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Internalization and localization of basal insulin peglispro in cells

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ABSTRACT

Background: Basal insulin peglispro (BIL) is a novel, PEGylated insulin lispro that has a large hydrodynamic size compared with insulin lispro. It has a prolonged duration of action, which is related to a delay in insulin absorption and a reduction in clearance. Given the different physical properties of BIL compared with native insulin and insulin lispro, it is important to assess the cellular internalization characteristics of the molecule.

Methods and materials: Using immunofluorescent confocal imaging, we compared the cellular internalization and localization patterns of BIL, biosynthetic human insulin, and insulin lispro. We assessed the effects of BIL on internalization of the insulin receptor (IR) and studied cellular clearance of BIL.

Results: Co-localization studies using antibodies to either insulin or PEG, and the early endosomal marker EEA1 showed that the overall internalization and subcellular localization pattern of BIL was similar to that of human insulin and insulin lispro; all were rapidly internalized and co-localized with EEA1. During ligand washout for 4 h, concomitant loss of insulin, PEG methoxy group, and PEG backbone immunostaining was observed for BIL, similar to the loss of insulin immunostaining observed for insulin lispro and human insulin. Co-localization studies using an antibody to the lysosomal marker LAMP1 did not reveal evidence of lysosomal localization for insulin lispro, human insulin, BIL, or PEG using either insulin or PEG immunostaining reagents. BIL and human insulin both induced rapid phosphorylation and internalization of human IR.

Conclusions: Our findings show that treatment of cells with BIL stimulates internalization and localization of IR to early endosomes. Both the insulin and PEG moieties of BIL undergo a dynamic cellular process of rapid internalization and transport to early endosomes followed by loss of cellular immunostaining in a manner similar to that of insulin lispro and human insulin. The rate of clearance for the insulin lispro portion of BIL was slower than the rate of clearance for human insulin. In contrast, the PEG moiety of BIL can recycle out of cells.

1. Background/introduction

Exogenous biosynthetic human insulin (human insulin) is effective therapy for returning blood glucose to normal levels in patients with diabetes mellitus; however, it is a short-acting agent with a delay in onset of action after a subcutaneous (SC) injection and is associated with hypoglycemia, a treatment-limiting adverse event (Borgono and Zinman, 2012; Cryer, 2002). To improve treatment outcomes in patients with Type 1 diabetes mellitus (T1DM) or Type 2 diabetes mellitus (T2DM), basal insulin analogs with prolonged durations of action, including detemir, glargine, and degludec have been developed (Owens et al., 2014; Singh and Gangopadhyay, 2014). Although these agents improve upon the profile of earlier basal insulin therapies, among the most important findings are the duration of action with detemir being generally <24 h (Vora and Heise, 2013), the intra-patient variability with glargine (Vora and Heise, 2013), and the higher plasma concentrations needed with degludec compared with other novel insulin formulations (Bolli et al., 2011). There remains a need for sustained consistent glycemic control with reduced intra-patient pharmacodynamic variability in order to decrease the occurrence of

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hypoglycemia.

Polyethylene glycol (PEG) is a synthetic polymer that has been used successfully for many years in a wide range of therapeutic products, including injected biopharmaceuticals, to improve the therapeutic efficacy (Fruijtier-Polloth, 2005; Milla et al., 2012; Webster et al., 2007, 2009). Basal insulin peglispro (BIL) comprises insulin lispro covalently bound, via a urethane bond, to a 20 kDa PEG moiety at lysine B28 (Owens et al., 2016). The addition of the PEG moiety increases the hydrodynamic size of the insulin lispro molecule; the large hydrodynamic size of BIL is believed to slow absorption and reduce clearance, which may contribute to the prolonged duration of action. The hydrodynamic diameter of BIL is similar to that of human serum albumin, a molecule that is not readily cleared by the kidneys (Hansen et al., 2012). It has recently been shown that the increased hydrodynamic size of BIL results in a hepatopreferential activity profile in both dogs and humans owing to the differential access of BIL in the capillary endothelium and hepatic sinusoidal endothelium (Moore et al., 2014; Mudaliar et al., 2015). A Phase 1 study in healthy volunteers found that BIL had a prolonged duration of action of ≥36 h after a single dose (Sinha et al., 2014). In a Phase 2, randomized, open-label, crossover trial of BIL versus insulin glargine, plus prandial insulin, for 8 weeks in patients with T1DM, treatment with BIL was associated with statistically significantly better glycemic control than insulin glargine (Rosenstock et al., 2013). In a Phase 2, 12-week, randomized, open-label, parallel study of BIL versus insulin glargine in patients with T2DM, it was demonstrated that mean fasting blood glucose and HbA1c were similar with BIL and insulin glargine, but that mean intra-day blood glucose variability was statistically significantly lower with BIL (Bergenstal et al., 2012). In these trials of patients with T1DM and T2DM, respectively, the rates and types of adverse events were similar with BIL and insulin glargine.

An assessment of the *in vitro* pharmacological properties of BIL showed that BIL had reduced binding affinity and functional potency compared with insulin lispro and human insulin. BIL had similar affinity and functional activation of IR isoforms A and B and demonstrated improved selectivity for human IR (hIR) compared with human IGF-1R (Owens et al., 2016). Furthermore, BIL showed a more rapid rate of IR-A and IR-B dephosphorylation following maximal stimulation, as well as reduced mitogenic potential in the SAOS-2 cellular model (Owens et al., 2016).

With the development of novel insulin analogs, it is of critical importance to assess the cellular action for appropriate activity of the novel insulin. Given the different physical properties of BIL, with PEGylation of insulin lispro significantly increasing the hydrodynamic size compared to native insulin lispro, it is important to assess the cellular trafficking of the molecule. Early studies described the internalization of insulin into distinct intracellular compartments, later termed endosomes, that served to concentrate the ligand (Khan et al., 1981; Posner et al., 1980). Subsequent studies have refined the understanding of insulin internalization and processing and described its key role in cellular insulin action. Upon insulin binding, the activated phosphorylated IR internalizes to endosomes where it remains active until subsequent dephosphorylation and ligand dissociation (Bevan et al., 2000). Endocytosis of receptor and ligand complexes is important to the function of many receptor signaling systems, and a recent review of the current evidence suggests an even greater role (Hupalowska and Miaczynska, 2012), stressing the importance of proper internalization of the IR to the endosome (Bevan et al., 1996; Faure et al., 1992). Endosomal signaling has been demonstrated for the IR where activation of the receptor in the absence of prior cell surface activation, can recapitulate many aspects of signaling downstream of the receptor (Bevan et al., 1995). Dephosphorylation of the IR by tyrosine phosphatases takes place at the endosome and is necessary for appropriate termination of signaling and availability of the receptor for subsequent restimulation (Faure et al., 1992; Posner and Bergeron, 2014). In the low pH environment of the endosome insulin dissociates from the IR and is largely degraded in the late endosome and lysosome (Authier and Desbuquois, 2014; Bevan et al., 1996; Desbuquois et al., 1992).

Proper endosomal localization can influence downstream signaling from the IR and receptor recycling to the plasma membrane, where aberrant acidification of the endosomal compartment has been shown to perturb insulin metabolism in a manner that mimics aspects of that seen in cells from patients with type 2 diabetes mellitus (Benzi et al., 1997; Bevan et al., 1996).

Unlike with human insulin, details regarding the internalization and processing of BIL relative to the cell are not known. For this report, we compared the intracellular localization of BIL with that of insulin lispro and human insulin and studied the *in vitro* clearance of BIL and PEG recycling.

2. Methods and materials

2.1. Insulin and insulin analogs

BIL was prepared in a zinc-formulated solution (3 moles zinc per mole of BIL hexamer) containing m-cresol (3.15 mg/mL) in a Tris buffer (7.5 mg/mL sodium chloride, 2 mg/mL Tris base, pH 7.3) and was stored at 4 °C. Human insulin (Eli Lilly and Company) was prepared in 0.01 N HCl and was stored at −20 °C in single-use aliquots. Insulin lispro (Eli Lilly and Company) was prepared as a zinc-formulated solution (3 moles zinc per mole of insulin lispro hexamer) containing m-cresol (3.15 mg/mL) in either a phosphate buffer (16 mg/mL glycerin, 1.88 mg/mL sodium phosphate, pH 7.3) or Tris buffer (7.5 mg/mL sodium chloride, 2 mg/mL Tris base, pH 7.3]; both solutions were stored at 4 °C. Human IGF-1 (PeproTech, Rocky Hill, NJ) was prepared as a 131 μM stock solution (1 mg/mL) in 0.01 N HCl and stored at −80 °C as single use aliquots.

2.2. Immunofluorescent confocal imaging studies

Fixed-cell immuno-fluorescent confocal microscopy was performed in human osteosarcoma U2OS cells co-expressing hIR isoform B (tagged at the C-terminus with an inactive fragment of beta galactosidase) and the SH2 domain of phospholipase C gamma1 (PLCγ) tagged with a complementary inactive fragment of beta galactosidase (PathHunter® U2OS INSR Functional Assay, DiscoveRx, Fremont, CA, herein referred to as U2OS-hIR). Cells were maintained in MEM medium supplemented with MEM non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin−100 μg/mL streptomycin, 10% certified FBS, 250 μg/mL hygromycin B and 500 μg/mL G418 sulfate. All cell culture reagents were from HyClone (GE Healthcare Life Sciences, Logan, UT), except fetal bovine serum (FBS) and bovine serum albumin (BSA), which were from Life Technologies (Thermo Fisher Scientific, Waltham, MA). Two days before the experiments, cells were seeded at 20,000 cells/well in growth medium in poly-D-lysine-coated, glass-bottom, black 96-well plates (PerkinElmer, Waltham, MA). Cells were incubated for 40 h at 37 °C in a 5% CO_2 incubator. On the day of the experiment, the medium was replaced with serum-free medium (MEM, non-essential amino acids, 1 mM sodium pyruvate, and 0.1% BSA); cells were returned to incubate at 37 °C in a 5% CO_2 incubator for 2 h. Cell medium was then aspirated and cells were treated with IR ligands (human insulin, insulin lispro, or BIL),