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Oral insulin (human, murine, or porcine) does not prevent diabetes in the non-obese diabetic mouse

Minh N. Pham [a], Claire Gibson [a], Anna K.E. Rydén [a], Nikole Perdue [a], Tamar E. Boursalian [a], Philippe P. Pagni [a,c], Ken Coppieters [b], Christian Skonberg [b], Trine Porsgaard [b], Matthias von Herrath [a,c], Jose Luis Vela [a,*]

[a] Type 1 Diabetes R&D Center, Novo Nordisk Research Center, Seattle, WA, USA
[b] Novo Nordisk A/S, Måløv, Denmark
[c] Type 1 Diabetes Center, La Jolla Institute for Allergy and Immunology, La Jolla, CA, USA

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ABSTRACT

Studies have shown oral insulin prevents type 1 diabetes (T1D) in mouse models, however human trials were inconclusive. We tested the ability of different insulins to prevent T1D in non-obese diabetic mice. Mice received oral insulin or PBS twice weekly and disease was monitored. Contrary to previous studies, no insulin tested showed significant ability to prevent T1D, nor did testing of linked suppression in a delayed type hypersensitivity model have reproducible effect. To investigate delivery of antigen within the GI tract, blue dye was fed to mice. Dye traveled 5–8 cm from stomach to small intestine within 10 s, suggesting orally administered antigen may not get digested in the stomach in mice. Insulin incubated with jejunum extracts was instantly digested. Thus, in humans large doses of insulin may be required to achieve tolerance as antigen may be more vulnerable to digestion in the stomach even before reaching the small intestine.

1. Introduction

The NOD mouse is considered a useful animal model of human type 1 diabetes, and can be used to study both the genetic and immunologic causes and to test the efficacy of treatments designed to prevent or reverse type 1 diabetes [1]. However, different NOD mouse colonies vary in type 1 diabetes incidence due to factors such as colony location and intestinal flora, which may therefore affect the reproducibility of data across different laboratories [1,2]. In addition, several treatments which have been successful at preventing or reversing type 1 diabetes in the NOD mouse have not resulted in positive outcomes in human clinical trials [1]. Thus more information is needed to help predict whether treatments which are able to prevent type 1 diabetes in NOD mice have a chance of successful translation to human disease.

The oral feeding of self antigens to promote tolerance and prevent type 1 diabetes appeared a viable and promising approach based on early studies in NOD mice [3–7]. When compared with PBS-fed mice, the onset of type 1 diabetes was delayed and incidence was reduced in 5 week old NOD mice when 1 mg of porcine insulin was fed twice weekly for 5 weeks and then weekly until the animals reached 1 year of age [5,6].

Several clinical trials have since investigated the efficacy of oral administration of insulin to prevent type 1 diabetes in children or adults at high risk of developing the disease [8,9] and in patients with recent onset type 1 diabetes [10]. None of these trials met their metabolic endpoint but they did provide evidence of subgroup effect and biomarker information [11]. The Immunotherapy Diabetes (IMDIAB) group investigated the efficacy of 5 mg oral insulin in patients recently diagnosed with type 1 diabetes and found no effect on residual beta-cell function (IMDIAB VII) [10]. No prevention of type 1 diabetes was observed and mean C-peptide, HbA1c, and insulin requirements in patients administered with oral insulin were similar to those treated with placebo. In the multi-center randomized, controlled Diabetes Prevention Trial-Type 1 (DPT-1) the effect of oral insulin compared with placebo was tested in relatives of patients with type 1 diabetes. This was a large study, however neither delay of onset, nor prevention of disease was observed [8], and a longitudinal data analysis indicated that treatment with oral insulin did not affect levels of insulin autoantibodies over time [12]. Trials of oral insulin in groups at risk of type 1 diabetes are ongoing and the evaluation of IAA affinity, autoantibody subtype and T cell response to insulin may identify an immunological response during these studies. Finally, a recent publication by the Pre-Point Study group investigated the immune responses and adverse events associated with orally administered insulin in auto-antibody negative, genetically at-risk children [9]. In this small pilot study (3 children in each treatment group and 10 in the placebo group), the investigators reported that more than 80% of children with high risk for developing type 1

* Corresponding author at: Novo Nordisk Research Center, 530 Fairview Avenue North, Seattle, WA 98019, USA.
E-mail address: jvl@novonordisk.com (J.L. Vela).

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diabetes who received daily high-dose insulin capsules of 67.5 mg, showed potential evidence of an immune response that could be protective for type 1 diabetes and no hypoglycemia after treatment when compared with placebo-treated children.

Oral administration of insulin to induce tolerance and prevent type 1 diabetes is an appealing route, but the results from human clinical trials have so far been inconclusive. The aim of this study was to repeat early oral tolerance studies in the NOD mouse and extend the scope of these trials to test the ability of different types of insulin to induce oral immune tolerance and prevent type 1 diabetes. Results from this study may help to explain why the initial positive results in the NOD mice have not translated into success in human clinical trials and may further inform the design of future clinical trials.

2. Materials and methods

2.1. Mice

Female NOD and Balb/c mice (8 weeks of age upon delivery), were purchased from the Jackson Laboratory, (Sacramento, CA, USA). Male C57BL/6J mice were purchased from Taconic Denmark. All mice were acclimatized for at least one week in-house prior to the start of experiments. The mice were housed with free access to filtered water and standard mouse chow (Purina Lab Diet 5053, irradiated) in a room with a 12 h day/night cycle. All procedures were approved by the Institution of Animal Care and Use Committee (IACUC) of Novo Nordisk Research Center (NNRC), Seattle, WA, USA, and Novo Nordisk, Måløv, Denmark.

2.2. Assessment of diabetes

Mice were weighed weekly and blood glucose values were monitored weekly by tail vein bleeding using a blood glucose meter (Bayer Contour USB, Whippany, NJ, USA) until the end of the experiment. Mice with a blood glucose level of ≥250 mg/dL on two consecutive days were defined as diabetic. Mice were sacrificed immediately upon reaching a blood glucose value of ≥600 mg/dL or when their overall condition was deteriorating, as determined by weight and/or overall appearance based on NNRC and IACUC guidelines. Mice that were diabetic at the start of the experiment were excluded from the study.

2.3. Oral administration of antigen

In the first experiment 9 week old NOD mice were fed *per os* (p.o.) through a blunt-ended curved oral gavage needle with one of the following treatments: sterile phosphate-buffered saline (PBS) (Life Technologies, Grand Island, NY) (N = 50); 1 mg porcine insulin (Sigma Aldrich, St. Louis, MO, USA) (N = 36); 1 mg murine insulin 1 (Novo Nordisk, Måløv, Denmark) (N = 23); 5 mg native human insulin (Novo Nordisk, Måløv, Denmark) (N = 21); or 1 mg inactive human insulin analog [7] (Novo Nordisk, Måløv, DK) (N = 26). In the second experiment 5 week old NOD mice were fed p.o. with sterile PBS (N = 31); or 1 mg porcine insulin (Sigma) (N = 29). The dosing volume for all treatment groups was 400 μL. All mice received a total number of 10 doses twice a week. They were monitored until 30 weeks of age for weight, blood glucose and health status.

2.4. Delayed-type hypersensitivity (DTH) response

Delayed-type hypersensitivity (DTH) responses in the skin are used to assess cell mediated immunity in vivo. An antigen is introduced intradermally (i.d.) and induration and erythema at 48–72 h post-injection indicate a positive reaction. In these experiments, in vivo tolerization was performed by oral administration of porcine insulin (Sigma) at 1 mg per dose in 0.4 ml PBS (pH 2.5), for 2 weeks (first experiment) or 3 weeks (second experiment), twice weekly with the final administration six days after sensitization, (the day before re-challenge). Mice were sensitized with either Keyhole Limpet Hemocyanin (KLH) (Thermo Fisher Scientific, Grand Island, NY) in Complete Freund's Adjuvant (CFA) (Difco, Detroit, MI), intraperitoneally (i.p.), or KLH plus 50 μg porcine insulin in CFA. Seven days after sensitization, mice were anesthetized with 5% isoflurane, and baseline ear thickness was measured with a micrometer gauge (Mitutoyo USA, Aurora, IL). For the re-challenge, 20 μl PBS was administered i.d. in the right ear pinnae, while the left ear was given either 25 μg KLH in 20 μl PBS, or 25 μg KLH plus 25 μg porcine insulin in 20 μl PBS (Experiment 1). In a second experiment, 25 μg KLH in 20 μl PBS was administered i.d. in the right ear pinnae, and 25 μg KLH plus 12.5 μg porcine insulin formulated in protamine sulfate (Sigma) was administered in the left ear pinnae. In all experiments, ear thickness was measured again at 24 and 48 h post-challenge to assess DTH response and expressed as the change from baseline ear thickness.

2.5. Transport of antigen within the murine intestinal tract

To measure how far insulin can potentially travel in the gastrointestinal tract, 10 male C57BL/6J mice (fasted prior to administration for 18 h) were fed p.o. with 5% Evans blue solution (Sigma) at the following doses: 1) 1 ml/kg (N = 3); 2) 4 ml/kg (N = 3); or 3) 10 ml/kg (N = 4). Mice were sacrificed by cervical dislocation. The abdominal cavity was immediately opened and intestines were gently taken out in order to measure how far the 5% Evans blue solution traveled down the intestine.

2.6. Insulin degradation within the intestinal tract

Human insulin (10 μM) was incubated with pepsin (10 U/ml), from porcine gastric mucosa (Sigma-Aldrich Chemie GmBH (Darmstadt, Germany) in 0.05 M glycine-HCl buffer with 0.005% BSA (pH 2.0, 37 °C) for 90 min. At selected time-points, aliquots were taken from the incubations and the enzymatic reaction was stopped by the addition of three volumes of ice-cold 96% ethanol. Samples were then centrifuged at 4400 *g*, and the supernatants were stored at −20 °C until analysis by LC–MS as above. This experiment was performed once with three replicates.

Human insulin was also incubated with luminal extracts from the small-intestine. Jejunum extracts from rats were procured in-house at Novo Nordisk after euthanizing male Sprague–Dawley rats (Charles River Laboratories, Germany). The peritoneum was opened and a piece of approximately 20 cm of the mid-jejunum (measured between pylorus and caecum) was resected and kept in ice-cold isotonic saline. The resected pieces of intestine were flushed with 2 ml ice-cold isotonic saline and lightly squeezed in the longitudinal direction in order to push out chyme and mucus. The contents were collected in centrifuge tubes and centrifuged at 4500 *g* for 10 min (4 °C). The resulting supernatant (the 'extract') was aliquoted into eppendorf tubes and stored at −80 °C until use.

Incubations of human insulin with intestinal extracts were performed by diluting the respective extracts ten times with incubation buffer (HBSS with Ca/Mg, 10 mM HEPES and 0.005% BSA, pH 6.5), and adding human insulin to a final concentration of 10 μM. Incubations were performed in 96-semi-deep-well plates (Costar, Corning, NY, USA) at 37 °C on a Thermo-mixer (Eppendorf AG, Hamburg, Germany). At selected time-points aliquots were taken from the incubations and transferred to three volumes of cold ethanol with 1% formic acid. Samples were centrifuged at 14,000 *g* and the supernatant was stored at −20 °C until analysis by liquid chromatography–mass spectrometry (LC–MS) on a Waters Acquity UPLC (Waters corp., Milford, MA, USA) coupled to a MaXis 4G q-ToF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The experiments were performed four times with 2–3 replicates per experiment.