Original article

MOLECULAR METABOLISM

Insulin-like peptide 5 is a microbially regulated peptide that promotes hepatic glucose production

Ying Shiuan Lee [1], Filipe De Vadder [1,2,3,4], Valentina Tremaroli [1], Anita Wichmann [1,6], Gilles Mithieux [2,3,4], Fredrik Bäckhed [1,5,*]

ABSTRACT

Objective: Insulin-like peptide 5 (INSL5) is a recently identified gut hormone that is produced predominantly by L-cells in the colon, but its function is unclear. We have previously shown that colonic expression of the gene for the L-cell hormone GLP-1 is high in mice that lack a microbiota and thus have energy-deprived colonocytes. Our aim was to investigate if energy deficiency also affected colonic *Insl5* expression and to identify a potential role of INSL5.

Methods: We analyzed colonic *Insl5* expression in germ-free (GF), conventionally raised (CONV-R), conventionalized (CONV-D) and antibiotic-treated mice, and also assessed the effect of dietary changes on colonic *Insl5* expression. In addition, we characterized the metabolic phenotype of *Insl5*−/− mice.

Results: We showed that colonic *Insl5* expression was higher in GF and antibiotic-treated mice than in CONV-R mice, whereas *Insl5* expression in the brain was higher in CONV-R versus GF mice. We also observed that colonic *Insl5* expression was suppressed by increasing the energy supply in GF mice by colonization or high-fat feeding. We did not observe any differences in food intake, gut transit or oral glucose tolerance between *Insl5*−/− and wild-type mice. However, we showed impaired intraperitoneal glucose tolerance in *Insl5*−/− mice. We also observed improved insulin tolerance and reduced hepatic glucose production in *Insl5*−/− mice.

Conclusions: We have shown that colonic *Insl5* expression is regulated by the gut microbiota and energy availability. We propose that INSL5 is a hormone that could play a role in promoting hepatic glucose production during periods of energy deprivation.

Keywords Insulin-like peptide 5 (INSL5); Gut microbiota; Liver; Colon

1. INTRODUCTION

Insulin-like peptide (INSL) 5 is a member of the relaxin/insulin family [1], which comprises insulin, insulin-like growth factor (IGF) 1 and 2 [2—4], relaxin 1 and 2, and INSL3-7 [5], and has been recently identified in colonic and brain tissue [1,6—8]. Although other members of the relaxin/insulin family are known to have roles in glucose metabolism, reproductive physiology and remodeling of connective tissue [5,9—12], the function of INSL5 is not clear. One study based on observations in *Insl5*−/− mice indicated that INSL5 may regulate glucose metabolism by affecting pancreatic beta cell number, but the *Insl5*−/− phenotype was mild and dependent on the genetic background of the mice [13]. Another study reported that INSL5 enhances glucose-stimulated insulin secretion, both *in vivo* and *in vitro* [14]. A more recent study suggested that INSL5 is an orexigenic gut hormone that is upregulated after fasting and calorie restriction [15]. In summary, these studies suggest that INSL5 may have a role in regulation of host energy metabolism.

The gut microbiota is known to contribute to efficient energy harvest from the diet by degrading plant polysaccharides, such as cellulose, xylan, pectin and resistant starch [16—18], and to promote energy storage by modulating the expression of host genes [19]. The microbially produced short-chain fatty acid (SCFA) butyrate is the primary energy source for colonocytes, and thus germ-free (GF) mice (i.e. mice that lack a microbiota) have energy-deprived colonocytes [20]. We recently reported that a lack of microbiota reduced the energy availability in the colon which increased the expression of *Gcg* [the gene for proglucagon, the precursor of glucagon-like peptide-1 (GLP-1)], and proposed that colonic GLP-1 plays an important role in slowing intestinal transit under conditions of calorie restriction [21]. A previous

[1]The Wallenberg Laboratory, Department of Molecular and Clinical Medicine, University of Gothenburg, 41345 Gothenburg, Sweden [2]Institut National de la Santé et de la Recherche Médicale, U855, Lyon 69372, France [3]Université de Lyon, Lyon 69008, France [4]Université Lyon 1, Villeurbanne 69622, France [5]Novo Nordisk Foundation Center for Basic Metabolic Research, Section for Metabolic Receptology and Enteroendocrinology, Faculty of Health Sciences, University of Copenhagen, 2200 Copenhagen, Denmark

[6] Present address: Health and Nutrition Division-Innovation, Chr Hansen A/S, Hørsholm 2970, Denmark.

*Corresponding author. The Wallenberg Laboratory, Department of Molecular and Clinical Medicine, University of Gothenburg, 41345, Gothenburg, Sweden. E-mail: Fredrik.Backhed@wlab.gu.se (F. Bäckhed).

Received December 29, 2015 • Revision received January 16, 2016 • Accepted January 18, 2016 • Available online 25 January 2016

http://dx.doi.org/10.1016/j.molmet.2016.01.007

microarray screen of tissues from GF and conventionally raised (CONV-R) mice revealed that colonic *Insl5* expression is significantly regulated by the microbiota [22]. Because both GLP-1 and INSL5 are secreted from colonic enteroendocrine L-cells, we hypothesized that colonic *Insl5* expression is similarly modulated by energy availability.

Here we investigated how the gut microbiota and energy deficiency affect the colonic expression of *Insl5* and used *Insl5*−/− mice to explore the role of INSL5. We provide evidence that INSL5 plays a role in promoting hepatic glucose production during periods of fasting.

2. MATERIAL AND METHODS

2.1. Mice and diets

GF Swiss Webster and C57Bl/6J mice were maintained in flexible film isolators under a strict 12-hour light cycle. GF status was monitored regularly by anaerobic culturing and PCR for bacterial 16S rRNA. *Insl5*−/− mice on a C57Bl/6J background (Deltagen Target ID#65) were purchased from Jackson Deltagen (San Mateo, CA, USA), backcrossed with our C57Bl/6J mice for >10 generations, and bred in our facility to harmonize the gut microbiota. Thereafter, C57Bl/6 WT and *Insl5*−/− mice were maintained as separate colonies.

Unless otherwise indicated, experiments were performed with male mice aged 12–14 weeks that were fed an autoclaved low-fat polysaccharide-rich chow diet (LabDiet, St Louis, MO, USA) *ad libitum*. For high-fat diet experiments, mice were weaned onto a high-fat, high-sugar western diet with 40% of calories from fat (Adjusted Fat Diet TD.96132, Harlan Teklad, Indianapolis, IN, USA). At the end of the experiments and unless otherwise indicated, mice were fasted for 4 h before being killed, and organs were harvested and flash-frozen in liquid nitrogen. All mouse experiments were performed using protocols approved by the Research Animal Ethics Committee in Gothenburg, Sweden.

2.2. Colonization of GF mice

For colonization with an unfractionated microbiota, GF mice were colonized with total cecal content from a CONV-R donor. The cecal content was resuspended in 5 ml sterile PBS and 200 μl of cecal slurry was given by oral gavage to each GF mouse. The resulting conventionalized (CONV-D) mice were kept in standard cages for 1, 3 or 7 days. For monocolonization experiments, *Bacteroides thetaiotaomicron* VPI-5482 (ATCC 29148) in liquid culture was fed to GF mice. Monocolonized mice were housed in separate sterile isolators for 4 weeks. At the end of the colonization period, mice were fasted for 4 h before killing and tissue harvest. Colonization density by *B. thetaiotaomicron* was verified by culture enumeration.

2.3. Antibiotic treatment

A cocktail of bacitracin, neomycin, and streptomycin (200 mg/kg bodyweight of each antibiotic) (Sigma Aldrich, St Louis, MO, USA) or water (vehicle control) was given by oral gavage to mice daily for 3 days. Colonic tissue from the mice was analyzed on day 4.

2.4. Quantitative RT-PCR (qRT-PCR)

Mouse tissues were homogenized in RLT buffer supplemented with 2-mercaptoethanol using 5 mm steel beads and TissueLyser (Qiagen, Hilden, Germany). RNA was isolated using the RNeasy Kit with on-column DNase I treatment (Qiagen). cDNA was synthesized from the total RNAs using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's manual. qRT-PCR reactions were prepared in a 25 μl volume containing 1× SYBR Green Master Mix buffer (Thermo Scientific, Waltham, MA, USA) and 900 nM specific primers targeting gene of interest (or 300 nM directed against the L32 gene expression). Reactions were run on a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Gene expression data were normalized to the expression level of the ribosomal protein L32 using the $\Delta\Delta C_T$ method and analyzed by calculating relative gene expression. Primer sequences are listed in Supplementary Table 1.

2.5. Immunohistochemistry

Colon tissues were fixed in 4% paraformaldehyde in PBS for 24 h and washed and dehydrated with ethanol. Paraffin-embedded sections (8 μm) were prepared. For staining, sections were deparaffinized and exposed to antigen unmasking in antigen retrieval 2100 using 10 mM sodium citrate buffer pH 6.0. After rinsing, sections were incubated in blocking buffer (10% goat serum, 1% bovine serum albumin and 0.1% Triton X-100 in PBS) for 1 h at room temperature. Sections were stained with anti-GLP-1 mouse monoclonal subtype IgG1 antibody (ab26278, Abcam, Cambridge, UK) diluted 1:400 or anti-peptide YY (PYY) chicken polyclonal antibody (ab15879, Abcam) diluted 1:800, and anti-INSL5 rabbit polyclonal antibody (G-035-40, Phoenix Pharmaceuticals, Burlingame, CA, USA) diluted 1:200 in blocking buffer overnight at 4 °C. Primary antibodies were targeted with immunofluorescent dye labeled secondary antibodies Alexa Fluor 568 anti-mouse IgG1 (γ1) (A21124) or Alexa Fluor 594 Goat anti-chicken (A11042) and Alexa Fluor 488 Goat anti-rabbit IgG (A11008), all diluted 1:1000 (Life Technologies, Carlsbad, CA, USA). Cell nuclei were counterstained with Hoechst 33342 nucleic acid stain (H1399, Life Technologies).

2.6. Measurements of body weight, total body fat content and food intake

WT and *Insl5*−/− mice were weaned on to standard chow diet at 3 weeks of age and were weighed once a week. For total body fat measurements, mice were anesthetized with isoflurane gas and dual-energy X-ray absorptiometry (DEXA) was performed by using the small animal densitometer (Lunar PIXImus Mouse, GE Medical Systems, Waukesha, WI, USA). Food intake was measured over 1 h in mice subjected to a previous 12 h fast.

2.7. Upper GI transit

WT and *Insl5*−/− mice were fasted overnight with *ad libitum* access to water. In the morning, mice were gavaged with 200 μl of 1.5% methylcellulose containing 5% Evans blue dye (Sigma–Aldrich). After 15 min, mice were killed, and the intestine from the region of the pyloric sphincter to the ileo-caecal junction was removed. The gut transit is presented as the distance the Evans blue dye traveled as a percentage of the whole length of the small intestine.

2.8. Oral and intraperitoneal glucose tolerance tests

Mice were fasted for 6 h and given either an oral gavage of glucose (2 g/kg body weight) or an intraperitoneal injection of glucose (1 g/kg body weight). Tail blood was collected and blood glucose measured with HemoCue 201+ analyzer (HemoCue, Ängelholm, Sweden) before (30 and 0 min) and after (15, 30, 60, 90 and 120 min) gavage or injection. Tail blood was also collected with Microvette CB 300 Z (Sarstedt, Nümbrecht-Rommelsdorf, Germany) for serum insulin analysis using the Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) according to the manufacturer's protocol.

2.9. Insulin tolerance test and pyruvate tolerance test

Tolerance tests were performed in WT and *Insl5*−/− mice given an intraperitoneal injection of insulin (0.75 U/kg bodyweight after a 6 h

