75-1 cells do not express IRS-1 (Gliozzo et al. 1998) and BT474 show only small IRS-1 levels (Mayer et al. 2008) which may explain the little or lack of growth response to insulin. However, other breast cancer lines tested, though clearly expressing IR, IGF-IR and IRS-1 (Mayer et al. 2008), did not show growth response to insulin, IGF-I and insulin analogues. Although the reason for this is unclear, it may be hypothesized that signaling pathways are corrupted in these long-term established cell lines.

Modification of the insulin molecule at B26–B30 positions may result in stronger affinity for the IGF-IR resulting in increased mitogenic potency of the compound (Slieker et al. 1997). Three insulin analogs investigated Lantus (A21GlyB31ArgB32Arg), Novorapid (B28Asp), and Humalog (B28LysB29Pro) show sequence modifications at this region. In our experiments on MCF7 cells, Novorapid showed slightly stronger proliferative effects than regular insulin at > 15 nmol/l concentration. This may be explained by its prolonged interaction with the IR (Hansen et al. 1996). Out of all
insulin analogs studied, only Lantus that has been described to have six- to eight-fold higher IGF-IR affinity than regular insulin (Kurtzhals et al. 2000), showed significantly stronger proliferative potency compared with regular insulin on MCF7 cells. These results are at variance with those of Staiger et al. (2007) and Liefvendahl & Arqvist (2008) who did not see this effect with their approach.

Interestingly, MCF7 cells are characterized by high IGF-IR/IR ratios (7:1 (Liefvendahl et al. 2008) to 4:1 (Gammeltoft et al. 1999)). Strong mitogenicity of insulin glargine was also observed in malignant SaOS-2 cells (IGF-IR/IR ratio ~ 10:1 (Liefvendahl et al. 2008)) and SaOS/B10 cells (IGF-IR/IR ratio > 30:1 (Kurtzhals et al. 2000)) as well as in benign HMEC (high IGF-IR/IR ratio (Slieker et al. 1997)) and primary normal human fibroblasts and smooth muscle cells expressing high levels of IGF-IR and IRS-1 proteins (Eckardt et al. 2007). By contrast, benign MCF10A cells which show similar growth response to normal insulin and Lantus have a low IGF-IR/IR ratio (0.8:1; Gammeltoft et al. 1999). Also in benign rat-1-fibroblasts (Berti et al. 1998), human coronary artery cells (Staiger et al. 2005), muscle cells (Bähr et al. 1997), and human skeletal muscle cells (Ciaraldi et al. 2001), mitogenic potency of insulin glargine was similar to that of regular insulin. The IGF-IR/IR ratio of these cells has not been reported.

To understand the molecular basis of the mitogenic effect of regular insulin and Lantus in MCF7 cells, we studied the activation of insulin and IGF-I signaling pathways by investigating the phosphorylation state of key signaling molecules. Insulin preferentially activates PI3K pathway and to some extent MAPK pathway (White & Kahn 1994). Interestingly, unlike insulin that induced strong phosphorylation of Akt, GSK3α/β, and weak phosphorylation of Erk1/2, Lantus induced not only strong phosphorylation of Akt, GSK3α/β but also of Erk1/2 suggesting that Lantus activates both PI3K and MAPK pathways in MCF7 cells. The use of specific inhibitors for each pathway permitted to identify MAPK pathway as the
major pathway involved in the stimulation of MCF7 proliferation by Lantus (Fig. 4). GSK3α/β, as a component of the insulin/IGF signaling pathway, is not considered to be strongly correlated with cell proliferation but rather with metabolic processes triggered by insulin. Therefore, the reason for the strong phosphorylation of GSK3α/β by Lantus and IGF-I requires further clarification.

Surprisingly, treatment of MCF10A cells with Lantus caused significantly stronger phosphorylation of Akt compared with regular insulin, whereas no significant difference between Lantus and regular insulin was observed with respect to phosphorylation of GSK3α/β. The reason of this result is unclear. It may be hypothesized that in MCF10A cells, the signal downstream of Akt level diverges and GSK3α/β is not the main target of Akt. Conversely, it can be argued that signal transduction from Akt to GSK3α/β is so strong in MCF10A cells that differences between the two treatments cannot be detected by immunoblotting. However, the complexity of the findings requires a more detailed analysis. We did not study which signaling pathway is responsible for proliferation in MCF10A cells. Nevertheless, the similar proliferative ability of Lantus and Actrapid and similar activation of MAPK pathway but not PI3K pathway by Lantus and Actrapid indicate that like in MCF7 cells, in MCF10A cells the MAPK pathway is the major proliferation-linked pathway too.

Another interesting observation was that the ratio of phosphorylated to total proteins of signaling molecules (Akt, GSK3α/β, and Erk1/2) in MCF10A cells treated with regular insulin or insulin analogue or IGF-I was much higher than the ratios observed in similarly treated MCF7 cells. This suggests that benign MCF10A cells respond much stronger to stimulation by insulin, insulin analogs, and IGF-I than malignant MCF7 cells. However, the proliferation levels observed in MCF7-treated cells were significantly higher than in MCF10A-treated cells. Although more work is necessary to unravel the reason for these discrepancies, care should be taken to present high-phosphorylation levels of key signaling molecules as primary readout for proliferative potency of any compound.

As activation of both IR and IGF-IR results in cell proliferation (Milazzo et al. 1992, Dufourny et al. 1997, Shymko et al. 1999, Pollak et al. 2004, Samani et al. 2007), we analyzed the role of these receptors in MCF7 cell proliferation induced by Lantus. Our experiments clearly demonstrate that under IGF-IR knockdown conditions, Lantus behaves like regular insulin, the phosphorylation levels of Akt and Erk1/2 proteins achieved by Lantus being now similar to those obtained with Actrapid. On the contrary, knockdown of IR compromises insulin ability to phosphorylate Akt and Erk1/2 but has no negative effect on the ability of Lantus to phosphorylate Akt and Erk1/2. The observation that under IGF-IR knockdown conditions, cell proliferation induced by Lantus drops to the levels induced by Actrapid provides additional conclusive evidence for the involvement of IGF-IR in Lantus mediated cell proliferation. In addition, immunoprecipitation analysis showed that Lantus-treatment phosphorylates IGF-IR much stronger than Actrapid treatment and confirmed the role of IGF-IR in Lantus action. Finally, Lantus induced the cell-cycle protein cyclin D1 which is known to be an IGF-responsive gene (Furlanetto et al. 1994, Sachdev & Yee 2001).

In summary, our results show that regular insulin activates mainly IR and the PI3K/Akt pathway. IGF-I, through the IGF-IR, activates both PI3K/Akt and MAPK pathways. Lantus stimulates both IR and IGF-IR resulting in activation of both PI3K and MAPK pathways at similar high levels. Activation of the IGF-IR by Lantus explains its strong mitogenic effect on MCF7 cells.

Long-term treatment of healthy mice and rats with high doses of insulin glargine did not result in a significant increase in the incidence of mammary tumors compared with regular insulin (Stammberger et al. 2002). Nevertheless, the strong proliferative effect of Lantus on human MCF7 breast cancer cells expressing high levels of IGF-IR may be regarded as a
matter of concern as high IGF-IR levels are observed in epithelial cells of normal human mammary gland and even higher levels are frequently observed in well and moderately differentiated primary breast cancer (Schnarr et al. 2000 and references therein). As described above, Lantus concentrations in the picomolar range had a significantly increased proliferative effect on MCF7 cells compared with regular insulin. Only few data are available on serum concentrations of Lantus. Free serum insulin levels of healthy volunteers and of patients with type-1 diabetes who received Lantus injections for 1–11 days varied between 70 and 90 pmol/l, with peak levels of about 220 pmol/l after injection (Heise et al. 2002, 2004, Gerich et al. 2006, Becker et al. 2008). Data on serum levels in patients long-term treated with Lantus are not available. Tissue levels have not been reported.

Presently, there is an increasing tendency to treat diabetic patients with insulin analogs including Lantus. It is important to note that women suffering from type-2 diabetes have slightly increased risk to develop breast cancer (Larsson et al. 2007). In the light of our data presented, it may be wise to use Lantus with caution in diabetics with breast cancer or with potentially increased breast cancer risk, since there may be a possibility for stimulation of proliferation of undetected cancer cells.

Declaration of interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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