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Proliferative and signaling activities of insulin analogues in endometrial cancer cells

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ABSTRACT

Insulin analogues have been developed to achieve further improvement in the therapy of diabetes. However, modifications introduced into the insulin molecule may enhance their affinity for the insulin-like growth factor-1 receptor (IGF1R). Hyperinsulinemia has been identified as a risk factor for endometrial cancer. We hypothesized that insulin analogues may elicit atypical proliferative and signaling activities in endometrial cancer cells. Our results demonstrate that glargine, but not detemir, stimulated cell proliferation, displayed an anti-apoptotic effect, and had a positive effect on cell cycle progression in endometrial cancer cell lines ECC-1 and USPC-1. In addition, we showed that glargine and detemir induced dual activation of the insulin receptor (INSR) and IGF1R in both cell types. Furthermore, we showed that glargine elicited signaling events that are markedly different from those induced by insulin. In conclusion, our data support the concept that, although insulin analogues were designed to display insulin-like metabolic effects, glargine and, possibly, additional analogues exhibit IGF1-like activities and, accordingly, may function as IGF1 analogues.

1. Introduction

Endometrial cancer is the most frequently diagnosed cancer in the female genital tract in the Western world. Women have a 2–3% lifetime risk of developing the disease, and more than 40,000 new cases were diagnosed in 2009 in the United States alone (Jemal et al., 2009). The incidence of the disease has been increasing in recent years, presumably because of the growing obesity epidemic. Endometrial carcinomas are classified into two major groups: type I and type II. Type I tumors, which account for about 80% of the cases, occur mostly in pre- and peri-menopausal women (Lax, 2004). These tumors are typically well differentiated, minimally invasive into the uterine wall, generally estrogen dependent, and have a good prognosis. In contrast, type II tumors, which are typically poorly differentiated, occur in older women, are usually diagnosed at advanced stage of the disease, and are characterized by an aggressive behavior and a worse prognosis (Hamilton et al., 2006). Uterine

serous carcinoma (USC) constitutes the predominant histological class among type II tumors.

Exogenous insulin administration is the only treatment available for type 1 diabetes patients and is one of the main therapeutic approaches for type 2 diabetes patients. Over the last decade, several insulin analogues have been developed to improve diabetes therapy. By recombinant DNA technology, the insulin molecule has been modified to either prolong or shorten its action time, therefore improving its pharmacokinetic properties (Vajo et al., 2001). Insulin analogues can be divided into two major subgroups: short-acting and long-acting, according to their absorption rates and action kinetics. Modifications of the insulin molecule are generally made into the C-terminus of the B-chain [a molecular domain important for dimer formation but not involved in insulin receptor (INSR) binding]. It appears, however, that this domain is important for insulin-like growth factor-1 receptor (IGF1R) binding (Zib and Raskin, 2006). Therefore it has been postulated that certain analogues may display IGF1-like activities (Hansen, 2008; Kurtzhals et al., 2000; Le Roith, 2007).

The insulin-like growth factors (IGFs) constitute a network of ligands, cell-surface receptors, and binding proteins with important roles in cell cycle progression as well as in tumorigenesis. The biological actions of IGF1 and IGF2 are mediated by the IGF1R, a transmembrane tetrameric receptor which resembles the INSR. Most experimental and clinical evidence is consistent with the notion that

INSR activation (mainly by insulin) is primarily involved in mediating metabolic types of action, whereas IGF1R activation (mainly by IGF1 or IGF2) predominantly mediates growth and differentiation activities (Werner et al., 2008). Nevertheless, there is a certain degree of cross-talk within this growth factor family. Furthermore, the IGF1R mediates strong anti-apoptotic signals and is usually overexpressed in endometrial tumors (Werner and Maor, 2006).

Recent epidemiologic studies provide evidence that chronic hyperinsulinemia, hyperglycemia, excess body weight, lack of physical activity and alterations in endogenous hormone metabolism confer an increased risk of endometrial cancer throughout lifetime (Kaaks and Stattin, 2010). In addition, studies have shown that components of the IGF system have a major role in endometrial cancer development and progression (Gunter et al., 2008; McCampbell et al., 2006; Pavelic et al., 2007; Petridou et al., 2003; Sarfstein et al., 2011). Given that diabetes is a chronic disease and, for most patients, increasing insulin dosage is required during time, the potential mitogenic effect of insulin analogues may increase the risk of developing various cancers (Friberg et al., 2007; Larsson et al., 2006; Shukla et al., 2009; Sommerfeld et al., 2010; Varewijck and Janssen, 2012). It is also possible that insulin analogues might worsen the situation of patients with diabetes in whom a cancer has already begun to develop. For these reasons the connection between diabetes, insulin treatment and cancer risk has raised, in recent years, serious concerns regarding the safety of particular insulin analogues (Werner et al., 2011).

Our previous studies have shown that glargine and detemir, unlike regular insulin, exhibit in vitro IGF1-like proliferative and anti-apoptotic activities in colon, breast and prostate cancer cell lines. Based on the recent literature regarding insulin analogues, and in view of the correlation between obesity and diabetes with endometrial cancer, we hypothesized that insulin analogues might elicit atypical proliferative and signaling activities in endometrial cancer. For this purpose, we analyzed in the current study the biological actions of short-acting insulin lispro (LysB28, ProB29 human insulin) and insulin aspart (AspB28 human insulin), and long-acting insulin glargine (GlyA21, ArgB31, ArgB32 human insulin) and insulin detemir [LysB29 (*N*-tetradecanoyl) des (B30) human insulin] analogues in types I and II endometrial cancer cell lines. Results obtained indicate that while glargine displays a potent proliferative effect, detemir as well as short-acting analogues lispro and aspart exhibit reduced mitogenic potentials. Furthermore, different analogues activate the INSR and IGF1R pathways in cell-type specific manners. These results emphasize the crucial need for careful follow-up evaluation of insulin analogues in the clinics.

2. Material and methods

2.1. Cell cultures

Human endometroid endometrial carcinoma (ECC-1; type I-derived) and uterine serous papillary endometrial carcinoma (USPC-1; type II-derived) cell lines were used in this study. The ECC-1 cell line was obtained from Dr. Y. Sharoni, Ben Gurion University, Beer-Sheba, Israel. The USPC-1 cell line was provided by Dr. A. Santin, Yale University School of Medicine, New Haven, CT, USA. ECC-1 cells were grown in DMEM and USPC-1 cells in RPM-1640 media (Biological Industries, Kibbutz Beit Haemek, Israel). Both media were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml gentamicin sulfate and 5.6 mg/l amphotericin B. Cells were treated with the following hormones and analogues: regular insulin (Humulin R®, Lilly France S.A.S.; Lilly Pharma, Germany), insulin glargine (Lantus®, Sanofi Aventis, Germany), insulin detemir (Levemir®, Novo Nordisk, Denmark), insulin lispro (Humalog®, Lilly France S.A.; Lilly Pharma, Germany), insulin aspart (NovoRapid®, Novo Nordisk, Denmark) and IGF1 (PeproTech Ltd, Rocky Hill, NJ, USA).

2.2. Cell proliferation assays

Cells were seeded at a density of 5×10^4 cells/well for USPC-1 and 4×10^4 cells/well for ECC-1 into 24-well plates with full media. After 24 h, cells were starved and treated with short- or long-acting analogues, IGF1 or regular insulin at a dose of 20 ng/ml for 72 h, in triplicate wells. Cell viability was assessed using a standard thiazolyl blue tetrazolium bromide (MTT) assay (Brasaemle and Attie, 1988). Cell proliferation was expressed as a fold of untreated cells (control).

2.3. Western immunoblot analyses

Cells were serum-starved overnight and then treated with insulin analogues, IGF1 or insulin at the indicated concentrations. After incubation, cells were harvested and lysed in a buffer containing protease inhibitors. Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard. Samples were electrophoresed through 10% SDS–PAGE, followed by transfer of the proteins to nitrocellulose membranes. Membranes were blocked with either 5% skim milk and/or 3% BSA and incubated overnight with antibodies against: phospho-IGF1R/INSR (#3024), IGF1R β-subunit (#3027), INSR β-subunit (#3025), phospho-Akt (#9271), Akt (#9272) and phospho-Erk1/2 (#9106). Antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). An antibody against Erk1/2 (#K-23) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After incubation, blots were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected using the SuperSignal West Pico® Chemiluminescent Substrate (Pierce, Rockford, IL, USA). The expression of actin was measured as a loading control.

2.4. Apoptosis analyses

Cells were serum-starved overnight, after which they were treated with insulin analogues, insulin, or IGF1 at a dose of 50 ng/ml and incubated for an additional 24 h. At the end of the incubation period cells were lysed and levels of poly-ADP ribose polymerase (PARP) were measured by Western blots. Appearance of an ~85 kDa band, a proteolytic cleavage product of the full-length ~116 kDa PARP protein, is considered an early marker of apoptosis (Duriez and Shah, 1997).

2.5. Cell cycle analyses

Cells were seeded in 10-cm Petri dishes until reaching 100% confluence. Cells were then serum-starved for 24 h and incubated in the presence of insulin analogues, insulin, or IGF1 at a dose of 50 ng/ml for 168 h. After incubation, cells were washed with phosphate-buffered saline, trypsinized, centrifuged, resuspended in citrate buffer and stored at –80 °C prior to analysis. The cells were then thawed, permeabilized and stained with propidium iodide according to Vindelov et al. (1983). Stained cells were analyzed using a FacsCalibur Flow Cytometer (Cytek Development Inc., Fremont, CA, USA).

2.6. Immunoprecipitation assays

Cells were grown to 100% confluence and serum starved for 24 h, after which they were treated with insulin analogues, regular insulin, or IGF1 at a dose of 50 ng/ml for 10 min. Then, cells were lysed and extracts (80 μg) were immunoprecipitated by incubating overnight with anti-IGF1R β-subunit or anti-INSR β-subunit. Protein A–G agarose beads (Santa Cruz Biotechnology) were added to the samples and further incubated at 4 °C for 2 h. Immunoprecipitates were pelleted by centrifugation, washed, mixed with sample buffer, boiled for 5 min at 95 °C, and electrophoresed through 10% SDS–PAGE.