Effects of glucose-dependent insulinotropic peptide on osteoclast function

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Submitted 21 July 2006; accepted in final form 21 September 2006

Our laboratory has previously reported that GIP receptors are present on osteoblasts, osteocytes, and chondrocytes and that addition of GIP to osteoblast-like cells leads to an increase in collagen type I expression and alkaline phosphatase activity, consistent with an anabolic effect (3). In addition, intermittent injection of GIP was shown to prevent bone loss in an ovariectomized rat model (2), while absence of the GIP receptor in GIP receptor knockout mice resulted in decreased bone formation, increased bone breakdown, and significantly lower bone mass (24, 25). To further evaluate a potential role for GIP in suppressing postprandial bone breakdown, we examined GIP effects on osteoclasts. We report that GIP receptors are expressed by osteoclasts and that activation of these receptors decreases osteoclastic activity. Thus GIP, in addition to other gastrointestinal hormones, may play a role in mediating nutrient-related effects on bone turnover, including the postprandial reduction in the rate of bone resorption.

MATERIALS AND METHODS

Methods

Tissue and cell preparation. The murine monocytic/macrophage (osteoclast) cell line RAW 264.7 was a kind gift from Dr. F. R. Bringhurst (Massachusetts General Hospital, Boston, MA). The cells were grown in RPMI 1640 with l-glutamine, without phenol red; all were supplemented with 10% fetal calf serum (vol/vol) (HyClone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (3 mg/ml) and utilized 3–7 days post-confluence. Cells were grown in four-chamber slides.

RT-PCR. Transcript levels for GIP receptor (GIPR) were determined by RT-PCR. Total RNA was isolated using TRIzol reagent (Gibco-BRL). First-strand cDNA was reverse transcribed from 5 μg of total RNA using a cDNA synthesis kit (SuperScript III First-Strand Synthesis System) from Invitrogen (San Diego, CA). PCR amplification was performed in 50 μl of Platinum PCR SuperMix (Invitrogen) that contained 220 μM dNTP, 1.65 mM MgCl2, 55 mM KCl, and 0.02 U/μl Taq DNA polymerase with 0.2 μM primers and 2 μl of first-strand cDNA and was subjected to initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min.

The PCR products were fractionated by electrophoresis on a 1% agarose gel and then transferred overnight to a nylon membrane. The membrane were probed for GIPR using a cloned PCR fragment amplified with GIPR-F (forward CGGCGTCCGCGAGCGCGCCA-GAT) and GIPR-R (reverse CGGAGCGAGCTAGCGAGCGG-GTAA) and verified by sequence analysis. The probe was labeled with 32P by random primer labeling (Amersham Pharmacia Biotech, Piscataway, NJ).

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Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>PCR Condition</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-fms</td>
<td>Forward AGCTCTGACTTCCAGG</td>
<td>95–3 min</td>
<td>~300 bp</td>
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<tr>
<td></td>
<td>Reverse CTAAAGCAGGGCTC</td>
<td>94–1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTAGA</td>
<td>60–1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72–1.20 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(20 cycles)</td>
<td></td>
</tr>
<tr>
<td>TRAP</td>
<td>Forward AGCATGGGCCTGACCTCA</td>
<td>95–3 min</td>
<td>~200 bp</td>
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<tr>
<td></td>
<td>Reverse GGCTTGGAGATGCTTT</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>AGAGT</td>
<td>60–1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>72–1.20 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(25 cycles)</td>
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<tr>
<td>Cathepsin-K</td>
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<td>~200 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse CTAACGGTGGTGG</td>
<td>94–1 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATGCCAAGCTTTGCG</td>
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<td></td>
<td>TGAT</td>
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<td></td>
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<td>(27 cycles)</td>
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</table>

For studies involving osteoclast-related genes, primers were based on previously published (4) sequences. TRAP, tartrate-resistant acid phosphatase.

NJ). The mouse brain cDNA was used as a positive control, and mouse spleen cDNA was used as a negative control for GIPR.

For studies involving osteoclast-related genes, primers were based on previously published (4) sequences (see Table 1).

Immunohistochemistry. Three-month-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were killed and bones removed. This protocol was approved by the Medical College of Georgia Institutional Animal Care and Use Committee. Rats were anesthetized and perfused transcardially with 4% paraformaldehyde and 0.2% gluteraldehyde. Femoral bones were harvested and fixed in 4% paraformaldehyde overnight at 4°C. The bones were decalcified in 10% EDTA for 4–6 days, dehydrated in ethanol and xylene, and embedded in paraffin; 7-μm-thick sections were cut, deparaffined, and rehydrated.

The immunohistochemical localization analyses were carried out using the ABC Staining System with diaminobenzidine (DAB) chromogen as the peroxidase substrate (Santa Cruz Biotechnology, Santa Cruz, CA). The bone sections were incubated with primary GIPR polyclonal antibody (MBL International, Woburn, MA) at 1:100 dilutions for 1 h at room temperature. Developed slides were dehydrated in ethanol, cleared in xylene, and embedded in paraffin; 7-μm-thick sections were cut, deparaffined, and rehydrated.

Since mature osteoclasts cannot be isolated in sufficient numbers for biochemical analyses, we used RNA isolated from mature OCLs cultured in vitro as described above. As shown in Fig. 1, an amplicon of the expected size for the GIPR transcript was generated by RT-PCR using OCL RNA. The size of the osteoclast GIPR amplicon is comparable to that generated using RNA prepared from osteoblasts and brain. Spleen tissue

Results are expressed as means ± SE. Data were analyzed using either ANOVA with Bonferroni post hoc testing or unpaired t-tests, using a commercial statistical package (Instat; GraphPad, San Diego, CA).

RESULTS

GIPR Transcript is Expressed by Osteoclasts

We have previously reported that osteocytes and osteoblasts resident in bone express the GIPR (3) but had not examined osteoclasts. We therefore initially sought to determine whether osteoclasts expressed the transcript for the GIPR. The bone sections were incubated with primary GIPR polyclonal antibody (MBL International, Woburn, MA) at 1:100 dilutions for 1 h at room temperature. Developed slides were dehydrated in ethanol, cleared in xylene, and counterstained with Gill's formulation no. 2 hematoxylin.

Fetal Bone-Resorbing Assay

The fetal rat long bone assay was performed as previously described (9, 10). Pregnant Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) were injected with 200 μCi of 45Ca on day 18 of gestation and killed 1 day later to remove the radiolabeled fetal radius and ulna. Bone explants were precultured for 24 h in BGB medium containing 2 mM L-glutamine, 0.5 mM ascorbate, 0.1 mg/ml BSA, and 50 U/ml penicillin-streptomycin, followed by 5 days of culture in the same media with or without the substances to be tested. At the end of the culture period, the 45Ca released into the media of control and treated bones was calculated by counting the media and the bones. Resorption is expressed as the ratio of 45Ca released from treated vs. control bones.

Marine Osteoclast-Like Cells for RNA Preparation

Osteoclast pit formation assay. C57/B16 mouse osteoclast-like cells (OCLs) were prepared by coculturing osteoblasts and bone marrow on collagen-coated 10-cm dishes in the presence of 10−8 M 1,25-dihydroxyvitamin D3 and 10−6 M prostaglandin E2 for 6 days. The collagen was digested, and the mature osteoclasts were plated onto calcium phosphate-coated Osteologic discs (BD Biosciences, Bedford, MA) in the presence of vehicle, receptor activator of NF-κB (RANK) ligand (RANKL; 100 ng/ml), or RANKL (100 ng/ml) plus increasing concentrations of GIP. After 5 days, the cells were removed with bleach, and the resorbed area (of ~120 different fields/condition) was quantitated with image analysis software (NIH Image, version 1.34k).

Statistics

For studies involving osteoclast-related genes, primers were based on previously published (4) sequences. TRAP, tartrate-resistant acid phosphatase.

Fig. 1. Isolated osteoclasts express the glucose-dependent insulinotropic peptide receptor. Isolated rat osteoclasts, prepared as described in Methods, were examined for GIP receptor transcript expression by RT-PCR. Shown is a representative blot from 3 separate experiments. The amplicon is of the expected size, which is 468 bp. OCL, osteoclasts; OBL, osteoblasts; GIPR, glucose-dependent insulinotropic peptide receptor. Brain tissue was used as a positive control. Mouse spleen was used as a negative control.
GIPR Protein is Expressed by Osteoclasts Resident in Bone

To evaluate whether the GIPR protein is present in osteoclasts, we performed immunocytochemistry experiments. Shown in Fig. 2A is a section of normal rat bone with a tartrate-resistant acid phosphatase (TRAP) stain demonstrating a labeled osteoclast (arrow). In sequentially sectioned bone, the contiguous bone sample was then used for immunocytochemistry experiments. The latter were performed using a commercially available GIPR polyclonal antibody. Shown by the arrow in Fig. 2B is the osteoclast positive for GIPR. As we have previously reported, osteoblasts and osteocytes also are positive for the GIPR (3). A higher-magnification image of a GIPR-labeled osteoclast is shown in Fig. 2C. To further evaluate GIPR immunopositivity in osteoclasts, we isolated normal bone marrow osteoclasts from mouse bone marrow. These cells were positive for TRAP stain (Fig. 2D), demonstrating that they are in fact osteoclasts. Shown in Fig. 2E are unlabeled cells, and, in Fig. 2F, these isolated osteoclasts were labeled with the same GIPR antibody, demonstrating the presence of GIPR protein on the osteoclast.

GIPDose Dependently Inhibits Parathyroid Hormone-Induced Bone Resorption

In an effort to determine whether GIPR expression in osteoclasts had physiological relevance in terms of osteoclast activity, the effect of GIP was determined in the fetal rat long bone resorption assay. Activity in this assay largely reflects effects on mature osteoclasts and late preosteoclasts. Parathyroid hormone (PTH) is the principal hormonal regulator of skeletal resorption on a day-to-day basis and shows dose-dependent activity in the long bone assay (11). As shown in Fig. 3, PTH at 10 nM induced a greater than twofold increase in bone-resorbing activity. In contrast, when increasing doses of GIP were added concurrently with PTH, there was a dose-dependent reduction in bone resorption such that, at 50 nM, GIP reduced the rate of resorption below that seen in vehicle-treated bones even though PTH was present in the medium. As an additional control, we utilized transforming growth factor-β (TGF-β) with PTH. TGF-β by itself can stimulate bone resorption (17) but has no additional effect to that of PTH by itself in the resorption assay. The GIP concentrations utilized were supraphysiological, but so was the concentration of PTH used for these experiments (10 nM). The high concentrations...
needed for both of these peptides reflect limitations in the sensitivity of the assay. Since the fetal rat long bone assay is an organ culture system, it is not possible to determine the cellular site of action of GIP from these data. To determine whether GIP could act directly on mature osteoclasts, studies were undertaken using mature OCLs generated in coculture.

**GIP Directly Inhibits Osteoclast-Induced Pit Formation**

For these experiments, the activity of mature OCLs was quantified by measuring resorptive area using Osteologic discs as described in *Osteoclast pit formation assay*. The OCLs were activated by using RANKL, which acts via its receptor RANK, expressed on mature osteoclasts. Therefore, this assay is an assessment of the direct actions of GIP on mature osteoclasts. As seen in Fig. 4, GIP was able to dose dependently decrease osteoclast resorbed area, consistent with a direct inhibition of mature osteoclast activity. As in the case of the fetal long bone resorption assay, a high concentration of GIP was used with incomplete suppression of RANKL-induced resorption. However, the concentrations of RANKL utilized were pharmacological, rather than physiological, concentrations. Thus, at a concentration of 10 mM, GIP reduced the resorptive effect of a quite high dose of RANKL by 48% (P < 0.02 by 1-way ANOVA).

**GIP Modulates Osteoclastic Gene Expression**

The OCL line RAW 264.7 was grown in the presence of RANKL and macrophage colony-stimulating factor (MCSF) to induce differentiation. We have shown that these cells express the GIPR. Some of the cells were exposed to 0.1 nM GIP added daily to the medium. As shown in Fig. 5, three markers of osteoclastic differentiation, c-fms (the gene that encodes for the receptor for colony-stimulating factor-1, CSF-1), TRAP (a marker for osteoclasts), and cathepsin K (involved in bone matrix resorption), were examined in response to differentiation over time in the presence or absence of 0.1 nM GIP. Expression of all three markers increased at 5 and 7 days.
compared with the 3-day time period. Addition of GIP decreased expression of both c-fms and TRAP (but not cathepsin K) at the 5- and 7-day time periods.

**DISCUSSION**

The data presented demonstrate that GIP receptors are present on osteoclasts and that GIP dose dependently inhibits osteoclast resorptive activity and inhibits the expression of some markers of osteoclastic differentiation.

GIP expression in the proximal small intestine positions the hormone for a rapid response to nutrient ingestion. As such, GIP is potentially involved in multiple metabolic processes in the body including modulating carbohydrate absorption through effects on insulin secretion (13), lipid breakdown through effects on adipocytes (26), enteric blood flow through effects on vascular endothelial cells (12), and bone turnover through effects on osteoblasts and osteoclasts (2).

We have previously demonstrated that high-affinity GIP receptors are present in osteoblasts and that activation of these receptors in isolated osteoblasts leads to an increase in collagen type I synthesis and alkaline phosphatase activity, consistent with an anabolic effect (3). Furthermore, we have shown that GIP receptor knockout mice have significantly lower bone mass than control mice, with reduced indexes of bone formation and increased indexes of bone breakdown (25). In addition, we reported that daily injection of GIP to ovariectomized rats prevents bone loss (2), which is consistent with an anabolic effect and anti-resorptive effect or both.

The current study clearly demonstrates that GIP has direct anti-resorptive activity. The mechanism of GIP effect on osteoclasts is not clear. We have previously shown that GIP, as a member of the seven-transmembrane domain G protein-coupled family of receptors, increases both intracellular calcium and cellular cAMP content (3). Other investigators have demonstrated that cAMP or agents that raise cAMP, such as calcitonin, inhibit osteoclastic activity (14). Thus we speculate that the effect of GIP on osteoclasts is mediated primarily through cAMP. The fact that we observed a GIP effect on osteoclasts both in organ culture and isolated cells supports both the validity and relevance of our findings. The effect was dose dependent and potent, with effects seen as low as 0.1 nM in isolated osteoclasts. Our results would appear to be contrary to those recently reported by Tsukiyama et al. (24). These investigators did bone histomorphometric analysis of GIP receptor knockout mice; they reported that, in the absence of GIP, the bones of these mice demonstrated a marked increase in the number of mature osteoclasts and a decrease in osteoblastic bone formation. However, using dentin slices, they found that GIP did not inhibit osteoclastic pit formation and thus concluded that there is no direct GIP effect on osteoclasts. However, these results are actually consistent with our own findings. As shown in Fig. 3 in the fetal long bone resorption assay, GIP by itself had no effect on bone resorption; however, GIP had dramatic effects on PTH-induced bone resorption, suggesting that the major effect of GIP is in inhibiting active bone resorption. Pathophysiologically, this is consistent with the postulated role of GIP in vivo. GIP goes up only after a meal, and the arrival of nutrients to the bone would be a signal for a stop to the active bone resorption that occurs during a fast.

The rate of skeletal resorption is known to decrease with nutrient ingestion, and multiple hormones have been suggested to mediate this effect, including GIP, GLP-1 and -2, ghrelin, growth hormone, and insulin and glucagon, as recently reviewed by Clowes et al. (8) and Reid et al. (21). Insulin infusion is known to decrease markers of bone breakdown. However, this effect is only ~30% of the decline in resorption markers that occurs postprandially. Furthermore, it has been suggested that this effect is due to hypoglycemia and the attendant impairment in skeletal cellular activity rather than a direct anti-resorptive effect (1). Similar to our findings with GIP, GLP-2 receptors are present on both osteoblasts and osteoclasts, and infusion of GLP-2 inhibits bone breakdown (10, 15). Therefore, it is likely that a combination of several hormones, incretin and perhaps pancreatic, is responsible for the observed postprandial fall in bone turnover, forming part of what we have termed the “entero-osseous” axis (2).

Another area of potential clinical relevance for our findings relates to patients with diabetes mellitus (DM). Studies of bone density in patients with type 1 DM generally have shown that these patients have lower bone mass, with cortical bone (hip and long bones) reduced to a greater extent than trabecular bone (vertebra, distal radius) (5, 16, 18–20). In contrast, patients with type 2 DM have generally been reported to have normal or high bone mass (5, 22), including both cortical and trabecular bone. Despite these reported differences in bone density, both patients with type 1 DM and those with type 2 DM have been reported to have a higher incidence of fracture, particularly of cortical bone (hip fractures) (5). Multiple potential factors have been implicated in the pathogenesis of the increased fracture rates: alterations in insulin levels, alterations in IGF-I levels, hypercalciuria related to the osmotic diuresis from hyperglycemia, alterations in vitamin D levels, alterations in fat content, which impacts the levels of other hormones such as estrogen and leptin, and alterations in glycation products (6, 11, 22). Interestingly the GIP receptor is known to be downregulated in DM because of the persistent elevations in GIP (23). Thus, as discussed above, it would be tempting to speculate that the loss of the protective effect of the GIP receptor on bone in patients with DM could contribute to their higher fracture rates.

In summary, our present findings demonstrate that GIP directly modulates osteoclastic activity and are consistent with a role for GIP as a mediator in the postprandial suppression of skeletal resorption and in the abnormal bone turnover seen in patients with DM and further support its role as an integrative hormone that maximizes ingested nutrient utilization.

**GRANTS**

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant ROI-DK-058680 to C. M. Isales and by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-46032, NIDDK Grant DK-45228, and National Institute of Dental and Craniofacial Research Grant DE-12459 to K. Insogna.

**REFERENCES**


