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Long-acting insulin analog detemir displays reduced effects on adipocyte differentiation of human subcutaneous and visceral adipose stem cells

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Abstract *Background and aims:* Since treatment with insulin detemir results in a lower weight gain compared to human insulin, we investigated whether detemir is associated with lower ability to promote adipogenesis and/or lipogenesis in human adipose stem cells (ASC).
Methods and results: Human ASC isolated from both the subcutaneous and visceral adipose tissues were differentiated for 30 days in the presence of human insulin or insulin detemir. Nile Red and Oil-Red-O staining were used to quantify the rate of ASC conversion to adipocytes and lipid accumulation, respectively. mRNA expression levels of early genes, including *Fos* and *Cebpb*, as well as of lipogenic and adipogenic genes, were measured at various phases of differentiation by qRT-PCR. Activation of insulin signaling was assessed by immunoblotting. ASC isolated from subcutaneous and visceral adipose tissue were less differentiated when exposed to insulin detemir compared to human insulin, showing lower rates of adipocyte conversion, reduced triglyceride accumulation, and impaired expression of late-phase adipocyte marker genes, such as *Pparg2*, *Slc2a4*, *Adipoq*, and *Cidec*. However, no differences in activation of insulin receptor, Akt and Erk and induction of the early genes *Fos* and *Cebpb* were observed between insulin detemir and human insulin.
Conclusion: Insulin detemir displays reduced induction of the *Pparg2* adipocyte master gene and diminished effects on adipocyte differentiation and lipogenesis in human subcutaneous and visceral ASC, in spite of normal activation of proximal insulin signaling reactions. These characteristics of insulin detemir may be of potential relevance to its weight-sparing effects observed in the clinical setting.

Abbreviations: Acad, acyl-CoA dehydrogenase; *Acox1*, acyl-CoA oxidase 1; *Acox2*, acyl-CoA oxidase 2; ACC2, acetyl-CoA carboxylase 2; *Adipoq*, adiponectin; ANOVA, analysis of variance; ASC, adipose stem cell; BMI, body mass index; BSA, bovine serum albumin; *Cebp*, CCAAT/enhancer binding protein; *Cidec*, cell death-inducing DFFA-like effector c; DAPI, 4′,6-diamidino-2-phenylindole; Erk, extracellular-signal-regulated kinases; FABP4, fatty acid binding protein 4; FWD, forward; GPD1, glycerol-3-phosphate dehydrogenase 1; GUSB, glucuronidase beta; IGF-I, insulin-like growth factor 1; IR, insulin receptor; *Lipe*, lipase, hormone-sensitive; LPL, lipoprotein lipase; NPH, neutral protamine Hagedorn; PLIN, perilipin; *Pparg2*, peroxisome proliferator-activated receptors gamma 2; qRT-PCR, quantitative real-time polymerase chain reaction; REV, reverse; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; *Slc2a4*, glucose transporter type 4.

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Introduction

In recent years, the basal insulin analog B29Lys-myristoyl des-B30 human insulin (NN-304), known as detemir, has been introduced in clinical practice [1]. Insulin detemir was developed to improve upon the limitations of NPH insulin and other conventional basal insulins, which have inadequate duration of action, marked peak glucose-lowering effect, and variability in response from one injection to another. Detemir represents human insulin with a B-chain shortened by the C-terminal threonine B30 and acylated with myristic acid at the ε-amino group of lysine B29 [1]. The protracted action of this soluble insulin analog *in vivo* is due to self-association into hexamers and dihexamers at the injection site and to reversible binding to serum albumin in the circulation (97–98% of circulating detemir is estimated to be albumin-bound), both being mediated by the myristic acid moiety [2]. Despite similar glucose-lowering effects, a series of clinical studies carried out in both type 1 and type 2 diabetic patients has demonstrated that detemir is weight-neutral or provokes only minor body weight gain when compared to long-acting insulins based on the human insulin structure and insulin glargine [3–8]. The mechanisms behind this unique weight-sparing effect of detemir are still unclear. A relative shift in liver vs. peripheral distribution, potentially resulting in greater hepatic vs. adipose tissue effects [9], and increased brain signaling, possibly associated with greater anorexic effect [10], have been postulated. However, whether detemir may regulate adipogenesis and/or lipogenesis in human adipose stem cells (ASC) differently than human insulin has not been investigated.

In this study, we have assessed the adipogenic and lipogenic potential of insulin detemir compared to human insulin using human subcutaneous and visceral ASC induced to differentiate into mature adipocytes. We show that detemir induces human adipocyte differentiation to a lower extent compared to human insulin.

Methods

Materials

Human insulin was purchased from Roche Diagnostics (Mannheim, Germany). Insulin detemir was kindly provided by Novo Nordisk (Copenhagen, Denmark). Rosiglitazone was kindly provided by GlaxoSmithKlein. Anti-phospho-Akt (pAkt S^{473}), anti-Akt, anti-phospho-Erk (pErk-1/2 T^{202}/Y^{204}), anti-Erk, anti-phospho-HSL (pHSL S^{552}), anti-HSL, anti-phospho-ACC (pACC S^{79}) and anti-ACC antibodies were supplied by Cell Signaling Technology (Frankfurt, Germany); anti-phospho-Tyr antibody (PY99), anti-insulin receptor (IR) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). All tissue culture reagents were from Life Technologies. Reagents for SDS–PAGE were supplied by Bio-Rad (Munich, Germany). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, US) unless otherwise stated.

ASC isolation and culture

Paired biopsies of the abdominal subcutaneous (subcutaneous) and omental (visceral) adipose tissue were obtained from 17 male [average age 66 yr (range 32–82), average BMI 31 kg/m^2 (range 21–49)], and 8 female [average age 51 yr (range 24–75), average BMI 41 kg/m^2 (range 24–61)] subjects undergoing elective open-abdominal surgery. None of the patients had diabetes or severe systemic illness, and none was taking medications known to affect adipose tissue mass or metabolism. The protocol was approved by the Independent Ethical Committee at the Azienda Ospedaliero-Universitaria Policlinico Consorziale, University of Bari School of Medicine, and all patients gave their written informed consent. Adipose tissue biopsies were processed, as previously reported [11,12], in order to obtain ASC. Cell cultures were grown in serum-containing medium and maintained at 37 °C with 5% CO_2. Confluent cells at passage 4 were changed to a chemically defined serum- and BSA-free medium containing 100 nM human insulin, 100 nM detemir, 1 μM detemir, or no insulin before induction of adipocyte differentiation; these insulin concentrations, although supraphysiological, are required to fully induce adipogenesis in this experimental setting. For differentiation into adipocytes, two days after confluence, the cells were washed and incubated in DMEM/F-12 medium containing 2% (vol./vol.) equine serum, insulin, 100 nM dexamethasone (Sigma Chemical), 1.0 nM triiodothyronine (Sigma Chemical), 10 μM rosiglitazone (kindly provided by S. Smith, GSK Pharmaceuticals, Harlow, Essex, UK), and, for the first 4 days of culture, 25 μM/l 3-isobutyl-1-methylxanthine (Sigma Chemical). This medium was changed every 2–3 days, until full differentiation was achieved (in about 30 days), in line with recently published methods for differentiation of human pre-adipocytes, as previously described [11–13].

Oil-Red-O staining of triglyceride-containing cells and analysis of lipid droplet size

Oil-Red-O staining of tissue cell cultures was performed to monitor progression of adipocyte differentiation, as described previously [11]. The stained lipid droplets were visualized by light microscopy. To yield a quantitative measure of triglyceride accumulation (i.e. lipogenesis), the optical density at 510 nm of the eluted Oil-Red-O stain was determined using AD340S absorbance detector (Beckman Coulter, Inc., Brea, CA, US). Fixed cells were also photographed at 20× magnification to perform a morphometric analysis on lipid droplets using ImageJ software, as previously described [12].

Nile Red staining and assessment of ASC conversion to adipocytes

Fixed cells were stained with Nile Red according to a published protocol [14] using DAPI as nuclear counterstain. The adipocyte conversion rate was assessed by