Bone 125 (2019) 178–185

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

GLP-2 and GIP exert separate effects on bone turnover: A randomized, placebo-controlled, crossover study in healthy young men

Kirsa Skov-Jeppesen[a,b], Maria S. Svane[b,c], Christoffer Martinussen[b,c], Maria B.N. Gabe[a], Lærke S. Gasbjerg[a,b], Simon Veedfald[a,b], Kirstine N. Bojsen-Møller[c], Sten Madsbad[c], Jens J. Holst[a,b], Mette M. Rosenkilde[a,b], Bolette Hartmann[a,b,*]

[a] Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark
[b] Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Copenhagen, Denmark
[c] Department of Endocrinology, Hvidovre University Hospital, Hvidovre, Denmark

ARTICLE INFO

Keywords:
Bone resorption
Bone formation
PTH
CTX
P1NP

ABSTRACT

Background: Glucagon-like peptide-2 (GLP-2) and glucose-dependent insulinotropic polypeptide (GIP) both inhibit bone resorption in humans but the underlying mechanisms are poorly understood. In vitro, GLP-2 activates the GIP-receptor (GIPR).

Objective: Based on in vitro studies, we hypothesized that the antiresorptive effect of GLP-2 was mediated through the GIPR. This was tested using the selective GIPR-antagonist GIP(3-30)NH_2.

Methods: The study was a randomized, single-blinded, placebo-controlled, crossover study conducted at Hvidovre University Hospital, Denmark.

Eight healthy young men were included and studied on four study days: GIP (200 μg), GLP-2 (800 μg), GIP(3-30)NH_2 (800 pmol/kg/min) + GLP-2 (800 μg), and placebo.

The main outcomes were bone resorption measured as collagen type 1 C-terminal telopeptide (CTX) and bone formation measured as procollagen type 1 N-terminal propeptide (P1NP).

Results: CTX (mean ± SEM) significantly decreased after both GIP (to 55.3 ± 6.3% of baseline at $t = 90$ min) and GLP-2 (to 60.5 ± 5.0% of baseline at $t = 180$ min). The maximal reduction in CTX after GIP(3-30)NH_2 + GLP-2 (to 63.2 ± 3.1% of baseline) did not differ from GLP-2 alone ($p = 0.95$) nor did net $AUC_{0–240}$ (−6801 ± 879%*min vs −6027 ± 648%*min, $p = 0.56$). At $t = 30$ min, GIP significantly ($p < 0.0001$) increased P1NP to 115.1 ± 2.2% of baseline compared with 103.1 ± 1.5% after placebo. Both GLP-2 and GIP(3-30)NH_2 + GLP-2 significantly ($p < 0.0001$) decreased P1NP to 91.3 ± 1.1% and 88.1 ± 3.0% of baseline, respectively (at $t = 45$ min) compared with placebo.

Conclusions: GIPR antagonism did not inhibit the GLP-2-induced reduction in bone resorption (CTX) in healthy young men. In contrast to GLP-2, GIP increased P1NP despite decreasing CTX indicating an uncoupling of bone resorption from formation. Thus, GLP-2 and GIP seem to exert separate effects on bone turnover in humans.

Clinical trials information: ClinicalTrials.gov (NCT03159741).

1. Introduction

Bone is remodeled throughout life to maintain calcium homeostasis and bone strength. Bone remodeling involves two tightly coupled processes; the osteoclastic bone resorption and the osteoblastic bone formation regulated by numerous systemic and local factors [1,2]. Markers of bone turnover can be used to assess the bone remodeling with the recommended markers being collagen type 1 C-terminal telopeptide (CTX) for bone resorption and procollagen type 1 N-terminal propeptide (P1NP) for bone formation [3]. CTX exhibits a diurnal variation with high levels during the night and low levels during the daytime [4,5]. A large part of this variation relates to gastrointestinal hormones secreted upon nutrient ingestion [6–8]. Thus, a gut-bone axis has been proposed [5–8] in which the two gastrointestinal hormones glucagon-like peptide-2 (GLP-2) and glucose-dependent insulinotropic polypeptide (GIP) have been suggested as mediators [7–12]. In healthy humans,

* Corresponding author at: University of Copenhagen, Department of Biomedical Sciences, The Panum Institute, Building 12.2, Blegdamsvej 3, DK-2200 Copenhagen, Denmark.
E-mail address: bhartmann@sund.ku.dk (B. Hartmann).

https://doi.org/10.1016/j.bone.2019.05.014
Received 22 January 2019; Received in revised form 15 April 2019; Accepted 11 May 2019
Available online 15 May 2019

GLP-2 reduces bone resorption (measured as CTX) when administered by subcutaneous (s.c.). injection in the morning [8,13,14] as well as at bedtime [9]. Moreover, GLP-2 treatment shows no sign of tachyphylaxis or generation of GLP-2 antibodies [10,11] and four months of GLP-2 treatment has been demonstrated to increase bone mineral density (BMD measured by DEXA) [11]. GIP infusion decreases bone resorption (measured as CTX) in healthy humans and in patients with type 1 diabetes (which shows that the effect is independent of endogenous insulin) [12,15]. Furthermore, in a large prospective cohort study, human carriers of a common mutation in the GIP receptor (GIPR) (Glu35Gln (rs1800437)) had a significantly lower BMD at a 10-year follow up, and a > 50% increased risk of non-vertebral fractures after 16 years of follow up compared to persons without the mutation [16]. Thus, GLP-2, GIP, as well as the GIPR seem to play important roles in the regulation of bone turnover and with a clinically relevant impact.

In vitro studies have shown that GIPRs are expressed on osteoclasts, osteoblasts, and osteocytes [17,18] suggestive of a direct effect of GIP. The importance of this is reflected in the finding that GIPR knock-out mice have lower bone mass and poorer bone quality compared with wild type mice [19,20]. GLP-2 receptors (GLP-2R) have so far not been identified on bone cells and the mechanism underlying the anti-resorptive effect of GLP-2 remains unknown.

In a preliminary in vitro screen for cross-reactivity among ligand:receptor pairs belonging to class B receptors, we recently discovered that GLP-2 activates the GIPR. Combined with the common promiscuity among class B receptors [21,22] also observed among peptide GPCRs belonging to class A receptors [23], this observation led us to speculate that the antiresorptive effect of GLP-2 might be mediated through the GIPR. The aim of the present study was to investigate whether antagonizing the GIPR would prevent the GLP-2-induced reduction in bone resorption (measured as CTX) in humans. This was tested using a newly developed selective GIPR-antagonist, GIP(3-30)NH_2 [24–26].

2. Materials and methods

2.1. In vitro transfection and tissue culture

COS-7 cells were cultured at 10% CO_2 and 37 °C in Dulbecco's modified Eagles medium 1885 supplemented with 10% FBS, 2 mmol/L glutamine, 180 units/mL penicillin, and 45 g/mL streptomycin. Transient transfection was performed using the calcium phosphate precipitation method [27].

2.2. In vitro competition binding and cAMP measurements

Transiently transfected COS-7 cells were seeded in 96-well plates one day after transfection (10.000 cells/well in clear plates for the binding and 35.000 cells/well in white plates for cAMP measurements) and the experiments carried out the following day. Binding: Cells were washed twice in binding buffer (50 mmol/L HEPES buffer (pH 7.2) supplemented with 0.5% bovine serum albumin) and assayed by competition binding for 4 h at 4 °C using 15–40 pmol/L ^{125}I-human GIP(1-42) as well as unlabeled ligand in binding buffer. After incubation, cells were washed twice in ice-cold binding buffer and lysed using 200 mmol/L NaOH with 1% SDS for 30 min. Nonspecific binding was determined as the binding in the presence of 100 nmol/L unlabeled human GIP. The samples were analyzed for radioactivity using a Wallac Wizard 1470 Gamma Counter. cAMP: cells were washed twice with HEPES-buffered saline (HBS) buffer and incubated with HBS and 1 mmol/L 3-isobutyl-1-methylxanthine for 30 min at 37 °C [28] and measured using the HitHunterTM cAMP XS assay (DiscoveRx, Herlev, Denmark) according to the manufacturer's instructions. To test agonistic properties, ligands were added and incubated for 30 min at 37 °C. In order to test for antagonistic properties, the cells were pre-incubated with antagonist for 10 min followed by addition of the agonist and incubation for 20 min. The in vitro pharmacological experiments were evaluated using GraphPad Prism. Sigmoid curves were fitted logistically with a Hill slope of 1.0. Calculations of K_i values were based on the Cheng Prussoffs formula [29].

2.3. Human study design and participants

The study protocol was approved by the Scientific Ethical Committee of the Capital Region of Denmark (protocol no. H-16047626) and the Danish Data Protection Agency (journal no. SUND-2017-21), and registered at ClinicalTrials.gov (NCT03159741). The study complies with the World Medical Association Declaration of Helsinki. All participants gave written informed consent before inclusion. The study was conducted using a randomized, placebo-controlled, single-blinded, crossover design. Sequences were computer-generated using a Williams design and participants were randomly assigned to the sequences. Each participant was studied on the four days separated by at least five days. The participants were blinded to the sequences by the use of placebo infusions/injections. On all study days, GIP(3-30)NH_2 or placebo was infused intravenously (i.v.) ($t = -20$ to 240 min) and a s.c. injection of GIP, GLP-2, or saline was administered ($t = 0$) in the following combinations: GIP day (placebo infusion + s.c. GIP); GLP-2 day (placebo infusion + s.c. GLP-2); GIP(3-30)NH_2 + GLP-2 day (GIP(3-30)NH_2 infusion + s.c. GLP-2); and placebo day (placebo infusion + s.c. saline).

The study was conducted at the Department of Endocrinology, Hvidovre University Hospital, Denmark. Study participants were recruited from the website www.forsøgsperson.dk and by advertisement at the University of Copenhagen. Inclusion criteria were male gender; age between 20 and 40 years old; and body mass index (BMI) between 18.5 and 24.9 kg/m^2. Exclusion criteria were chronic disease; present or past smoking; alcohol or drug abuse; use of any medication within the last 3 months; weight changes of > 3 kg within the last 3 months; hemoglobin level below 8.0 mmol/L; decreased renal function (eGFR < 90 mL/min/1.73 m^2); and any previous disease, medication, or surgery believed to influence the study outcome.

2.4. Study procedure

At a separate screening visit, demographic and baseline characteristics (age, height, weight, BMI, blood pressure, heart rate, Hb1Ac, hemoglobin, and vitamin D) were collected. Participants were informed to avoid strenuous physical activities and abstain from alcohol 48 h prior to each study day.

On study days, participants arrived at 8–9 am after an overnight fast (at least 9 h). Participants were recumbent in a hospital bed during the experiments. Catheters were inserted in cubital veins: one for the infusion and one for blood sampling. Participants then rested for 10 min before baseline blood sampling ($t = -30$ min and $t = -25$ min). Previous studies report changes in blood flow and cardiac parameters in response to GIP and GLP-2 [25,30,31] and therefore, we measured heart rate and blood pressure before each blood sampling. At $t = -20$ min, a continuous infusion of either GIP(3-30)NH_2 or placebo was commenced. At $t = 0$ min, GIP, GLP-2, or saline was injected s.c. in the umbilical region over approximately 1 min. Blood was sampled frequently for a total of 240 min. After the last blood sample ($t = 240$ min), the infusion was discontinued and the study day ended.

2.5. Intravenous infusions of GIP(3-30)NH_2 or placebo

The GIPR-antagonist, synthetic human GIP(3-30)NH_2 (Caslo ApS, Lyngby, Denmark) (structure and purity > 98% confirmed by mass, sequence, and HPLC analysis) was formulated by the Capital Region Pharmacy (Herlev, Denmark). GIP(3-30)NH_2 was dissolved in a sodium hydrogen carbonate buffer with 0.5% human serum albumin (HSA, CSL Behring, Marburg, Germany) followed by sterile filtration and distribution into vials. The formulation was tested for sterility and

endotoxins (LAL, Ph.Eur). On study days, GIP(3-30)NH_2 was diluted in sodium chloride (NaCl 9 mg/mL, Fresenius Kabi AB, Uppsala, Sweden) containing 0.5% HSA, to a final volume of 500 mL with a concentration appropriate for an infusion of 800 pmol/kg/min. Placebo infusions consisted of 500 mL NaCl 9 mg/mL containing 0.5% HSA. The i.v. infusions were infused with a rate of 1.92 mL/min using an infusion pump (Infusomat®, B. Braun Melsungen AG, Melsungen, Germany).

2.6. Subcutaneous injections of GIP, GLP-2, or saline

Synthetic human GIP(1-42) (PolyPeptide Group, Strasbourg, France) and synthetic human GLP-2(1-33) (Bachem, Bubendorf, Switzerland) (purity > 97%) were dissolved in a sodium hydrogen carbonate buffer with 0.5% HSA in NaCl 9 mg/mL to a final concentration of 100 μg/mL and 400 μg/mL, respectively, and distributed into 1 mL vials. Solutions were prepared and tested (LAL, Ph.Eur) by the Capital Region Pharmacy (Herlev, Denmark). All vials were stored at −20 °C.

For placebo, saline (NaCl 9 mg/mL) was injected. On each study day, a total volume of 2 mL was s.c. administered as two times 1 mL corresponding to doses of 200 μg GIP(1-42) and 800 μg GLP-2(1-33), respectively. The dose of 200 μg GIP(1-42) was selected based on our previous study where a significant decrease in CTX was seen during infusion at physiological concentrations [12]. The dose of 800 μg GLP-2 was also based on previous observations showing 30–40% reduction in CTX using this dose [12].

2.7. Blood samples

Blood samples were drawn at the following time points relative to the s.c. injection: $t = -30, -25, -10, -5, 7, 15, 30, 45, 60, 90, 120, 150, 180, 210$, and at 240 min. Blood for plasma was collected into chilled tubes containing EDTA and dipeptidyl peptidase-4 inhibitor (valine pyrrolidide, final concentration 0.01 mmol/L, a gift from Novo Nordisk). Blood for serum was collected into clot activator tubes and left at room temperature for 30 min for coagulation. All tubes were centrifuged for 10 min (1200 × *g*, 4 °C). Plasma glucose was analyzed using an YSI model 2300D STAT plus analyzer (YSI Incorporated, Yellow Springs, Ohio, USA). Plasma and serum were stored at −20 °C.

2.8. Measurements

Blood pressure and heart rate were measured before each blood sampling using a standard blood pressure monitor (Omron M6, Intelli Sense, Omron Healthcare Europe B. V., Hoofddorp, The Netherlands). Serum CTX, intact P1NP, osteocalcin (OC), and intact parathyroid hormone (PTH) were measured on an IDS-iSYS Multi-Discipline Automated System® (ImmunoDiagnosticSystems, Frankfurt am Main, Germany) by the Automated Chemiluminescence Immunoassay method.

Serum insulin and C-peptide were analyzed by sandwich immunoassay using the Chemiluminescent Technology (Advia Centaur XP, Siemens). Plasma samples were extracted at a final concentration of 70% ethanol before GIP measurements and 75% ethanol before GLP-2 measurements. Total GIP concentration was measured with an in-house radio-immuno assay (RIA) using an antibody directed towards the C-terminal (code no. 80867), which reacts fully with intact and N-terminally truncated GIP [32]. Intact GIP was determined using antiserum no. 98171, which reacts with the N-terminus of intact GIP [33]. The standard was human GIP (Bachem, cat no. H-5645) and the tracer was ^{125}I-labeled human GIP (Perkin Elmer, cat no. Nex402). Intact GLP-2 was measured by in-house RIA [34] using the antiserum (code no. 92160), which is specific for the intact N-terminus of GLP-2. For standards, we used recombinant human GLP-2 and the tracer was ^{125}I-labeled rat GLP-2 with an Asp33 → Tyr33 substitution. GIP(3-30)NH_2 was measured using antibody (code no. 95234) and human GIP(1-30)NH_2 (Phoenix Pharmaceuticals, cat no. 027-30) as standard and the tracer was ^{125}I-labeled human GIP [24]. Sensitivity for all assays was below 5 pmol/L, and intra-assay coefficient of variation was below 10%.

2.9. Statistical analysis

Based on previous studies [8,13,14], we calculated that a minimum of 8 participants would be necessary to detect a difference of 20% in CTX with a power of 90%, two-sided 5% significance level, and a SD of 13%. CTX, P1NP, OC, and PTH are expressed as percentage of baseline (baseline calculated as mean of $t = -30$ and $t = -25$). Differences at time points between groups were analyzed by two-way repeated-measures ANOVA and when relevant Tukey's post hoc test. Net AUC was defined as the algebraic sum of AUCs above and below baseline values. For CTX the net AUC were evaluated for the time periods (0–180 min) and (0–240 min) because of the difference in the timing of the effects of the two hormones. Likewise, the net AUC were evaluated for the time periods (0–60 min) and (0–240 min) for P1NP. For CTX and P1NP, one-way repeated-measures ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparisons test were used post hoc to compare differences in net AUC between study days. Concentrations of GIP, GLP-2, GIP(3-30)NH_2, insulin, C-peptide, and glucose as well as blood pressure and heart rate are presented as absolute values. Results are reported as mean ± SEM. For demographic and baseline characteristics, data are presented as median and range. A two-sided p-value < 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism Version 7.00 for Windows (GraphPad Software, LA, Jolla California USA).

3. Results

3.1. GLP-2 binding and activation of the human GIP receptor in vitro

The most common signaling pathway for class B1 G protein-coupled receptor involves cAMP production as a result of receptor-mediated activation of adenylate cyclase via Gαs. Using this readout, we found that human GIP and GLP-2 activated their cognate receptors with potencies (EC_{50}) of 24 and 107 pmol/L, respectively (Fig. 1a and b). Whereas GIP displayed no activation of the GLP-2R (Fig. 1b), GLP-2 activated the GIPR with an EC_{50} of 108 nmol/L (Fig. 1a). To analyze the interaction of the two agonists on the GIPR, we used the novel selective GIPR-antagonist GIP(3-30)NH_2 [26,35–37] and observed that it inhibited both the GIP and the GLP-2-induced activation of the GIPR with similar potencies (IC_{50} of 12 and 24 nmol/L, respectively) (Fig. 1c). Finally, we determined the affinity of GLP-2 for the human GIPR. Using the same cellular background as for the cAMP experiments and ^{125}I-GIP (1-42) as radioligand, we observed a Ki of 50 nmol/L, thus only 16-fold lower than the Kd for GIP (3.2 nmol/L) (Fig. 1d). Intrigued by these in vitro data showing GLP-2 binding and activation of the human GIPR, we continued with the human study.

3.2. Participants

Eight healthy, non-smoking, Caucasian men with a median age of 27 years (range 20–34 years), and a median body mass index of 22.6 kg/m^2 (range 21.1–23.9 kg/m^2) were included in the study, and all participants completed the four study days within three months from inclusion (no drop-outs). The study was conducted between May 16, 2017 and September 7, 2017. All participants were included in the final analysis. Peptide administrations (GIP, GLP-2, and GIP(3-30)NH_2) were well tolerated with no side effects reported by the participants. Demographic and baseline characteristics of participants are summarized in Table 1.

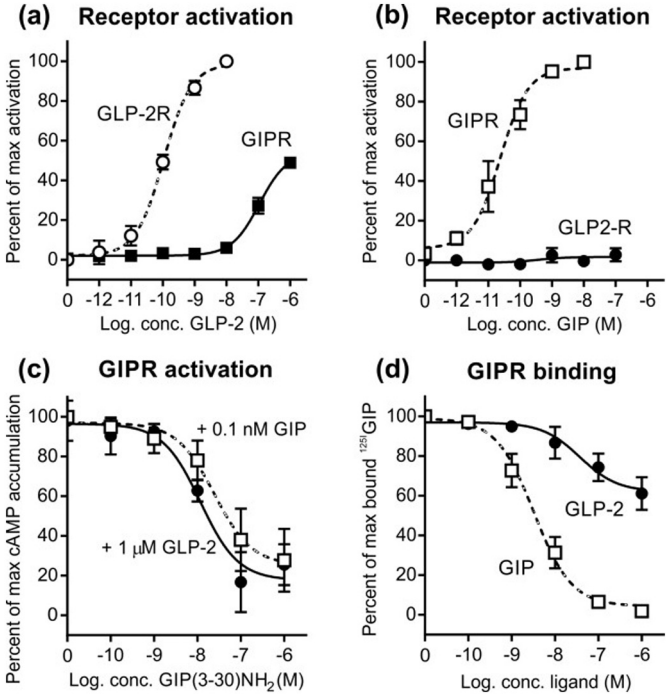

Fig. 1. Binding and signaling of GLP-2 and GIP.
(a) cAMP measurements using GLP-2 on the GLP-2 receptor (dashed line, open circles) and on the GIP receptor (solid line, black squares). (b) cAMP measurements using GIP on the GIP receptor (dashed line, open squares) and on the GLP-2 receptor (solid line, black circles). (c) Inhibition by increasing concentration of GIP(3-30)NH_2 of a GIP-induced activation (dashed line, open squares) and of GLP-2-induced activation (solid line, black circles). (d) Competition binding using ^{125}I-GIP as radioligand displaced with increasing concentration of GIP (dashed line, open squares) and GLP-2 (solid line, black circles). Data are presented as mean ± SEM of 3–5 independent experiments performed in duplicates.

Table 1
Demographic and baseline characteristics.

Demographic and baseline characteristics (n = 8)	Median	Range
Age (years)	27	20–34
Height (cm)	181.9	175.0–188.5
Weight (kg)	74.5	69.0–85.0
Body mass index (kg/m^2)	22.6	21.1–23.9
Systolic blood pressure (mmHg)	131.5	110–152
Diastolic blood pressure (mmHg)	72.5	57–84
Heart rate (bpm)	63	47–68
Hb1Ac (mmol/mol)	31	26–36
Hemoglobin (mmol/L)	9.7	8.7–10.6
Vitamin D (nmol/L)	73	38–112

Data are median and range.

3.3. Plasma concentrations of total and intact GIP, intact GLP-2, and GIP (3-30)NH_2

After GIP administration, total GIP reached a peak concentration of 321 ± 40 pmol/L after 45 min. Intact GIP reached a peak concentration of 155 ± 19 pmol/L after 15 min and was almost back at baseline at t = 90 min (Fig. 2a). On the study days with GLP-2 and GIP(3-30)NH_2 + GLP-2, intact GLP-2 concentrations reached 3165 ± 263 pmol/L and 2925 ± 390 pmol/L, respectively, after 60 min, and elevated concentrations were seen until t = 240 min (Fig. 2b). Finally, after i.v. infusion of GIP(3-30)NH_2, a mean steady state plasma concentration of 81 ± 2.5 nmol/L was reached within 30 min (Fig. 2c).

3.4. Bone resorption measured by serum CTX

Fig. 3a presents CTX as percentage of baseline during the four study days. Injection of GIP resulted in a decrease in CTX to 55.3 ± 6.3% of baseline compared with 82.3 ± 3.2% after placebo treatment (at t = 90 min; $p < 0.0001$) (Fig. 3a). The net $AUC_{0–180}$ of CTX were more decreased after GIP than placebo (−5174 ± 928%*min vs −2565 ± 475%*min; $p = 0.02$). GLP-2 decreased CTX to 60.5 ± 5.0% of baseline after 180 min compared with 85.4 ± 4.2% after placebo ($p < 0.001$) (Fig. 3a). The net $AUC_{0–240}$ of CTX was more decreased after GLP-2 compared with placebo (−6801 ± 879%*min vs −3351 ± 718%*min; $p = 0.002$). GIP(3-30)NH_2 + GLP-2 decreased CTX to 63.2 ± 3.1% after 180 min, and net $AUC_{0–240}$ of CTX amounted to −6027 ± 648%*min, both significantly different from placebo (Fig. 3a). Neither maximal decrease in CTX ($p = 0.79$) nor net $AUC_{0–240}$ of CTX ($p = 0.56$) differed between GLP-2 and GIP(3-30)NH_2 + GLP-2 (Fig. 3a). Absolute baseline values of CTX were 0.75 ± 0.1, 0.86 ± 0.1, 0.81 ± 0.1, and 0.73 ± 0.1 ng/mL on the study days with GIP, GLP-2, GIP(3-30)NH_2 + GLP-2, and placebo, respectively (one way rm. ANOVA $p = 0.23$).

3.5. Bone formation measured by serum P1NP and OC

GIP increased P1NP to 115.1 ± 2.2% of baseline after 30 min compared with 103.1 ± 1.5% after placebo ($p < 0.0001$) (Fig. 3b) and P1NP returned to placebo level at t = 45 min. GIP increased net $AUC_{0–60}$ of P1NP more than placebo (537 ± 104%*min vs 207 ± 81%*min; $p = 0.0001$). GLP-2 decreased P1NP to 88.1 ± 3.0% of baseline after 45 min compared with 105 ± 2.4% after placebo ($p < 0.0001$) and P1NP returned to placebo level at t = 150 min (Fig. 3b). Net $AUC_{0–240}$ of P1NP were more decreased after GLP-2 compared with placebo (−569.1 ± 512.7%*min vs 1898 ± 502.4%*min; $p = 0.005$). GIP(3-30)NH_2 + GLP-2 decreased P1NP to 91.3 ± 1.1% of baseline after 45 min, and net $AUC_{0–240}$ of P1NP amounted to −511 ± 358%*min, both significantly different from placebo (Fig. 3b). Neither maximal decrease in P1NP ($p = 0.50$) nor net $AUC_{0–240}$ of P1NP ($p > 0.99$) differed between GLP-2 and GIP(3-30)NH_2 + GLP-2 (Fig. 3b). Absolute baseline values of P1NP were 71.9 ± 6.6, 71.1 ± 8.3, 69.8 ± 6.7 and 70.0 ± 6.1 ng/mL on the GIP, GLP-2, GIP(3-30)NH_2 + GLP-2, and placebo day, respectively (one way rm. ANOVA, $p = 0.81$). OC also increased significantly after GIP and decreased after GLP-2, however, the changes were less pronounced than seen for P1NP (Fig. 3b and d).

3.6. Changes in serum PTH

PTH measurements revealed that both GIP and GLP-2 induced an acute suppression of PTH.

GIP resulted in a short-lasting (< 60 min) decrease in PTH compared with placebo, at t = 15 min (71.2 ± 4.0% vs 103 ± 4.8% of baseline, $p = 0.0003$) (Fig. 3c). GLP-2 rapidly decreased PTH to 71.2 ± 4.9% of baseline after 15 min compared with placebo ($p = 0.0004$), and returned to placebo level at t = 150 min. GIP(3-30)NH_2 + GLP-2 decreased PTH to 73.7 ± 3.0% of baseline at 15 min significantly different from placebo. After both GLP-2 and GIP(3-30)NH_2 + GLP-2, the level of PTH were sustained at a significant lower level than placebo until t = 120 min (Fig. 3c). Maximal decrease in PTH did not differ between GLP-2 and GIP(3-30)NH_2 + GLP-2 ($p = 0.99$) (Fig. 3c).

3.7. Serum insulin, C-peptide, and glucose

GIP resulted in a rapid short-lived increase in insulin and C-peptide followed by a decrease in glucose from 5.3 ± 0.1 at baseline to 4.6 ± 0.1 mmol/L at t = 45 min. The effect of GIP on glucose disappeared at 90 min (Fig. 4a–c). Concentrations of insulin, C-peptide,

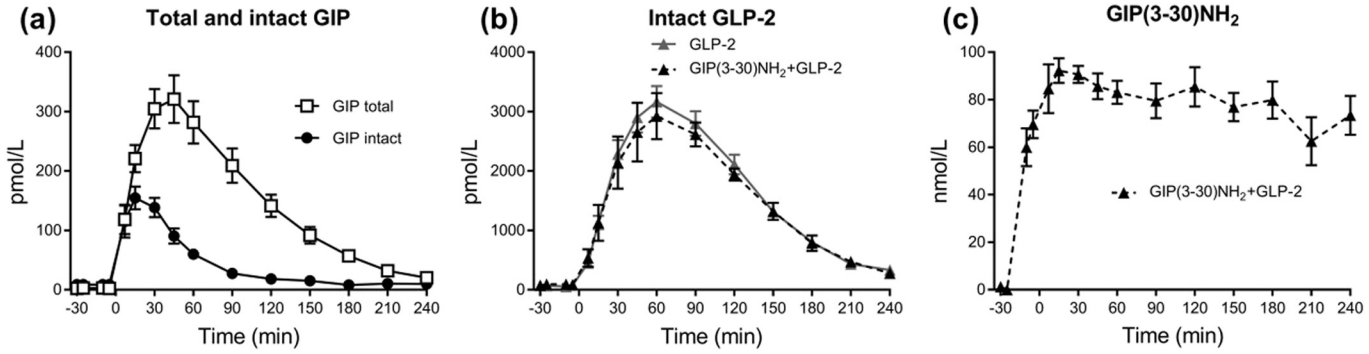

Fig. 2. Plasma levels of GIP, GLP-2 and GIP(3-30)NH_2.
Plasma concentrations of total and intact GIP (a), intact GLP-2 (b), and GIP(3-30)NH_2 (c). Total GIP (white squares), intact GIP (black circles), intact GLP-2 on the GLP-2 day (grey triangles), intact GLP-2 on the GIP(3-30)NH_2 + GLP-2 day (black triangles and dashed line), and GIP(3-30)NH_2 (black triangles and dashed line). Data are mean ± SEM.

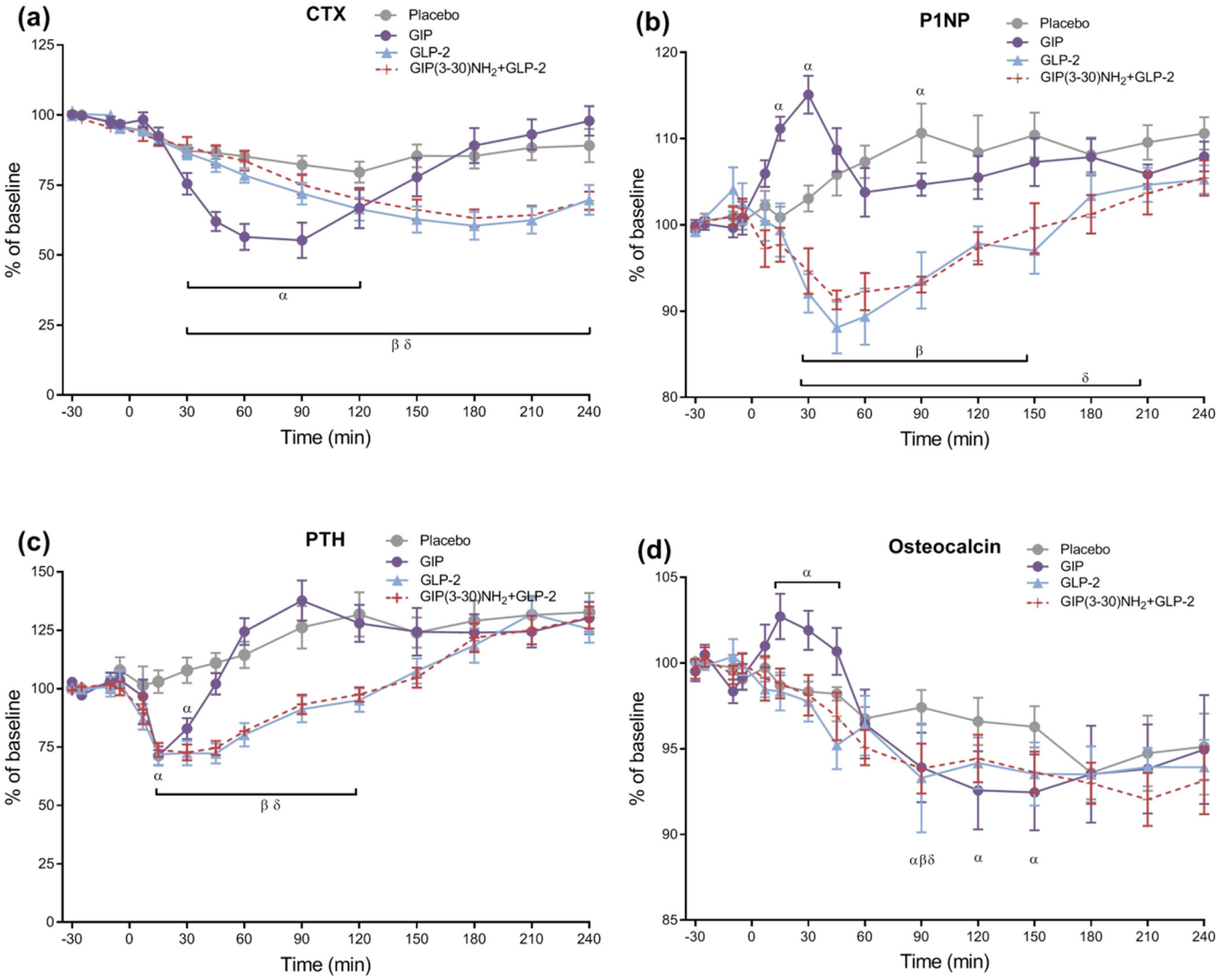

Fig. 3. Bone markers.
Serum CTX (a), P1NP (b), PTH (c), and OC (d) presented as percentage of baseline. GIP (purple circles), GLP-2 (blue triangles), GIP(3-30)NH_2 + GLP-2 (red crosses and dashed line), and placebo (grey circles). Differences between groups at time points are indicated as α ($p < 0.05$ for GIP vs placebo), β ($p < 0.05$ for GLP-2 vs placebo), δ ($p < 0.05$ for GIP(3-30)NH_2 + GLP-2 vs placebo). Brackets indicate a significant difference between relevant groups for all time points within the bracket. Data are mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

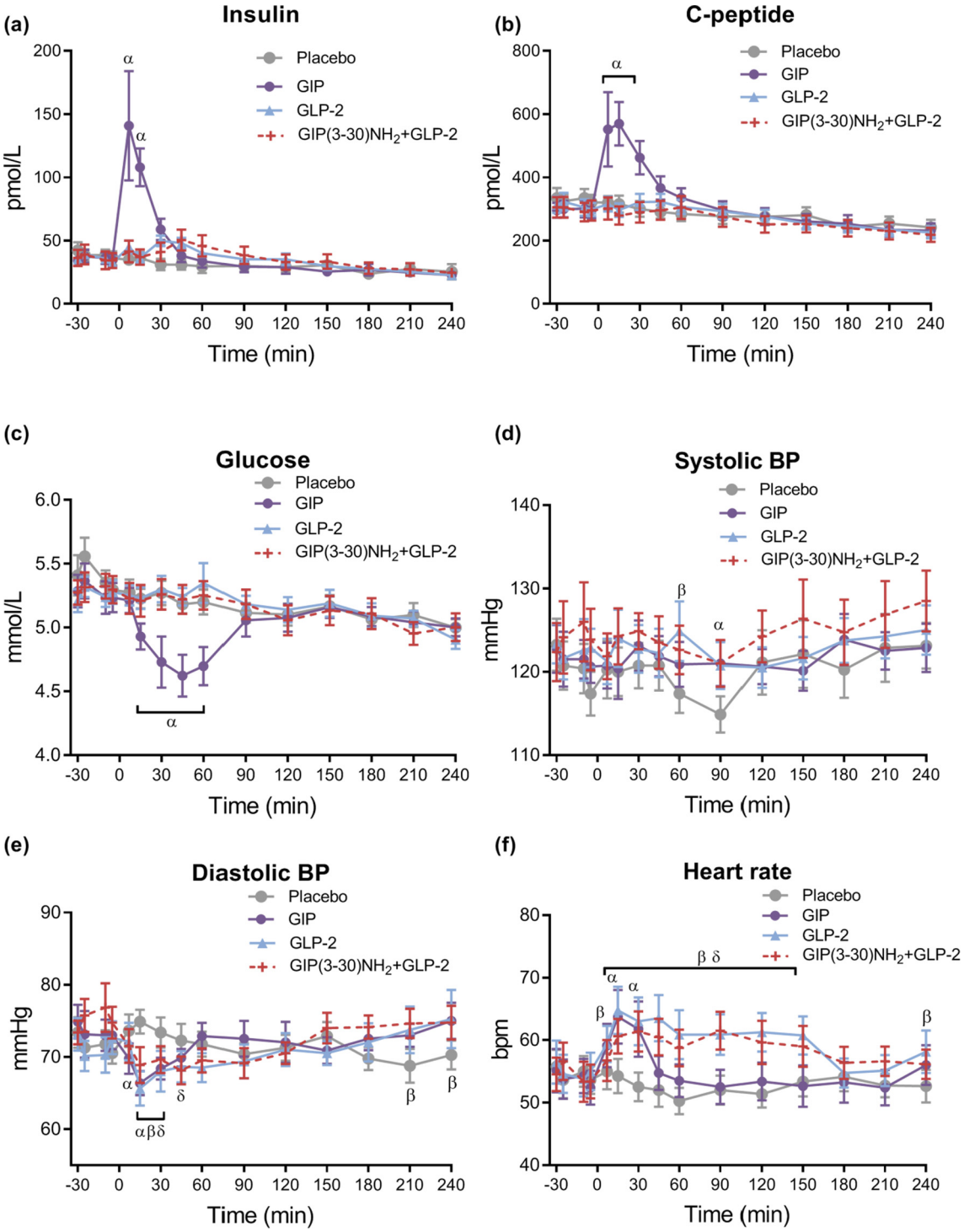

Fig. 4. Insulin, C-peptide, glucose, blood pressure, and heart rate.
Serum insulin (a), c-peptide (b), glucose (c), systolic blood pressure (d), diastolic blood pressure (e), and heart rate (f). GIP (purple circles), GLP-2 (blue triangles), GIP(3-30)NH_2 + GLP-2 (red crosses and dashed line), and placebo (grey circles). Differences are indicated as α ($p < 0.05$ for GIP vs placebo), β (p < 0.05 for GLP-2 vs placebo), δ (p < 0.05 for GIP(3-30)NH_2 + GLP-2 vs placebo). Brackets indicate a significant difference between relevant groups for all time points within the bracket. Data are mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and glucose during both GLP-2 and GIP(3-30)NH_2 + GLP-2 did not differ from placebo (Fig. 4a–c).

3.8. Blood pressure and heart rate

Systolic blood pressure did not differ between study days (two-way rm ANOVA: time $p = 0.03$, treatment $p = 0.35$, interaction $p = 0.85$) (Fig. 4d). Diastolic blood pressure acutely decreased from baseline after GIP, GLP-2, and GIP(3-30)NH_2 + GLP-2 but was comparable to baseline within 60 min (two-way rm. ANOVA: time $p < 0.0001$, treatment $p = 0.40$, interaction p < 0.0001) (Fig. 4e). Heart rate increased 5, 9, and 9 beats per minute from baseline to $t = 15$ min after GIP, GLP-2, and GIP(3-30)NH_2 + GLP-2, respectively (two-way rm. ANOVA: time p < 0.0001, treatment p < 0.0001, interaction p < 0.0001) (Fig. 4f).

After GIP, the heart rate was maintained significantly above placebo level until t = 45 min. For GLP-2 and GIP(3-30)NH_2 + GLP-2, the heart rate was elevated significantly above placebo level until t = 150 min (Fig. 4e).

4. Discussion

We found that GIP(3-30)NH_2 did not prevent the effect of GLP-2 on bone turnover. Thus, GLP-2 seems to regulate bone resorption independently of the GIPR in healthy young men.

Placebo injection resulted in a small but significant decrease in CTX (to 79.6% of baseline at 120 min) also observed by others during placebo/fasting probably explained by maintenance of some circadian variation despite fasting [10,12,38].

Our study is the first human study where GIP has been injected subcutaneously. The s.c. injection of 200 μg GIP resulted in a peak plasma concentration of intact GIP of 155 pmol/L reached after 15 min and sustained for only 1 h. A maximal reduction in CTX to 55% of baseline after 90 min was observed. With comparable plasma concentrations of intact GIP during a continuous i.v. infusion, Nissen et al. [12] also reported a significant decrease in CTX with a maximal reduction to ~65% of baseline after 90 min (at euglycemic conditions). In contrast, Henriksen et al. [8] found that i.v. injected GIP, with a peak plasma concentration of ~800 pmol/L (total GIP), resulted in a non-significant decrease in CTX during a study duration of 48 min. However, because of the short study duration, an effect on CTX might not have been observed.

Surprisingly, we found that GIP, in addition to decreasing bone resorption (CTX), also acutely increased the bone formation marker P1NP to 115% of baseline indicating an acute uncoupling of bone resorption from bone formation. P1NP has not been reported in previous studies investigating effects of exogenous GIP in healthy humans [8,12], but recently Christensen et al. [15] also reported an increase in P1NP after i.v. GIP in patients with type 1 diabetes during low glycemia. In addition, we also found that OC acutely increased after administration of GIP.

On the study days with GLP-2 alone and GIP(3-30)NH_2 + GLP-2, plasma concentrations of intact GLP-2 reached ~3000 pmol/L after 60 min and remained elevated above baseline until the end of the study day. The CTX response to GLP-2 had a slower onset than to GIP, but eventually CTX decreased to a similar level of almost 60% of baseline (at t = 180). This is comparable to findings from previous studies with s.c. administration of GLP-2 in healthy humans where 800 μg GLP-2 (with peak plasma concentration of ~2700 pmol/L) reduced CTX to ~65% of baseline [8], and 1600 μg GLP-2 (with peak plasma concentration of ~3800 pmol/L [13] and ~3000 pmol/L [14]) reduced CTX to ~65% [13] and ~80% of baseline [14], respectively. In our study, GLP-2 also significantly decreased P1NP (and to a lesser extent OC), which is in contrast to previous studies, where there were reported no significant effects of GLP-2 on bone formation (measured as OC or P1NP) [10,12,39]. One explanation for this discrepancy could be the study population being healthy young men in our study in contrast to postmenopausal women in other studies [8,10]. We found that serum PTH decreased within minutes after administration of both GIP, GLP-2, and GIP(3-30)NH_2 + GLP-2 which is in agreement with previous studies [15,39]. The exact mechanism behind the rapid decrease in PTH and whether it affects the bone turnover is unclear. Our findings could reflect a direct inhibitory effect of GIP and GLP-2 on parathyroid cells resulting in a decrease in bone resorption (CTX). While bone formation (measured as P1NP) increased after GIP, it decreased after GLP-2 and GIP(3-30)NH_2 + GLP-2 implying that distinct pathways are involved in the effects of GIP and GLP-2 on bone formation (P1NP), neither of which appear to involve PTH.

Diastolic blood pressure decreased and heart rate increased sustained for 1 h after GIP administration and 2–3 h after GLP-2 both with and without GIP(3-30)NH_2 corresponding to the time periods where elevated concentrations of the intact hormones were measureable in plasma. This effect is presumably due to increased blood flow to various tissues e.g. intestinal and adipose tissue [25,40] and consistent with previous observations [25,30,31,41]. These changes in blood flow are unlikely to explain the drastic change in bone turnover markers.

We chose the doses of 800 μg GLP-2 and 200 μg GIP based on previous studies. GLP-2 seems to dose-dependently inhibit bone resorption (CTX) but only with supra-physiological plasma concentrations [8], whereas GIP seems to be efficacious at lower plasma concentrations [12]. For the doses chosen in our experiment, it seems that s.c. injected GLP-2 produce a relatively higher and more sustained exposure in plasma than s.c. GIP. The explanation for this is unknown and could be related to the different doses and/or different release from the sub-cutis to the circulation. Although the time courses of the effects of GIP and GLP-2 on CTX and other outcome parameters seem to differ, this may mainly reflect the different plasma profiles of the two hormones. Another explanation might be that GIP acts directly on the bone cells (mediated by activation of the GIPR) whereas GLP-2 acts by an indirect pathway, e. g. by changing another hormone or local factor. The difference in the bone formation marker, P1NP, discussed above, supports that separate mechanisms are being activated by the two hormones.

We assume that the GIPR was blocked by GIP(3-30)NH_2 in our experiments. Previous studies, clearly indicate that GIP(3-30)NH_2 reaches steady state within 20 min and efficiently block the GIPR. Gasbjerg et al. [24] infused GIP(3-30)NH_2 800 pmol/kg/min and reached a steady state of 54 ± 12 nmol/L after < 20 min and found that the GIP-induced insulin secretion was markedly reduced by 82% during co-infusion with GIP(3-30)NH_2 concluding that it is an efficacious antagonist in humans. Asmar et al. [25] infused GIP(3-30)NH_2 1000 pmol/kg/min and reached a steady state of 66.4 ± 10.7 nmol/L and found GIP(3-30)NH_2 to be an effective antagonist inhibiting the liporegulatory and vasodilatory effects of GIP in humans. Furthermore, our previous in vitro studies showed that GIP(3-30)NH_2 is selective and does not bind to the GLP-2R or related receptors [24]. In addition we show that in vitro GIP(3-30)NH_2 inhibits the GIP-induced and GLP-2-induced GIPR activity equally (similar IC_{50} values). We therefore assume that the GIPR was adequately blocked in our experiments.

Although a control experiment showing the effectiveness of the GIP (3-30)NH_2 would have been advantageous, we think that the findings of this study strongly indicate that the primary effect of GLP-2 is mediated independent of the GIPR in humans. A limitation of this study is that we did not measure plasma ionized calcium, which might have been relevant in relation to the observed acute decrease in PTH. However, Clowes et al. observed a similar transient decrease in PTH after glucose ingestion without any changes in ionized calcium [7]. A strength of this study is the crossover design eliminating the inter-individual variability in CTX and P1NP, but further experiments are necessary to confirm the results in other study populations. Despite the short half-life of GIP, we report that the s.c. route of administration is equally effective to an i.v. infusion in inhibiting the bone resorption (measured as CTX) and in addition, that it leads to an increase in bone formation (measured as P1NP and OC).

5. Conclusion

The main outcome of this study was the bone turnover markers (CTX and P1NP) in response to GLP-2 and whether this was inhibited by the GIPR-antagonist, GIP(3-30)NH_2. We found no effect of GIPR antagonism on the GLP-2-induced reduction in CTX and we therefore conclude that the antiresorptive effect of GLP-2 does not seem to be mediated through the GIPR in humans. Surprisingly, the most interesting finding of the study was that GIP not only reduced CTX, but also, in contrast to GLP-2, acutely increased the bone formation (P1NP and OC) in healthy young men, indicating an acute uncoupling of bone resorption and bone formation. This also suggests that the regulatory mechanisms of GIP and GLP-2 on the bone turnover are mediated by

separate molecular pathways in humans.

Author contributions

KSJ, LSG, SV, JJH, MMR, and BH conceptualized and designed the study; MBNG and MMR performed the in vitro studies; KSJ, MSS, and CM performed the clinical study; KSJ, MSS, CM, MBNG, LSG, SV, KNBM, SM, JJH, MMR, and BH contributed to the analysis and interpretation of the data; KSJ drafted the manuscript; MSS, CM, MBNG, LSG, SV, KNBM, SM, JJH, MMR, and BH critically revised the manuscript for important intellectual content. All authors have provided approval of the final version to be published. BH is responsible for the integrity of the work as a whole.

Funding

Novo Nordisk Foundation Center for Basic Metabolic Research, University of Copenhagen, Denmark (Grant no NNF18CC0034900) and The Novo Nordisk Foundation (Grant no NNF16OC0023916).

Declaration of Competing Interest

JJH, MMR and BH are shareholders of Bainan Biotech (a small biotech company founded in October 2018 focused on developing GIP/GLP-2 dual agonist for the treatment of bone diseases). KSJ, MSS, CM, LSG, SV, MBNG, KNBM and SM declare no potential conflicts of interest relevant to this article.

References

[1] D.J. Hadjidakis, I.I. Androulakis, Bone remodeling, Ann. N. Y. Acad. Sci. 1092 (2006) 385–396.
[2] L.J. Raggatt, N.C. Partridge, Cellular and molecular mechanisms of bone remodeling, J. Biol. Chem. 285 (33) (2010) 25103–25108.
[3] S. Vasikaran, et al., International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine position on bone marker standards in osteoporosis, Clin. Chem. Lab. Med. 49 (8) (2011) 1271–1274.
[4] C. Hassager, et al., Diurnal variation in serum markers of type I collagen synthesis and degradation in healthy premenopausal women, J. Bone Miner. Res. 7 (11) (1992) 1307–1311.
[5] S. Christgau, Circadian variation in serum CrossLaps concentration is reduced in fasting individuals, Clin. Chem. 46 (3) (2000) 431.
[6] N.H. Bjarnason, et al., Mechanism of circadian variation in bone resorption, Bone 30 (1) (2002) 307–313.
[7] J.A. Clowes, et al., Octreotide abolishes the acute decrease in bone turnover in response to oral glucose, J. Clin. Endocrinol. Metab. 88 (10) (2003) 4867–4873.
[8] D.B. Henriksen, et al., Role of gastrointestinal hormones in postprandial reduction of bone resorption, J. Bone Miner. Res. 18 (12) (2003) 2180–2189.
[9] D.B. Henriksen, et al., Reduction of nocturnal rise in bone resorption by subcutaneous GLP-2, Bone 34 (1) (2004) 140–147.
[10] D.B. Henriksen, et al., Disassociation of bone resorption and formation by GLP-2: a 14-day study in healthy postmenopausal women, Bone 40 (3) (2007) 723–729.
[11] D.B. Henriksen, et al., Four-month treatment with GLP-2 significantly increases hip BMD: a randomized, placebo-controlled, dose-ranging study in postmenopausal women with low BMD, Bone 45 (5) (2009) 833–842.
[12] A. Nissen, et al., Glucose-dependent insulinotropic polypeptide inhibits bone resorption in humans, J. Clin. Endocrinol. Metab. 99 (11) (2014) E2325–E2329.
[13] C. Askov-Hansen, et al., Effect of glucagon-like peptide-2 exposure on bone resorption: effectiveness of high concentration versus prolonged exposure, Regul. Pept. 181 (2013) 4–8.
[14] I.B. Gottschalck, et al., Reduction in bone resorption by exogenous glucagon-like peptide-2 administration requires an intact gastrointestinal tract, Scand. J. Gastroenterol. 43 (8) (2008) 929–937.
[15] M.B. Christensen, et al., Glucose-dependent insulinotropic polypeptide (GIP) inhibits bone resorption independently of insulin and glycemia, J. Clin. Endocrinol. Metab. 103 (1) (2018) 288–294 (January 2018).
[16] S.S. Torekov, et al., A functional amino acid substitution in the glucose-dependent insulinotropic polypeptide receptor (GIPR) gene is associated with lower bone mineral density and increased fracture risk, J. Clin. Endocrinol. Metab. 99 (4) (2014) E729–E733.
[17] Q. Zhong, et al., Effects of glucose-dependent insulinotropic peptide on osteoclast function, Am. J. Physiol. Endocrinol. Metab. 292 (2) (2007) E543–E548.
[18] R.J. Bollag, et al., Osteoblast-derived cells express functional glucose-dependent insulinotropic peptide receptors, Endocrinology 141 (3) (2000) 1228–1235.
[19] A. Mieczkowska, et al., Glucose-dependent insulinotropic polypeptide (GIP) receptor deletion leads to reduced bone strength and quality, Bone 56 (2) (2013) 337–342.
[20] D. Xie, et al., Glucose-dependent insulinotropic polypeptide receptor knockout mice have altered bone turnover, Bone 37 (6) (2005) 759–769.
[21] R. Jorgensen, et al., Oxyntomodulin differentially affects glucagon-like peptide-1 receptor beta-arrestin recruitment and signaling through Galpha(s), J. Pharmacol. Exp. Ther. 322 (1) (2007) 148–154.
[22] J.J. Holst, et al., Oxyntomodulin: actions and role in diabetes, Peptides 100 (2018) 48–53.
[23] S. Thiele, M.M. Rosenkilde, Interaction of chemokines with their receptors—from initial chemokine binding to receptor activating steps, Curr. Med. Chem. 21 (31) (2014) 3594–3614.
[24] L.S. Gasbjerg, et al., GIP(3-30)NH2 is an efficacious GIP receptor antagonist in humans: a randomised, double-blinded, placebo-controlled, crossover study, Diabetologia 61 (2018) 413–423.
[25] M. Asmar, et al., The gluco- and liporegulatory and vasodilatory effects of glucose-dependent insulinotropic polypeptide (GIP) are abolished by an antagonist of the human GIP receptor, Diabetes 66 (9) (2017) 2363–2371.
[26] L.S. Hansen, et al., N-terminally and C-terminally truncated forms of glucose-dependent insulinotropic polypeptide are high-affinity competitive antagonists of the human GIP receptor, Br. J. Pharmacol. 173 (5) (2016) 826–838.
[27] A. Steen, et al., Biased and constitutive signaling in the CC-chemokine receptor CCR5 by manipulating the interface between transmembrane helices 6 and 7, J. Biol. Chem. 288 (18) (2013) 12511–12521.
[28] H.A. Hassing, et al., Biased signaling of lipids and allosteric actions of synthetic molecules for GPR119, Biochem. Pharmacol. 119 (2016) 66–75.
[29] A. DeBlase, K. O'Reilly, H.J. Motulsky, Calculating receptor number from binding experiments using same compound as radioligand and competitor, Trends Pharmacol. Sci. 10 (1989) 227–229.
[30] L. Bremholm, et al., The effect of glucagon-like peptide-2 on arterial blood flow and cardiac parameters, Regul. Pept. 159 (1–3) (2010) 67–71.
[31] M. Asmar, et al., Insulin plays a permissive role for the vasoactive effect of GIP regulating adipose tissue metabolism in humans, J. Clin. Endocrinol. Metab. 101 (8) (2016) 3155–3162.
[32] O. Lindgren, et al., Incretin hormone and insulin responses to oral versus intravenous lipid administration in humans, J. Clin. Endocrinol. Metab. 96 (8) (2011) 2519–2524.
[33] C.F. Deacon, et al., Degradation of endogenous and exogenous gastric inhibitory polypeptide in healthy and in type 2 diabetic subjects as revealed using a new assay for the intact peptide, J. Clin. Endocrinol. Metab. 85 (10) (2000) 3575–3581.
[34] B. Hartmann, et al., Structure, measurement, and secretion of human glucagon-like peptide-2, Peptides 21 (2000) 73–80.
[35] M.B.N. Gabe, et al., Human GIP(3-30)NH2 inhibits G protein-dependent as well as G protein-independent signaling and is selective for the GIP receptor with high-affinity binding to primate but not rodent GIP receptors, Biochem. Pharmacol. 150 (2018) 97–107.
[36] A.H. Sparre-Ulrich, et al., GIP(3-30)NH2 is a potent competitive antagonist of the GIP receptor and effectively inhibits GIP-mediated insulin, glucagon, and somatostatin release, Biochem. Pharmacol. 131 (2017) 78–88.
[37] L.S. Gasbjerg, et al., Glucose-dependent insulinotropic polypeptide (GIP) receptor antagonists as anti-diabetic agents, Peptides 100 (2018) 173–181.
[38] S. Westberg-Rasmussen, et al., Differential impact of glucose administered intravenously or orally on bone turnover markers in healthy male subjects, Bone 97 (2017) 261–266.
[39] I.B. Gottschalck, et al., Effects of treatment with glucagon-like peptide-2 on bone resorption in colectomized patients with distal ileostomy or jejunostomy and short-bowel syndrome, Scand. J. Gastroenterol. 43 (11) (2008) 1304–1310.
[40] L. Bremholm, et al., Glucagon-like peptide-2 increases mesenteric blood flow in humans, Scand. J. Gastroenterol. 44 (3) (2009) 314–319.
[41] J. Koffert, et al., Effects of meal and incretins in the regulation of splanchnic blood flow, Endocr Connect 6 (3) (2017) 179–187.