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GIP reduces osteoclast activity and improves osteoblast survival in primary human bone cells

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Abstract

Objective: Drugs targeting the glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR) are emerging as treatments for type-2 diabetes and obesity. GIP acutely decreases serum markers of bone resorption, whilst preserving bone formation.

Methods: Osteoclasts were differentiated from human CD14+ monocytes and osteoblasts from human bone. GIP expression was determined using RNA-seq in primary human osteoclasts and in situ hybridization in human femoral bone. Osteoclastic resorptive activity was assessed using microscopy. GIPR signaling pathways in osteoclasts and osteoblasts were assessed using LANCE cAMP and AlphaLISA phosphorylation assays, intracellular calcium imaging and confocal microscopy. The bioenergetic profile of osteoclasts was evaluated using Seahorse XF-96.

Results: GIP is robustly expressed in mature human osteoclasts. GIP inhibits osteoclastogenesis, delays bone resorption, and increases osteoclast apoptosis by acting upon multiple signaling pathways (Src, cAMP, Akt, p38, Akt, NfκB) to impair nuclear translocation of nuclear factor-κB. Osteoblasts also expressed GIPR, and GIP improved osteoblast survival. Decreased bone resorption and improved osteoblast survival were also observed after GIP treatment of osteoclast-osteoblast co-cultures. Antagonizing GIPR with GIP(3–30)NH2 abolished the effects of GIP on osteoclasts and osteoblasts.

Conclusions: GIP inhibits bone resorption and improves survival of human osteoblasts, indicating that drugs targeting GIPR may impair bone resorption, whilst preserving bone formation.

Keywords: GIPR, bone remodeling, osteoporosis, resorption, Akt1/2, c-Src, NFATc1, NfκB

Significance

Short-term treatment with glucose-dependent insulinotropic polypeptide (GIP) acutely decreases markers of bone resorption and transiently increases bone formation markers in humans. We aimed to investigate the mechanisms by which GIP mediates these effects. We demonstrated that GIP acts directly on primary human osteoclasts and osteoblasts to reduce osteoclast activity and differentiation and improve osteoblast survival through multiple GIP receptor (GIPR)-mediated signaling pathways. Targeting the GIPR is an emerging therapy for obesity and type-2 diabetes. Our data indicate that GIPR agonism may improve bone mass by decreasing bone resorption, but not bone formation. Thus, GIPR agonists could potentially be repurposed as treatment for patients with osteoporosis, or individuals with type-2 diabetes with increased fracture risk.

† C.M.G. and M.F. contributed equally to this manuscript and should be considered joint senior authors.

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Introduction

Bone is a dynamic tissue, remodeled throughout life by bone resorbing osteoclasts and bone forming osteoblasts to adapt to physiological or mechanical demands. Osteoporosis is caused by an imbalance in bone remodeling, characterized by decreased bone mass and increased fracture risk and affects >10 million individuals in the United States and >27 million in Europe.1,2 Deciphering how bone remodeling is regulated is central for the development of anti-osteoporotic treatments, which reduce fracture risk by decreasing bone resorption, stimulating bone formation, or through dual effects on bone resorption and formation.3

Rodent and human studies indicate that the gut-secreted hormone glucose-dependent insulinotropic polypeptide (GIP) regulates bone remodeling.4 GIP acts on the GIP receptor (GIPR), a G protein-coupled receptor, to mediate its primary function at pancreatic β-cells, stimulating glucose-dependent insulin secretion.4 Both GIPR agonists and antagonists prevent weight gain in preclinical models and have been pursued as anti-obesity therapies, and pharmacological agents targeting GIPR and the glucagon-like peptide-1 (GLP-1) receptor show promising results for the treatment of type-2 diabetes (T2D).5

In humans, GIP infusion suppressed the bone resorption marker C-terminal telopeptide of type-I collagen (CTX),6–9 and transiently increased the bone formation marker procollagen type 1 N propeptide (P1NP) in healthy men and type-1 diabetics8,10 but not in overweight or hypoparathyroid individuals.7,9 Importantly, pre-treatment with GIP(3–30)NH2, a high-affinity GIPR antagonist,11 abolished GIP-induced CTX and P1NP responses in healthy men.10 Consistent with this, homozygous transgenic mice with a Gip truncation had reduced bone volume and increased osteoclast surface12 and Gip overexpression increased bone mineral density, and an osteoblast activity marker, while reducing osteoclast numbers and bone resorption markers.13 By contrast, reports on the bone phenotypes of global Gipr knockout mice (Gipr−/−) are inconsistent. Gipr−/− mice with deletion of GIPR exons 1–6 have reduced BMD, decreased circulating markers of bone formation markers, and elevated osteoclast numbers and resorption markers;14–16 however, another murine model with deletion of GIPR exons 4–5 showed fewer osteoclasts, increased trabecular bone volume and more active osteoblasts, despite reductions in bone strength.17

Although clinical studies support that GIP acutely regulates bone resorption and formation, and murine models indicate GIP and GIPR influence bone mass and strength, the direct effects of GIP and GIPR antagonism on signaling and activity of primary mature human bone cells are unknown. We recently demonstrated GIPR mRNA expression on primary human osteoclasts and osteoblast-like cell-lines,8 and previous studies show GIP may activate cAMP and/or intracellular calcium (Ca2+) signaling in human and/or rodent cell-lines.18,19 Several GIPR targeting therapies are in development, and the recent FDA approval of tirzepatide, a dual GIP and GLP-1 receptor agonist, for use in T2D, highlights the importance of understanding how GIP regulates whole-body metabolism, including bone remodeling. We therefore conducted a comprehensive analysis of GIP effects on primary human osteoclasts and osteoblasts.

Methods

Detailed methods are in Supplementary Appendix. The number of replicates and statistical analyses are described in figure legends.

Cell culture

Primary human osteoclasts were differentiated from human CD14+ monocytes isolated from anonymous blood donations.20 Primary human osteoblast-lineage cells were obtained from bone specimens from patients receiving hip replacement surgery, as described.21 Informed consent was obtained with approval from local ethics committees in Denmark (S-2011-0114 and S-20120193) and the UK (ERN_14-0446).

Expression studies

Bone specimens were obtained from proximal femurs from adolescents during corrective surgery for coxa valga.22 Paraffin sections were subjected to in situ hybridization using an RNAscope 2.5 high-definition procedure using hybridized 20-ZZ-pair probes (477541, ACD Bioscience), directed against the human GIPR 384-1553 mRNA region.22,23

RNA was extracted using Trizol. For PCR, 500 ng of total RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem) and primers in Table S1. RNA-sequencing was performed according to manufacturer’s instructions (TruSeq2, Illumina). Differential gene expression was analyzed using DESeq2.24

Osteoclast activity assays

Osteoclasts were seeded on bone slices in monocultures or cocultures with osteoblasts and exposed to vehicle or GIP for 72 hours. Media was collected for TRAcP activity and bone resorption assays performed.25 For number of nuclei studies, differentiating osteoclasts were exposed to vehicle or GIP for 7 days, then fixed and stained with Giemsa and May–Grünwald.26

Functional assays

Cells were plated on bone slices in 96-well plates and exposed to either vehicle or GIP for 0–120 minutes for cAMP and 30 minutes for AlphaLISA assays. LANCE cAMP and AlphaLISA assays were performed following manufacturer’s instructions using a PheraStar (BMG Labtech) plate reader. Western blots were performed as described.27

Apoptosis and cell viability (ATP) assays were performed using Caspase-Glo 3/7 and CellTiterGlo (Promega) on cells incubated with vehicle or GIP for 3 days. For bioenergetic profiling, mature osteoclasts were seeded in a Seahorse 96-well plate (Agilent Technologies), incubated with GIP or vehicle and measurements performed as described.28

Microscopy

Time-lapse recordings of osteoclastic actin ring formation on bone slices were performed as described.29 For intracellular calcium imaging, osteoclasts were loaded with Fura-2 (ThermoFisher) and imaged on a Crest X-light spinning disk system.30 For NFATc1 and Nfkb imaging, cells were preincubated with inhibitors, then exposed to vehicle or GIP, followed by fixation and immunostaining with total NFATc1 or p-65 primary antibodies and secondary Alexa Fluor 488. TUNEL staining was performed using a TUNEL-FITC kit (Abcam), Images were captured using a Zeiss LSM780 confocal microscope.
Results

GIP acts directly on osteoclast GIPR to reduce bone resorption

We confirmed GIPR expression by in situ hybridization (Figure 1A) on osteoclasts differentiated from human CD14+ monocytes and showed GIPR mRNA expression increases during osteoclastogenesis (Figure 1B). GIP decreased osteoclastic resorptive activity in human osteoclasts and osteoclast–osteoblast co-cultures (Figure 1C; Figure S1A, B). Pre-treatment of mature osteoclasts with the GIPR antagonist, GIP(3–30)NH2, prevented GIP-mediated reductions in bone resorption (Figure 1D). GIPR expression also correlated with the resorptive activity of mature osteoclasts (Figure 1E). Osteoclasts resorb bone by forming pits (round cavitas made by osteoclasts that are immobile during bone resorption) or trenches (elongated excavations as a result of osteoclasts moving across the bone surface while resorbing). The ratio between pits and trenches was not affected by GIP, indicating that GIP is unlikely to affect osteoclast mobility or the bone resorption pattern (Figure S1). GIP reduced the number of nuclei per osteoclast during osteoclastogenesis (Figure 1F, G), and reduced TRAcP activity in mature osteoclasts, but not in pre-osteoclasts (Figure 1H), consistent with induction of GIPR mRNA expression during late osteoclastogenesis.

GIPR activation enhances cAMP signaling in human osteoclasts

GIP couples to Gs signaling pathways, which activate adenylyl cyclase and increase cAMP. To determine whether GIP activates these pathways, we measured cAMP accumulation using LANCE assays in osteoclasts exposed to vehicle or GIP. GIP, but not vehicle, increased cAMP levels after 30 minutes (Figure S1C) which was reversed by pre-treatment with GIP(3–30)NH2 (Figure 1I). GIP also increased cAMP in osteoclast–osteoblast co-cultures after 30 minutes, but not in those pre-treated with GIP(3–30)NH2 (Figure S1D).

Stimulation of cAMP can enhance the cAMP response element binding protein (CREB) pathway to increase bone resorption. No difference in the accumulation of phosphorylated CREB was identified in osteoclast monocultures or osteoclast–osteoblast co-cultures exposed to vehicle or GIP (Figure S1E, F), indicating GIPR and cAMP likely modify other signaling pathways in human osteoclasts.

GIPR activation reduces human osteoclast Src signaling

We next assessed signaling pathways known to affect osteoclast-activity. Phosphorylation of the tyrosine kinase c-Src is important for bone resorption via actin ring formation. AlphaLISA assays revealed that GIP suppresses the phosphorylation of Src-Tyr419 in osteoclast and osteoclast–osteoblast co-cultures, which was abolished by pre-treatment with GIP(3–30)NH2 (Figure 2A; Figure S2A).

Elevations in cAMP can reduce Src activity in a PKA-dependent pathway. We therefore hypothesized that activation of GIPR-cAMP pathways could enhance PKA and reduce p-Src generation (Figure 2B). However, pre-treatment of osteoclasts with the PKA inhibitor H-89, had no effect on GIP-induced reductions in p-Src (Figure 2C). Treatment with forskolin, which activates adenylyl cyclase and should phenocopy treatments that activate cAMP, also did not affect p-Src concentrations in osteoclasts (Figure 2D), indicating it is unlikely that canonical GIPR-Gs-cAMP-PKA pathway modulates p-Src levels. Moreover, pre-treatment of osteoclasts with a PKC inhibitor (GF109203X), a calcium chelator (BAPTA), and an Akt1/2 inhibitor had no effect on p-Src concentrations, suggesting GIP does not activate p-Src by these pathways, although a Src inhibitor (3,4-methylenedioxy-β-nitro styrene) significantly reduced p-Src concentrations (Figure S2B–D). Thus, GIP-mediated Src inhibition may occur by direct interaction with the receptor, components of its signaling pathway, or by crosstalk with other membrane receptors that recruit Src kinases.

As GIP reduced p-Src and bone resorption, we predicted that actin ring formation may be impaired. In osteoclasts exposed to GIP, the median time for initiation of actin ring formation was 26.2 hours, which was significantly longer than for vehicle-treated cells (10.9 hours) (Figure 2E, F; Movie S1). Thus, GIP impairs bone resorption by reducing p-Src signaling and delaying actin ring formation.

GIP reduces PI3K-Akt signaling in human osteoclasts

PI3K can promote bone resorption by c-Src recruitment and activation of Akt. To investigate if GIP affects PI3K signaling, we measured Akt1/2/3 phosphorylation using an Akt1/2/3 (p-Akt) AlphaLISA assay, following 30 minutes exposure of osteoclasts to GIP or vehicle. P-Akt was reduced by GIP in osteoclasts and osteoclast–osteoblast co-cultures, which was reversed by pre-treatment with GIP(3–30)NH2 (Figure 3A; Figure S3A). Pre-treatment of cells with the PI3K inhibitor wortmannin, phenocopied GIP actions on p-Akt, while co-treatment with GIP and wortmannin had an additive effect on p-Akt, which was reversed by pre-treatment with GIP(3–30)NH2 (Figure 3B). Thus, GIP acts on PI3K to reduce p-Akt generation in osteoclasts. However, as co-treatment with wortmannin and GIP could still further reduce p-Akt, GIP may activate additional pathways (Figure 3C).

To determine whether p-Akt is also reduced by GIP-mediated increases in cAMP, we pre-treated osteoclasts with H-89 (Figure 3D). This prevented GIP-induced reductions in p-Akt, indicating GIP also reduces p-Akt via a cAMP-PKA pathway in osteoclasts. Moreover, a Src inhibitor phenocopied these effects (Figure 3E), indicating that the GIP-c-Src pathway could also contribute to reductions in p-Akt.

GIP reduces calcium oscillations to impair p-Akt generation

Exposure to a stable GIP analog for 48 hours reduced Ca2+ signaling in mouse osteoclast-like cell-lines. We tested acute effects of GIP by single-cell microfluorimetry with the calcium-indicating dye Fura-2. Quantification of the 340/380 ratio showed that GIP induced a greater Ca2+ maximal stimulatory response (Emax), than observed in vehicle-treated osteoclasts (Figure 3F–H; Figure S3). However, vehicle-treated osteoclasts had more frequent Ca2+ oscillations of similar amplitude than GIP-treated osteoclasts (Figure 3I–K; Figure S3). The amplitude of these oscillations was significantly higher with GIP, consistent with elevated Emax (Figure 3L, M; Figure S3). Thus, GIP reduces Ca2+ oscillation frequency, which may reduce calcium-mediated gene expression, consistent with previous studies showing the frequency, rather than Emax of oscillations, is important for NFATc1 activation.

As p-Akt can be stimulated by elevations in Ca2+ we hypothesized that reductions in Ca2+ oscillations could contribute to the observed GIP-induced p-Akt reductions. Indeed,
p-Akt was lower in osteoclasts pre-treated with the calcium chelator BAPTA, such that p-Akt responses were not different to GIP-treated cells. However, BAPTA did not reduce p-Akt levels when compared to vehicle-treated cells (Figure 3N),
suggesting that multiple signaling pathways may act to reduce p-Akt downstream of GIPR in human osteoclasts.

GIP impairs NFATc1 nuclear translocation
Ca\textsuperscript{2+} oscillations and Akt can activate NFATc1, which upon nuclear translocation regulates expression of genes that regulate osteoclast differentiation and function.\textsuperscript{41} We hypothesized that GIP may reduce NFATc1 translocation by the Ca\textsuperscript{2+}-Akt and cAMP-PKA-Akt pathways. Osteoclasts exposed to vehicle or pre-treated with GIP(3–30)NH\textsubscript{2} had significantly higher concentrations of NFATc1 in the nuclear fraction compared to GIP treatment alone (Figure 4A–C; Figure S4). Pre-treatment of cells with H-89 prevented the GIP-induced impairment in NFATc1 nuclear translocation (Figure 4A–C; Figure S4) indicating GIPR-cAMP-PKA contributes to NFATc1 translocation. Co-treatment of cells with GIP and an Akt1/2 inhibitor further inhibited NFATc1 translocation, indicating GIP-mediated reductions in Akt1/2 may also contribute to this pathway (Figure 4A–D; Figure S4).

GIP reduces p38 signaling in human osteoclasts
Src can activate p38, which promotes NFATc1 phosphorylation and interacts with Akt pathways.\textsuperscript{42} GIP reduced phosphorylated p38 (p-p38) in osteoclasts and osteoclast-osteoblast co-cultures compared to vehicle or pre-treatment with GIP(3–30)NH\textsubscript{2} (Figure 5A, B; Figure S5A). Pre-treatment with H-89, forskolin or a Src inhibitor had no effect on p-p38 responses (Figure S5B–D), indicating that the cAMP-PKA and Src pathways are unlikely to regulate p-p38. In contrast, pre-treatment of osteoclasts with the calcium chelator BAPTA reduced p-p38 in vehicle-treated cells, and combined GIP and BAPTA treatment had similar effects to GIP (Figure 5C). The Akt1/2 inhibitor had no effect on p-p38 (Figure S5E), however, inhibition of p38 impaired the generation of p-Akt (Figure 5D). Thus, GIP-induced reductions in p-p38 contribute to the inhibition of p-Akt in osteoclasts.

GIP reduces NF\textsubscript{k}B signaling in human osteoclasts
NF\textsubscript{k}B regulates osteoclast differentiation and function\textsuperscript{43} and can be stimulated by PI3K-Akt and p38,\textsuperscript{44} which are reduced by GIP (Figures 3–5). We therefore assessed phosphorylation of the p65 NF\textsubscript{k}B subunit (p-p65) by AlphaLISA, and showed reduced p-p65 in GIP-treated osteoclasts and osteoclast–osteoblast co-cultures, which was reversed by pre-treatment with GIP(3–30)NH\textsubscript{2} (Figure 5E; Figure S5F).

Following activation, p65 translocates to the nucleus, where it regulates gene expression. Confocal imaging showed that GIP reduced the amount of p-p65 in nuclear fractions to similar levels compared to vehicle or pre-treatment with GIP(3–30)NH\textsubscript{2} (Figure 5A, B; Figure S5A). Pre-treatment with H-89, forskolin or a Src inhibitor had no effect on p-p38 responses (Figure S5B–D), indicating that the cAMP-PKA and Src pathways are unlikely to regulate p-p38. In contrast, pre-treatment of osteoclasts with the calcium chelator BAPTA reduced p-p38 in vehicle-treated cells, and combined GIP and BAPTA treatment had similar effects to GIP (Figure 5C). The Akt1/2 inhibitor had no effect on p-p38 (Figure S5E), however, inhibition of p38 impaired the generation of p-Akt (Figure 5D). Thus, GIP-induced reductions in p-p38 contribute to the inhibition of p-Akt in osteoclasts.
Figure 3. GIP reduces p-Akt signaling in human osteoclasts. (A) Quantification of phosphorylated Akt1/2/3 (p-Akt) generated in osteoclasts by vehicle (veh), GIP, or GIP with GIP(3–30)NH$_2$ measured by AlphaLISA. (B) Effect of pre-treatment with the PI3K inhibitor wortmannin. (C) Cartoon showing GIPR signaling pathways that may act on Akt. Inhibitors/activators are in red. (D–E) Effect of pre-treatment with (D) the PKA inhibitor H-89 and (E) a Src inhibitor on p-Akt responses (n = 9 in panels A and B, n = 10 in panel D and n = 6 donors in panel E). Each dot represents one donor measured with median shown in red in panels A, B, D, and E. (F) Normalized mean fluorescence intensity ratio (340/380 nm) of Fura-2-AM calcium imaging in an individual cell exposed to vehicle or GIP. Data are normalized to the 340/380 ratio at 0 seconds for each cell. (G–H) Maximal Ca$^{2+}$I responses in all osteoclasts. Panel (G) shows $E_{\text{max}}$ of all cells measured. Panel (H) shows average for each of the 4 donors. Panel (I) Close-up of oscillations in a cell exposed to vehicle (left) and GIP (right). (J–K) Total number of Ca$^{2+}$ oscillations and (L–M) amplitude of oscillations from all cells measured. Panels (K) and (M) show averages for each of the four donors. Data for individual donors are shown in Figure S3. (N) Effect of pre-treatment with the calcium chelator BAPTA on p-Akt (n = 6 donors). p-Akt was normalized to GAPDH in AlphaLISA assays. Each dot represents one cell measured with median shown in red. ****P < .0001, ***P < .001, **P < .01, *P < .05. Comparisons to vehicle-treated cells are labeled as (a) and to GIP-treated cells as (b). Statistical analyses were performed using Kruskal–Wallis one-way ANOVA with Dunn’s multiple comparisons test for panels A, B, D, E, N; Mann–Whitney test for panels G, J, L; unpaired t-test for panels H, K, and M.
concentrations to that observed in cells exposed to an inhibitor of NFκB nuclear translocation (Figure 5F–H; Figures S5, 6). Therefore, GIP impairs the phosphorylation of NFκB subunits and reduces their nuclear translocation. Pre-treatment of osteoclasts with H-89 or forskolin had no effect on vehicle or GIP-induced NFκB nuclear translocation, while inhibitors of Src, Ca$^{2+}$, and p38 reduced NFκB nuclear translocation compared to vehicle-treated cells, indicating these three pathways activate NFκB signaling in human osteoclasts (Figure 5G, H; Figures S5-6). Combined treatments with GIP and inhibitors of Ca$^{2+}$ and p38 phenocopied the GIP effects, indicating that Ca$^{2+}$ and p38 may act downstream of GIP to inhibit NFκB signaling pathways (Figure 5G, H; Figures S5-6).

**GIP reduces expression of genes involved in osteoclast activity and apoptosis**

As GIP reduces NFATc1 and NFκB nuclear translocation, we hypothesized that GIP may affect osteoclast gene expression. RNA-seq on mature osteoclasts showed 911 differentially expressed genes between osteoclasts exposed to vehicle or GIP, with enrichment of genes involved in bone resorption, ATP production and apoptosis, correlating with osteoclast activity data (Figure 6A–C). More than 40 genes were involved in lysosome and osteoclast function (Figure 6D). Among the downregulated genes we found those encoding TRAP, cathepsin K and the calcitonin receptor, which are known to be regulated by NFATc1. Using the ISMARA algorithm we quantified the impact of NFATc1 activity on global gene expression, which increases during osteoclastogenesis, and decreases upon GIP treatment in mature osteoclasts (Figure 6E). Genes predicted at higher confidence to be regulated by NFATc1 showed increasing specificity of osteoclast activity (Figure 6F) and higher susceptibility to be repressed by GIP treatment (Figure 6G, H).

Genes involved in apoptosis or mitochondrial function, which is important for osteoclast differentiation and resorptive activity, were differentially expressed in osteoclasts exposed to GIP (Figure 6B). Consistent with this, we showed that osteoclasts exposed to GIP had significantly more terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells and higher caspase-3/7 activity, which was not present in cells pre-treated with GIP(3–30)NH$_2$ (Figure 6K). Additionally, GIP reduced intracellular ATP concentrations in mature osteoclasts, but not immature cells, and reduced the total number of osteoclasts (Figure 6M). To determine whether alterations in osteoclast energy metabolism...
affect ATP production, we performed bioenergetic profiling using extracellular flux assays. Acute treatment with GIP for 30 minutes did not affect basal metabolism (Table S2). However, exposure of osteoclasts to GIP for 3 days produced a small but significant increase in basal respiration (Table S3; Figure 6N). No other differences were observed. Thus, GIP does not have a major effect on osteoclast bioenergetics despite lowering intracellular ATP concentrations.

GIP increases p-Akt via a CAMP-PKA pathway and decreases cell apoptosis in human osteoblasts

We confirmed GIPR expression in proliferating human osteoblasts and showed GIP significantly elevated CAMP following 15 and 30 minutes stimulation, which was prevented by pretreatment with GIP(3–30)NH₂, indicating direct activation of osteoblastic GIPR (Figure 7A; Figure S7A, B). Additionally, GIP increased p-Akt concentrations, which have previously been shown to enhance osteoblast differentiation. Pre-treatment with GIP(3–30)NH₂, H-89 or wortmannin inhibited GIP-induced increases in p-Akt, demonstrating GIPR activates PKA and PI3K to stimulate p-Akt in osteoblasts (Figure 7B, C). In contrast to osteoclasts, Ca²⁺, NFATc1 nuclear translocation, and p38 signaling were unaffected by GIP in osteoblasts (Figure 7D–H; Figure S7C–E).

As Akt signaling promotes cell survival pathways we assessed osteoblast apoptosis. GIP-treated osteoblasts had fewer TUNEL-positive cells when compared to vehicle (Figure 7I, J), and reduced caspase3/7 activity (Figure 7K). Pre-treatment with GIP(3–30)NH₂, H-89, wortmannin or an Akt1/2 inhibitor prevented the GIP-induced reduction in caspase3/7 activation (Figure 7K), indicating that GIPR activation of cAMP-PKA and PI3K-Akt signaling pathways are involved in GIP-mediated reductions in osteoblast apoptosis. Consistent with this, GIP increased ATP generation in osteoblasts, which was reduced by GIP(3–30)NH₂, H-89, wortmannin and an Akt1/2 inhibitor (Figure 7L). In line with our findings that GIP has opposing effects on cell viability in osteoclasts and osteoblasts, there was no significant difference in the viability of osteoclast–osteoblast co-cultures exposed to vehicle or GIP (Figure S7F). As cell viability was increased in
Figure 6. GIP reduces expression of genes involved in osteoclast function and induces apoptosis. (A) Heat map showing fold changes (FC) for genes with significant changes (P-value < 0.05) in expression upon 4 hours of GIP treatment in mature osteoclasts (n = 8 donors, DEseq2 P-values modeled for donor and treatment). (B) Heat map showing enrichment for gene ontology terms among the up- and downregulated genes. (C) Heat map showing enrichment for targets of the indicated signaling pathways (SPEED2) among the up- and downregulated genes. (D) Log fold-change of significantly differentially expressed genes with known roles in osteoclast activities, comparing mature osteoclasts exposed to GIP for 4 hours to vehicle-treated cells. (E) ISMARA-based motif activity of NFATc1 during osteoclast differentiation and in response to 4 hours GIP treatment in mature osteoclasts. (F) Heat map showing the enrichment of selected osteoclast-related terms among NFATc1 targets with increasing confidence. (G) Box plot quantifying log fold changes in gene expression of ISMARA-based NFATc1 targets that were grouped according to prediction score against 4 hours of GIP treatment. (H) Heat map showing the log fold changes for predicted NFATc1 target genes (ISMARA score above 4) in expression upon 4 hours of GIP treatment. (I) Representative images and (J) quantification of the percentage of TUNEL-FITC-positive (+ve) osteoclasts following 3 days of GIP exposure. DAPI was used to stain nuclei (n = 6 donors). (K) Caspase3/7 activity and (L) intracellular ATP levels, measured by CaspaseGlo and CellTiterGlo, respectively, in immature (day 8) and mature (day 10) osteoclasts exposed to vehicle, GIP, or GIP with GIP(3–30)NH₂ for 3 days (n = 7 for day 8, n = 9 for donor and day 10). (M) Total number of osteoclasts per bone slice following vehicle or GIP exposure (n = 4 donors). (N) Basal respiration and mitochondrial ATP production rate in osteoclasts exposed to vehicle and GIP (n = 6 donors). *P < 0.01, **P < 0.05. Each dot represents one donor measured, with mean or median shown in red in panels K–N. Statistical analyses were performed by unpaired t-test for panel J; Kruskal–Wallis one-way ANOVA with Dunn’s test for panels K and L; and paired t-test for panels M and N.

Discussion

Our studies demonstrated that GIPR is robustly expressed on primary human osteoclasts, increasing during differentiation, and utilizes multiple signaling pathways to reduce osteoclast differentiation and bone resorption (Figure 8). GIPR is also expressed on proliferating mature human osteoblasts, and GIP improves osteoblast survival via cAMP-Akt signaling.

Endogenous GIP contributes to the postprandial suppression of bone resorption by up to 25% in humans, and GIP abruptly decreases biomarkers of bone resorption. However, these studies did not determine whether changes in bone turnover were due to direct effects on GIPR on bone cells, or indirect effects via extra-skeletal GIPR. A major advantage of our studies is the use of the GIPR antagonist, GIP(3–30)NH₂, to demonstrate direct effects of GIP on primary human osteoclasts and osteoblasts. Other gut-derived hormones, including GLP-1 and GLP-2, have been described to affect bone, and adoption of a similar strategy to investigate bone cell signaling and activity with specific antagonists would help determine whether these hormones induce direct effects on human bone cells.

We showed that GIP stimulates the canonical GIPR-cAMP pathway in human osteoclasts, and that this pathway contributes to reduced osteoclast activity. This is consistent with previous studies that showed forskolin, which elevates cAMP, impairs bone resorption. Previous studies in an osteoclast mouse cell-line showed a stable GIP analog did not activate
Figure 7. GIP increases cAMP and p-Akt signaling, and reduces apoptosis in human osteoblasts. (A) Quantification of cAMP in osteoblasts exposed to vehicle (veh), GIP or GIP+GIP(3–30)NH₂ measured by LANCE assays (n=7 donors). (B–C) Quantification of phosphorylated Akt1/2/3 (p-Akt) in osteoblasts exposed to vehicle or GIP for 30 minutes measured by AlphaLISA. Cells were pre-treated with vehicle and (B) GIP(3–30)NH₂, (C) the PKA inhibitor H-89 and PI3K inhibitor wortmannin [n=7 for B, n=6 donors for C]. p-Akt concentrations were normalized to GAPDH in AlphaLISA assays. (D) Normalized mean fluorescence intensity ratio of Fura-2-AM. Data are normalized to the 340/380 ratio at 0 seconds for each cell. Data show mean ± SEM (n=4 donors). (E–F) Quantification of the maximal Ca²⁺ responses from data in panel D. Panel (E) shows Eₘₐₓ of all cells measured. Panel (F) shows average for each of the four donors. (G) Representative images of NFATc1 and (H) quantification of nuclear and cytoplasmic ratios in osteoblasts exposed to vehicle or GIP for 60 minutes. DAPI was used to label nuclei and AlexaFluor488 to fluorescently label NFATc1 (n=5 donors). (I) Representative images of osteoblasts stained with TUNEL to detect apoptotic cells and DAPI to detect nuclei, with zoomed images of cells indicated by a yellow box shown below. (J) Quantification of the percentage of TUNEL-FITC-positive cells (n=3 donors). (K) Caspase-3/7 activity and (L) ATP generation, measured by CaspaseGlo and CellTiterGlo, respectively, in osteoblasts exposed to vehicle, GIP or GIP +/− inhibitors of signaling (n=6 donors). Comparisons to vehicle-treated cells are labeled as (a), and to GIP-treated cells labeled as (b), in panels J and K. ***P < .001, **P < .01, *P < .05. Each dot represents one donor measured with mean or median shown in red in panels A–E and G–H. Statistical analyses were performed by Kruskal–Wallis one-way ANOVA with Dunn’s test for panels A, B, E, F, H, K; one-way ANOVA with Dunnett’s multiple comparisons test for C and L; and unpaired t-test for panels J and M.
and there are several possible reasons for these differences. Different signaling pathways could exist between species, or between primary and immortalized cells. However, we were able to verify other GIP-induced signaling effects (i.e., inhibition of Ca$^{2+}$ and impaired NFATc1 nuclear translocation) observed in these mouse cell-lines. Alternatively, experimental differences could explain why cAMP induction was not previously observed. We used a highly sensitive LANCE cAMP assay, which unlike the FRET probe used in the mouse cell-lines, does not require transfection. Therefore, it will be important to investigate the effects of GIPR analogs in primary human bone cells, rather than mouse cell-lines, to assess possible effects on osteoclast activity.

Our examination of multiple signal readouts demonstrated that GIP regulates several signaling pathways in human osteoclasts and that these have distinct effects on gene expression. Thus, NFATc1 nuclear translocation is regulated by cAMP-PKA and Ca$^{2+}$ signaling pathways, whereas NFkB is regulated by Src and Ca$^{2+}$-p38-Akt, but not by cAMP-PKA. This could explain why GIP affects multiple aspects of osteoclast function (actin ring formation, gene expression, differentiation, cell survival, resorption). We showed that GIP-induced signaling events in human bone cells occur rapidly (phosphorylation events by 30 minutes, nuclear translocation by 60 minutes and gene expression by 4 hours), consistent with known acute signaling by GIPR. Previous studies showed that NFATc1 nuclear translocation was impaired by chronic exposure (48 hours) to GIP; however, chronic GIPR activation may desensitize GIPR activity. We demonstrate that GIP-mediated effects on human osteoclasts are due to acute GIPR activation, rather than indirect effects of receptor desensitization. Our studies showed that osteoclasts differentiated in the presence of GIP have fewer nuclei per cell and a reduced total number of osteoclasts. It is possible that GIP could impair osteoclast fusion or adhesion during differentiation and this remains to be explored in future studies. However, it is unlikely that GIP has a significant effect on mobility of resorbing osteoclasts, as there was no evidence that GIP affected the extent of resorption pits (indicative of non-mobile cells) versus trenches (indicating more mobile cells), when compared to vehicle-treated cells.

Despite an enrichment in differentially expressed genes associated with metabolic pathways and oxidative phosphorylation, and lower ATP concentrations in osteoclasts exposed to GIP, we did not identify changes in ATP production. The most likely explanation for the observed lower ATP concentrations is that GIP reduces the total number of osteoclasts and the number of nuclei per osteoclast, possibly due to increased apoptosis. However, the differences between total ATP concentrations and ATP production in GIP-stimulated osteoclasts could reflect more complex dynamics between intracellular and extracellular ATP, previously shown to regulate osteoclast survival and bone resorption.

GIP had a more pronounced effect on osteoclasts than osteoblasts, presumably due to higher osteoclastic GIPR expression. Importantly, osteoblast GIPR remains functional, resulting in reduced apoptosis and improved survival of osteoblasts, which...
may explain the transient and small increase in bone formation markers observed in some human studies.\textsuperscript{6,10,49} We could not demonstrate any changes in osteoblast activity using two methodologies in osteoblast monocultures or osteoclast–osteoblast co-culture. However, it is possible that GIP affects osteoblast differentiation or other osteoclast–osteoblast co-factors (e.g., hormones, cytokines and growth factors that mediate crosstalk) and this remains to be explored in future studies. Current anti-resorptive treatments for osteoporosis inhibit osteoclast activity, and through coupling mechanisms impair osteoblast activity, leading to reduced bone remodeling, which may compromise skeletal integrity with long-term exposure.\textsuperscript{54} Bone anabolic treatments induce increases in bone formation and resorption, as observed with PTH-based therapies, or transiently increase bone formation, with a concomitant reduction in bone resorption, as seen with the sclerostin-antibody romosozumab.\textsuperscript{55}

Our co-culture studies demonstrate that GIP-mediated reductions in bone resorption can occur in a more physiologically relevant system, and indicate that GIP decreases bone resorption, while bone formation is maintained. This could lead to larger gains in bone mass and possibly lower fracture risk. Although it remains to be studied in clinical studies if these observed changes are maintained with long-term GIPR agonism, drugs targeting GIPR, including tirzepatide,\textsuperscript{56-58} could represent a new class of anti-osteoporotic therapeutics with long-term advantages over currently approved anti-resorptive drugs.

In conclusion, our studies show that GIP acts via multiple signaling pathways to reduce differentiation and bone resorption in primary human osteoclasts and activates GIPR to improve cell survival in human osteoblasts. Stable GIPR agonists may reduce bone resorption without impairing bone formation, thus supporting studies of long-term effects of GIPR agonism on bone and exploration of such agonists as treatments for patients with increased fracture risk associated with osteoporosis and T2D.

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Supplementary Data

Supplementary material is available at European Journal of Endocrinology online.

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Conflicts of interest: None declared.

Data Availability

Raw RNA-seq data and DESeq2 processed data that have been generated in this study are available under the GEO accession number GSE201100. Requests for other data and materials will be made available upon reasonable request to C.M.G. (cgovin@bham.ac.uk) or M.F. (mmfnielsen@health.sdu.dk).

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