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RESEARCH ARTICLE

Insulin analogues display IGF-I-like mitogenic and anti-apoptotic activities in cultured cancer cells

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Abstract

Background Insulin analogues are widely used in the treatment of diabetes mellitus. Some insulin analogues were reported to display atypical activities *in vitro* that resemble those of insulin-like growth factor-I (IGF-I). The aim of this study was to investigate whether two long-acting insulin analogues [glargine (Lantus, Sanofi Aventis, Germany) and detemir (Levemir, Novo Nordisk, Denmark)] and two short-acting analogues [lispro (Humalog, Eli Lilly, Indianapolis, USA) and aspart (Novolog, Novo Nordisk, Denmark)] exhibit IGF-I-like activities on cultured cancer cells in comparison with IGF-I and regular human insulin.

Methods HCT-116 (colorectal cancer), PC-3 (prostate cancer) and MCF-7 (breast adenocarcinoma) cell lines were treated with insulin, IGF-I or insulin analogues, and proliferation and protection from apoptosis were measured by cell counting and fluorescent-activated cell sorter (FACS) analysis, respectively. In addition, Western blots were used to identify signalling molecules activated by the analogues.

Results Glargine, detemir and lispro had proliferative effects that resemble IGF-I action. Insulin, however, did not stimulate cellular proliferation. In addition, glargine and detemir displayed an IGF-I-like anti-apoptotic activity. Glargine, like insulin and IGF-I, induced phosphorylation of both ERK and AKT, suggesting that the analogue is able to stimulate both the ras-raf-mitogen-activated protein kinase (MAPK) and PI3K-AKT pathways. Furthermore, glargine induced both insulin receptor (IR) and IGF-IR phosphorylation.

Conclusions Glargine, detemir and lispro, unlike regular insulin, exhibit *in vitro* proliferative and anti-apoptotic activities in a number of cancer cell lines. These actions resemble some of the effects of IGF-I, a growth factor involved in cancer initiation and progression. Insulin had no increased IGF-I activity. The specific receptor/s involved in mediating analogues actions remains to be identified. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords insulin; insulin-like growth factor-I; insulin analogues; glargine; detemir; lispro

Introduction

Exogenous insulin administration is the main treatment available for type 1 diabetes patients and is one of the main therapeutic approaches for type 2 diabetes patients. Development of new and more effective insulin analogues is a key issue in the field of modern diabetes therapy. In recent years, several

insulin analogues have been developed using DNA recombinant techniques. Specifically, point modifications were inserted into the human insulin primary structure, resulting in an array of analogues that exhibit different absorption rates into the circulation and, consequently, different onset and duration of action [1,2]. Accordingly, insulin analogues are divided into two subgroups known as long-acting and short-acting analogues. Although the clinical pharmacology of the insulin analogues in terms of glycemic control have been amply published [3], the long-term effects of most of these analogues have not yet been systematically evaluated [4,5].

Most of the structural modifications of insulin analogues are introduced into the *C*-terminus of the native insulin β-chain because this region does not participate in mediating insulin binding to the insulin receptor (IR). However, it is becoming increasingly clear that a potential safety risk of insulin analogues, including a putative carcinogenic activity, stems from the fact that these modifications, in addition to altering their absorption kinetics, may enhance their affinity for the related insulin-like growth factor-I (IGF-I) receptor (IGF-IR) [6–8]. IGF-IR is a transmembrane receptor that belongs to the receptor tyrosine kinase superfamily and that is responsible for mediating most of the biological activities of IGF-I and IGF-II. In addition to mediating physiological growth and metabolic actions, the IGF-IR has important roles in tumour biology [9–11].

The question whether insulin is capable of inducing or promoting mitogenic effects through its cognate receptor or via the IGF-IR has been a controversial issue for many years [12–14]. In fact, recent studies showed that some of the newly developed insulin analogues exhibit an increased affinity for the IGF-IR and display atypical activities, such as inhibition of apoptosis and abnormal post-receptor signalling compared with native insulin [15,16]. In view of the well-established role of IGF-IR in tumour development, and given that insulin-like peptides are most probably unable to elicit malignant transformation *per se*, it is very important to investigate the potential mitogenic activity of insulin analogues, including IGF-IR activation, in the context of already transformed cells. Therefore, the aim of this study was to investigate whether two long-acting insulin analogues [glargine (Lantus, Sanofi Aventis, Germany) and detemir (Levemir, Novo Nordisk, Denmark)], and two short-acting analogues [lispro (Humalog, Eli Lilly, Indianapolis, USA) and aspart (Novolog, Novo Nordisk, Denmark)] exhibit IGF-I-like activities in cultured cancer cells in comparison with IGF-I and regular human insulin. The activities that were evaluated included enhancement of cancer cell proliferation and protection from apoptosis. In addition, our study was aimed at identifying signal transduction molecules through which the proliferative activities of the insulin analogues are mediated.

Materials and methods

Cell cultures and treatment

The human colorectal cancer cell line HCT-116 and prostate cancer cell line PC-3 were grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 50 μg/mL gentamicin sulphate and 2.5 μg/mL fungizone. HCT-116 cells were provided by Dr Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD, USA) [17] and PC-3 cells were provided by Dr Gil Ast (Tel Aviv University, Tel Aviv, Israel). The human breast adenocarcinoma cell line MCF-7 was maintained in DMEM medium with 10% FBS and antibiotics. Cells were treated with the following hormones and analogues: regular insulin (Humalin R, Lilly France S.A.S., Fegersheim, France); glargine (Lantus, Sanofi Aventis); detemir (Levemir, Novo Nordisk); lispro (Humalog, Eli Lilly); aspart (Novolog, Novo Nordisk) and IGF-I (CytoLab, Rehovot, Israel). Stock concentration of native insulin and insulin analogues was 100 IU/mL and stock concentration of IGF-I was 1 mg/mL.

Proliferation assays

HCT-116, PC-3 and MCF-7 cells were plated in six-well plates (2×10^5 cells/well) in complete medium. After 24 h, the medium of HCT-116 and PC-3 cells was changed to serum-reduced medium [containing 1% FBS and 1% bovine serum albumin (BSA)], whereas the medium of MCF-7 cells was changed to serum-free medium. Hormones and analogues at the indicated concentrations were replenished every 24 h. Cells were counted daily by trypsin treatment and manual counting with a haemocytometer. At least four fields were counted at each time point and ligand dose. The short-acting analogues lispro and aspart were assayed only in HCT-116 cells using the MTT protocol. Briefly, 48 h after addition of the analogue, MTT (5 mg/mL) was added to the wells and incubated for 1 h. Supernatants were collected and absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm.

Apoptosis measurements

Cells were plated in six-well plates (5×10^5 cells/well) in complete medium. Upon reaching confluence, cells were serum-starved for 24 h, after which they were incubated in the presence of the hormones (20 nM, 100 nM) for the indicated time intervals. Cells were then harvested and apoptotic rates were measured using an Annexin V–fluorescein isothiocyanate (FITC) kit (Bender Med Systems GmbH, Vienna, Austria). Briefly, cells were washed in ice-cold phosphate-buffered saline (PBS) and resuspended in binding buffer. Annexin V–FITC was added to the cell suspension and incubated for 10 min in the dark. Propidium iodide (PI) was then added

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and stained cells were analysed using a FACSort Flow Cytometer (Beckton Dickinson Franklin Lakes, NJ, USA). Viable cells are primarily Annexin V–FITC and PI negative, PI positive staining indicates necrosis, Annexin V–FITC positive staining indicates early apoptosis, and cells that are Annexin V–FITC and PI positive are considered to be in late apoptosis.

Western immunoblots

Cells were serum-starved overnight, after which they were treated with the insulin analogues, IGF-I or human insulin at the indicated concentrations. After 24 h, cells were harvested with ice-cold PBS containing 5 mM EDTA and lysed in a buffer composed of 150 mM NaCl, 20 mM Hepes, pH 7.5, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, 2 μg/mL aprotinin, 1 mM leupeptin, 1 mM pyrophosphate, 1 mM vanadate and 1 mM DTT. Samples (50 μg protein) were subjected to 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer of the proteins to nitrocellulose membranes. Membranes were blocked with 3% milk in T-TBS (20 mM Tris–HCl, pH 7.5, 135 mM NaCl and 0.1% Tween-20) and then incubated with antibodies against total-ERK, phospho-ERK (Thr^{202}/Tyr^{204}), total-AKT and phospho-AKT (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were detected using the SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA).

Immunoprecipitation analysis

Cells were serum-starved overnight, after which they were treated with glargine (50 ng/mL) for 10 or 20 min. Cells were then lysed, immunoprecipitated with anti-IGF-IR or anti-IR antibodies, electrophoresed through 10% SDS-PAGE and blotted with anti-phosphotyrosine, anti-total IGF-IR or total-IR antibodies, as described above.

Results

Mitogenic effects of insulin analogues

The potential mitogenic nature of insulin analogues has been the focus of extensive and controversial research. Results of many studies suggested a remarkable overlap between the spectra of biological effects elicited by insulin analogues and IGF-I [16]. Therefore, we investigated the potential proliferative activities of a series of long- and short-acting insulin analogues in comparison with native insulin and IGF-I. Serum-starved HCT-116 (colorectal cancer), PC-3 (prostate cancer) and MCF-7 (breast adenocarcinoma) cell lines were treated daily with regular human insulin, IGF-I or long-acting analogues glargine or detemir at a dose of 100 nM, and compared with control, untreated cells. Cells were stained with trypan blue and counted every 24 h with a haemocytometer. As expected, IGF-I had a statistically significant ($p < 0.05$) mitogenic effect compared with control and insulin-treated cells (Figure 1A). Thus, after 96 h, a 24% elevation of cell number in IGF-I-treated cells was observed (32.3×10^5 cells/well in IGF-I-treated cultures *versus* 25.9 and 26.0×10^5 cells/well in control and insulin-treated cultures, respectively). Glargine and detemir also stimulated proliferation of HCT-116 cells by 22% and 17% in comparison with control and insulin-treated cells, respectively, at 96 h (31.7 and 30.5×10^5 cells/well, respectively). As with IGF-I treatment, stimulation by glargine and detemir was statistically significant *versus* control and insulin treatments ($p < 0.05$). The short-acting analogue lispro (100 nM) stimulated proliferation of HCT-116 cells at 48 h by ~20%, compared with ~22% stimulation by IGF-I and 7% stimulation by insulin (Figure 1B). The short-acting analogue aspart had no proliferative effect (data not shown).

Incubation of PC-3 cells with glargine or detemir for 72 h resulted in a ~16% and ~14% increases in proliferation rate, respectively, compared with control and insulin-treated cells (6.9 and 6.8×10^5 cells/well *versus* 5.9 and 6.0×10^5 cells/well, respectively; Figure 2A). These effects were statistically significant *versus* both control and insulin treatments ($p < 0.05$). IGF-I enhanced proliferation of PC-3 cells by 25% (7.5×10^5 cells/well). In addition, the proliferative potential of glargine and detemir was also demonstrated in MCF-7 cells. Thus, glargine and detemir stimulated MCF-7 proliferation at 72 h by 14% and 6%, respectively, compared with control cells. IGF-I treatment stimulated proliferation by 22%, whereas insulin had no major effect (Figure 2B).

To examine whether the proliferative effect of the analogues was correlated with the doses used, HCT-116 cells were exposed to increasing doses of glargine (0–200 nM) for 72 h. Results obtained showed that the proliferative effect of the analogue displayed a dose-dependent curve (Figure 3A). Thus, whereas lower doses (20–50 nM) showed minimal increases of cell numbers (3–4%) compared with control untreated cells, a dose of 100 nM resulted in a significant effect (34%) in proliferation, as shown above. Further stimulation of cell proliferation was obtained with higher doses of glargine (46% stimulation with 130 nM, 81% with 150 nM and 90% with 200 nM glargine). These effects were statistically significant *versus* both control and insulin treatments ($p < 0.05$). IGF-I, however, stimulated proliferation by an average of 21% at all doses tested, without any evident dose–response curve (Figure 3A). Regular human insulin had a negligible effect at all doses tested. Similar to glargine, the effect of detemir on HCT-116 cells displayed also a dose-dependent response (Figure 3B). Thus, lower doses (20–50 nM) led to minimal stimulation (3–8% compared with control cells) at 48 h, whereas doses of 130–200 nM led to proliferation rates of 25–34%. These effects were statistically significant *versus* both control and insulin treatments ($p < 0.05$).

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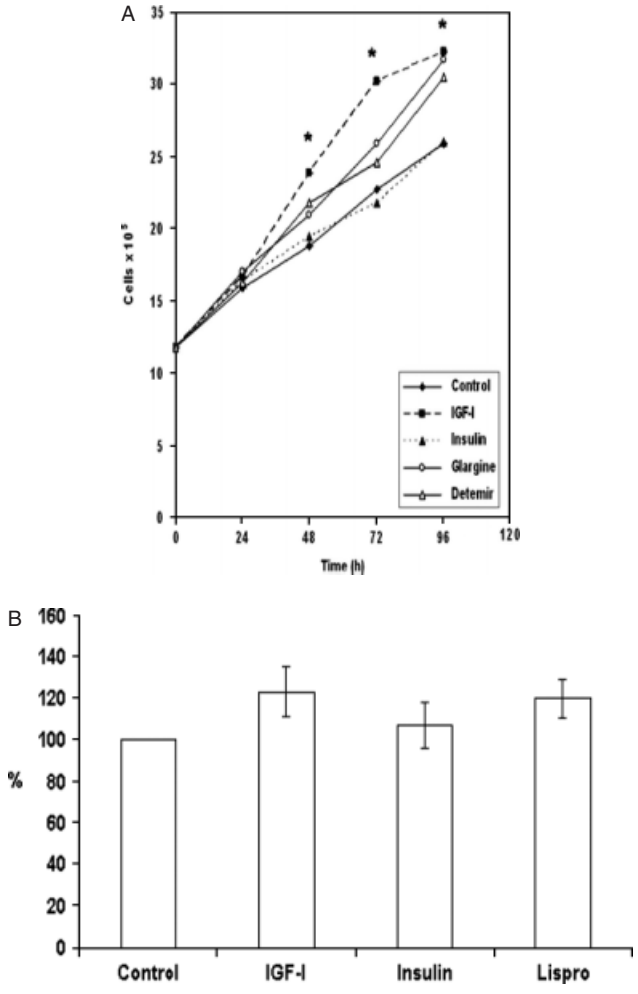

Figure 1. Proliferative effects of insulin analogues in the colorectal cancer cell line HCT-116. (A) HCT-116 cells were plated in six-well plates (2×10^5 cells/well) in complete medium. After 24 h, cells were transferred to serum-reduced medium (1% FBS + 1% BSA) and incubated for 96 h in the presence of 100 nM of IGF-I, insulin, glargine or detemir. Hormones were replenished on a daily basis. Cells were tripsinized every 24 h, stained with Trypan blue and counted using a haemocytometer. Results of a representative experiment repeated three times with similar results are shown. *, significantly different *versus* control and insulin-treated cells ($p < 0.05$). (B) HCT-116 cells were incubated with 100 nM of insulin lispro (or regular insulin or IGF-I, for comparison) for 48 h, and cell proliferation was assessed using the MTT protocol, as described in *Materials and Methods*. Values in the *y*-axis represent percentage of proliferation. A value of 100% was given to the number of control cells after 48 h

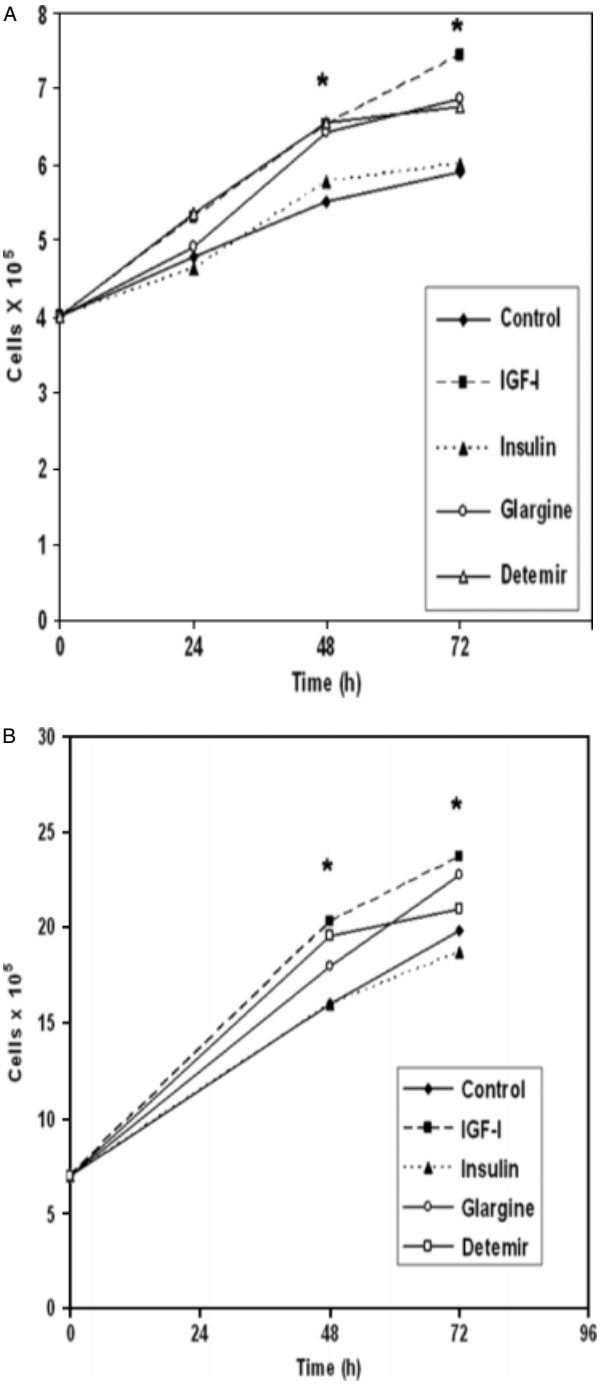

Figure 2. Proliferative effects of glargine and detemir in the prostate cancer cell line PC-3 and breast adenocarcinoma cell line MCF-7. PC-3 (A) and MCF-7 (B) cells were plated in six-well plates in complete medium. After 24 h, PC-3 cells were transferred to serum-reduced medium (1% FBS + 1% BSA) and MCF-7 cells to serum-free medium, in the presence of the indicated hormones (100 nM) for 72 h. Cells were treated and counted as described in the Legend to Figure 1. Results of a representative experiment repeated three times are shown. *, significantly different *versus* controls and insulin-treated cells ($p < 0.05$)

Anti-apoptotic activities of insulin analogues

To evaluate the potential involvement of long-acting insulin analogues in the regulation of apoptosis, HCT-116 cells were serum-starved overnight, after which they were incubated with glargine, detemir, regular insulin or IGF-I (20 and 100 nM) for 12 and 24 h. At the end of the incubation period, the extent of apoptosis was measured using an Annexin V–FITC kit. Results of fluorescent-activated cell sorter (FACS) analysis showed that the percentage of apoptotic cells at 12 h was significantly lower in cells treated with 100 nM of IGF-I (16.9%), glargine (14.9%) and detemir (18.1%) in comparison with insulin-treated (24.5%) and control

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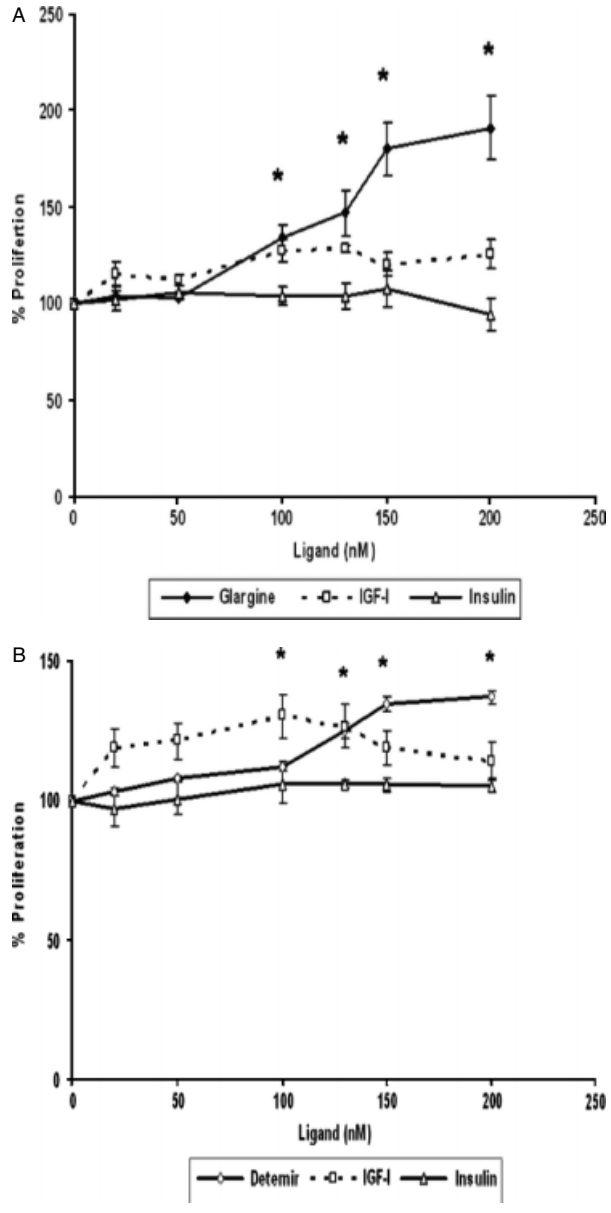

Figure 3. Dose–response effect of glargine and detemir in the HCT-116 cell line. HCT-116 cells were plated in six-well plates (2×10^5 cells/well) in complete medium. After 24 h, cells were transferred to serum-reduced medium (1% FBS + 1% BSA) and incubated in the presence of increasing doses (0–200 nM) of IGF-I, insulin or glargine for 72 h (A), or IGF-I, insulin or detemir for 48 h (B). Cells were treated and counted as described in the Legend to Figure 1. Results of a representative experiment repeated three times are shown. *, significantly different *versus* controls and insulin-treated cells ($p < 0.05$)

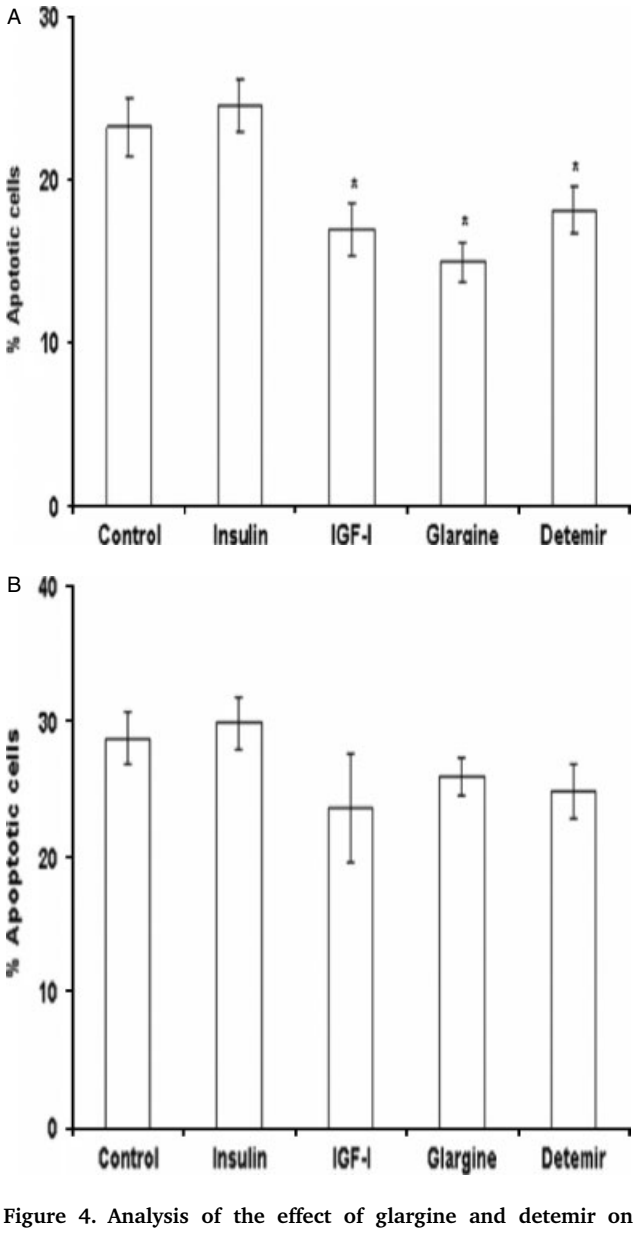

Figure 4. Analysis of the effect of glargine and detemir on apoptosis. Serum-starved HCT-116 cells were treated with 100 nM of IGF-I, insulin, glargine or detemir for 12 h (A) or 24 h (B) and apoptosis was evaluated using an Annexin–FITC kit, as described in *Materials and Methods*. Quantitative analysis of the flow cytometry data was performed using the WinMDI 2.8 software. Results of a representative experimented repeated three times with similar results are shown. *, significantly different *versus* insulin-treated cells ($p < 0.05$)

(23.2%) cells ($p < 0.05$; Figure 4A). Similarly, insulin analogues conferred protection from apoptosis at 24 h (Figure 4B). At this time, the percentage of apoptotic cells in IGF-I-, glargine- and detemir-treated cells were 23.6%, 25.9% and 24.9%, respectively. The percentage of apoptotic cells in insulin-treated cultures at 24 h was, again, similar to that in control cells (29.9%). These values, however, did not reach statistical significance. Finally, doses of 20 nM of glargine and detemir also conferred protection from apoptosis, although at this dose also regular insulin exhibited a protective effect similar to IGF-I and insulin analogues (data not shown).

Signalling pathways of insulin analogues

A key issue in the elucidation of the mechanisms of action of insulin analogues is the identification of the specific receptor/s through which their biological activities are mediated. To discard the possibility that HCT-116 cells express unequal numbers of IRs and IGF-IRs (thus leading to preferential activation of one specific

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receptor), we measured in initial experiments the relative expression of both receptors using Western blotting and immunofluorescent staining with specific anti-IR and anti-IGF-IR antibodies. Results obtained showed that both receptors are basally expressed at equivalent levels (data not shown). Next, we investigated the potential activation of IR and IGF-IR by glargine and detemir. To this end, cells were treated with the analogues for 10 or 20 min, after which lysates were prepared, immunoprecipitated with anti-IR (Figure 5A) or anti-IGF-IR (Figure 5B), electrophoresed and blotted with anti-phosphotyrosine, anti-total IR or total-IGF-IR. Results of immunoprecipitation experiments showed that glargine was able to phosphorylate both IR and IGF-IR. However, detemir led to IR, but not IGF-IR, phosphorylation in HCT-116 cells (data not shown).

Although the signalling events elicited by native IGF-I and insulin have been, for the most part, well delineated, little information is available regarding the cytoplasmic transduction cascades elicited by insulin analogues. Both IR and IGF-IR are linked to two major signalling pathways: the ras–raf–MAPK and PI3K–PKB/AKT [9,18,19]. To begin to investigate the downstream signalling mediators involved in insulin analogues action, HCT-116 cells were serum-starved overnight, and then treated with glargine (or diluent) for 20, 40 or 60 min. Activation of specific molecules was assessed by Western immunoblotting using antibodies against phospho-extracellular signal-regulated Kinase (ERK) and phospho-AKT. As shown in Figure 6A and B, glargine induced the phosphorylation of both ERK and AKT in a very rapid and strong manner. Maximal phosphorylation of both proteins was seen at 20 min, with significant reduction in phosphorylation intensity at 60 min. No changes were seen in the levels of total-ERK and total-AKT. To compare the potencies of both insulin analogues with that of regular insulin and IGF-I in induction of AKT phosphorylation, cells were treated for 30 min with 50 ng/mL of glargine, detemir, insulin or IGF-I, after which AKT phosphorylation was evaluated as described above. Results obtained showed that glargine, like regular human insulin, had a very potent stimulatory effect in comparison with IGF-I and detemir (Figure 7A). Scanning densitometric analysis of the phospho-AKT bands normalized to the corresponding total-AKT bands is presented in Figure 7B.

To compare the relative potencies of the analogues in the induction of ERK phosphorylation, HCT-116 cells were treated for 30 min with 50 ng/mL of glargine, detemir, insulin or IGF-I, after which ERK phosphorylation was evaluated by Western blotting with a specific anti-phospho-ERK antibody. Results obtained showed that regular insulin had a potent stimulatory effect on ERK phosphorylation. The effects of glargine and detemir were lower than that of insulin and were comparable with the effect of IGF-I (Figure 8A). Scanning densitometric analysis of the data is shown in Figure 8B.

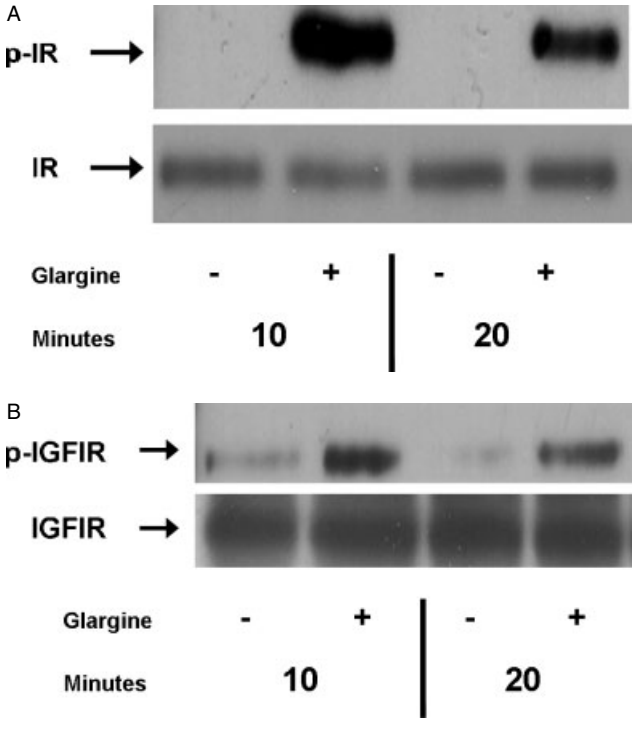

Figure 5. Activation of IR and IGF-IR by glargine. HCT-116 cells were serum-starved overnight, after which they were treated with 50 ng/mL glargine for 10 or 20 min. Cells were lysed as described in *Materials and Methods*, immunoprecipitated with anti-IR (A) or anti-IGF-IR (B) antibodies, electrophoresed through 10% SDS-PAGE and blotted with anti-phosphotyrosine (upper panels) or anti-total IR or total-IGF-IR antibodies (lower panels)

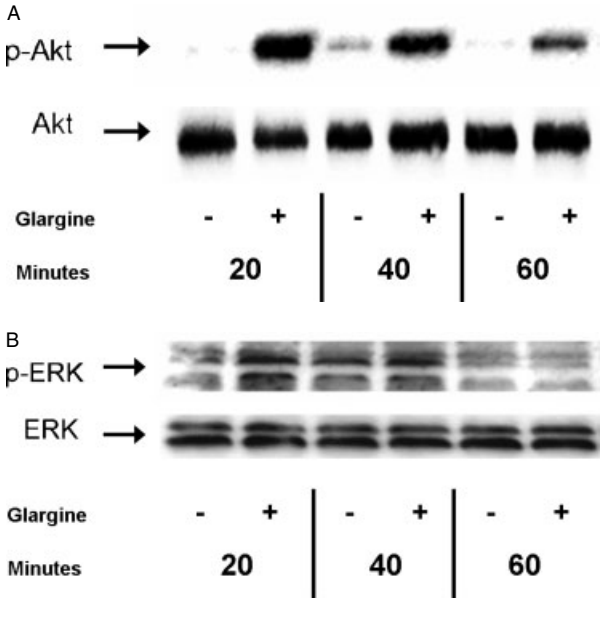

Figure 6. Activation of AKT and ERK by glargine. HCT-116 cells were serum-starved overnight, after which they were treated with 100 nM glargine for 20, 40 or 60 min. Cells were lysed as described in *Materials and Methods*. Cell extracts (50 μg protein) were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-phospho-AKT (A) or anti-phospho-ERK (B). Blots were stripped and re-probed with anti-total-AKT or anti-total ERK (lower panels)

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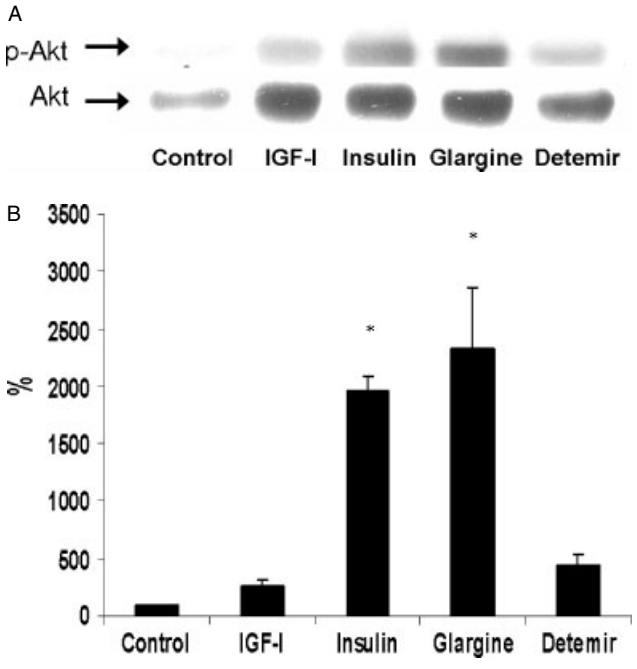

Figure 7. Comparison of the potencies of insulin analogues to activate AKT. (A) Serum-starved HCT-116 cells were treated with the indicated hormones and insulin analogues for 30 min at a concentration of 50 ng/mL. At the end of the incubation period, cells were lysed and the extent of AKT phosphorylation was evaluated by Western blotting as described in *Materials and Methods*. (B) Scanning densitometry of the phospho-AKT bands normalized to the corresponding total-AKT bands. A value of 100% was assigned to control untreated cells. *, significantly different *versus* control, IGF-I and detemir treatments ($p < 0.05$)

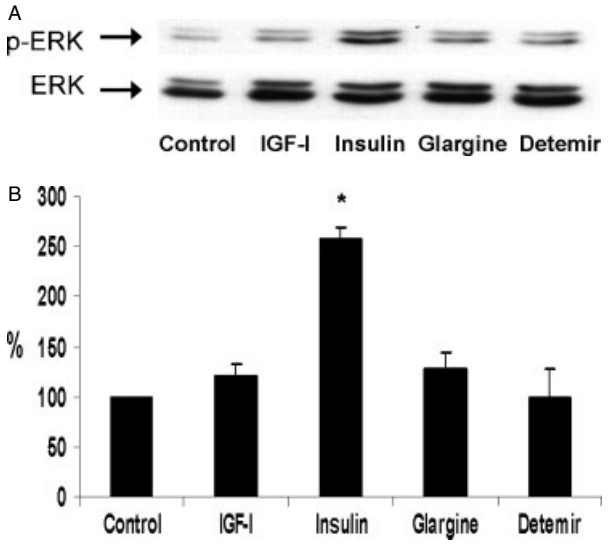

Figure 8. Comparison of the potencies of insulin analogues to activate ERK. (A) Serum-starved HCT-116 cells were treated with the indicated hormones and insulin analogues for 30 min at a concentration of 50 ng/mL. At the end of the incubation period, cells were lysed and the extent of AKT phosphorylation was evaluated by Western blotting as described in *Materials and Methods*. (B) Scanning densitometry of the phospho-ERK bands normalized to the corresponding total-ERK bands obtained. *, significantly different *versus* control, IGF-I, glargine and detemir treatments ($p < 0.05$)

Discussion

The involvement of the IGF system in the initiation and progression of human cancer has been well established [20,21]. Of particular relevance, epidemiological data identified a positive correlation between circulating IGF-I concentrations and incidence of multiple kinds of neoplasia, including breast, prostate and colorectal cancer [22–24]. The structural and functional similarities between insulin and IGF-I suggest that both molecules are derived from a common ancestral precursor that participated in food intake and regulation of cellular growth [25]. A divergence of functions most likely occurred before the appearance of the first vertebrates, with insulin mostly active in the regulation of metabolism and IGF-I in growth processes. In view of their common evolutionary origins and semiconserved architecture, however, there is a certain degree of crosstalk between insulin, IGF-I and their receptors [8,26]. Accordingly, insulin exhibits a number of IGF-I-like activities, including growth stimulation, and IGF-I, on the other hand, exerts certain metabolic effects [14]. Furthermore, and consistent with the reduced affinity displayed by each receptor for the opposite ligand, the crosstalk between insulin-like ligands and receptors is largely intensified at supra-physiological doses of the respective ligands. In addition, the fact that IGF-I, but not insulin, is carried in the circulation and extracellular fluid by IGF-binding proteins (IGFBPs) further contributes to the divergent actions of both ligands. Given the crosstalk between insulin and IGF-I signalling pathways, and in view of the modifications introduced into the structure of insulin analogues, the massive use of insulin and insulin analogues in recent years has raised a number of concerns among clinicians, patients and regulatory agencies, regarding the safety of the life-long use of these synthetic hormones.

The results presented here show that long-acting insulin analogues glargine and detemir and short-acting analogue lispro stimulated cell proliferation of cultured colorectal, prostate and breast cancer cells. Although the proliferative effects of the analogues in HCT-116, PC-3 and MCF-7 cells were somehow lower than that of IGF-I (17–22% stimulation by the analogues *versus* 24% enhancement by IGF-I in HCT-116 cells, 14–16% stimulation by the analogues *versus* 25% enhancement by IGF-I in PC-3 cells and 6–14% stimulation by the analogues *versus* 22% enhancement in MCF-7 cells), it significantly exceeded the effect of regular human insulin, which was essentially devoid of mitogenic capacity at the doses used. Of interest, a recent study showed that both regular insulin and glargine at doses of 50–100 nM significantly enhanced ^{3}[H]thymidine uptake and MTT-assayed proliferation of normal human breast epithelial cells (MCF-10) and MCF-7 breast cancer cells. No differences, however, were seen in this study between regular insulin and analogue [27].

In addition to their ability to enhance proliferation, both long-acting analogues prevented apoptosis in HCT-116 cells. Again, the effect of the analogues, at least at

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a concentration of 100 nM, resembled the typical pro-survival activity of IGF-I. Consistent with most published data, insulin did not display an anti-apoptotic activity. Taken together, our results indicate that pharmacological doses of insulin analogues can potentiate the intrinsic mitogenic capabilities of cancer cells *in vitro*. It is generally recognized that neither insulin nor IGF-I can induce DNA damage, lead to accumulation of mutations, activate oncogenes or elicit any other event associated with induction of malignant transformation [28,29]. However, it is also well established that once a transformation event has already occurred, the IGF-I/IGF-IR axis has a central role in the development of the cancer phenotype. Results of proliferation and apoptosis assays show that glargine and detemir have a significant IGF-I-like mitogenic activity, which is not shared by insulin. This IGF-I-like activity of the analogues in transformed cell lines may support the speculation that long-time exposure to elevated doses of insulin analogues may exacerbate the inherent malignant potential of neoplastic cells and lead to an aggravated phenotype. In addition, and given that diabetic patients are often required to increase the doses of their insulin injections to achieve better glycemic control, the results obtained in the present study with supra-physiological doses are of high clinical relevance. In contradistinction to our findings and those of Eckardt *et al*. [16], life-long glargine treatment of rats and mice injected daily with increasing doses of the analogue had no carcinogenic effect [5]. Although an increase in mortality rate was observed in male rats at every glargine dose and in female rats in the high-dose glargine group, these results are consistent with the prevailing view that regular insulin and insulin analogues, *per se*, do not induce malignant transformation. Furthermore, neither regular insulin nor glargine were shown to affect viability and proliferation of non-transformed human coronary endothelial and smooth muscle cells [30].

The biological significance of the crosstalk between insulin-like peptides and IR/IGF-IR family of receptors has not yet been entirely clarified. Data presented here show that glargine has the ability to activate both receptors. Although the entire collection of cytoplasmic molecules and nuclear transcription factors activated by insulin analogues is yet to be identified, and given that the similarities and differences between the signal transduction events elicited by IGF-I/insulin and insulin analogues are still unknown, the results presented here suggest that particular analogues can phosphorylate different cytoplasmic mediators in a more or less specific manner. Thus, glargine, similar to insulin, strongly induced the phosphorylation of AKT, a cytoplasmic kinase involved in the transduction of many important metabolic activities. However, the capacity of detemir to stimulate AKT phosphorylation was much lower than that of glargine. In addition, we showed that both glargine and detemir activate ERK, a cytoplasmic kinase mainly involved in the transduction of mitogenic signals, in a fashion similar to IGF-I, but not to insulin. Combined, these data suggest that, at least *in vitro*, glargine has the potential to act both as insulin and as IGF-I on cancer cells. The biological significance of these differential activities requires further investigation.

In conclusion, the data presented here show that the long-acting insulin analogues glargine and detemir, and the short-acting analogue lispro, unlike regular human insulin, exhibit *in vitro* proliferative and anti-apoptotic activities in a number of cancer-derived cell lines. These actions strongly resemble some of the typical biological effects of IGF-I, a growth factor whose involvement in cancer initiation and progression has been well established. Further studies are required to evaluate the signal transduction events elicited by insulin analogues as well as the impact of these synthetic hormones in cellular physiology. At the clinical level, the implications of long-term use of insulin analogues await a careful and critical follow-up.

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Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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