GENERAL REVIEW

Interplay between bone and incretin hormones: A review

Interaction entre l’os et les hormones incrétines : une revue

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Summary
Bone is a tissue with multiple functions that is built from the molecular to anatomical levels to resist and adapt to mechanical strains. Among all the factors that might control the bone organization, a role for several gut hormones called “incretins” has been suspected. The present review summarizes the current evidences on the effects of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) in bone physiology.
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Résumé
Le tissu osseux est un tissu conjonctif avec de multiples fonctions qui est organisé depuis l’échelle moléculaire jusqu’à l’échelle anatomique pour résister et s’adapter aux contraintes mécaniques. Parmi tous les facteurs qui pourraient contrôler son organisation, le rôle de certaines hormones intestinales appelées « incrétines » a émergé. La présente revue résume les connaissances actuelles sur les effets du polypeptide insulinotrope dépendant du glucose (GIP) et du glucagon-like peptide-1 (GLP-1) en physiologie osseuse.
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Introduction
Bone is a tissue with multiple functions:
• it supports the body weight and protects essential organs from potential mechanical injuries (mechanical function);
• it acts as a calcium, phosphate and sodium reservoir (metabolic function);
• it is a host tissue for hematopoietic bone marrow and;
• it is also an endocrine organ involved in the regulation of glucose metabolism, energy expenditure, regulation of testosterone production and phosphate homeostasis [1–4].

From the molecular to anatomical levels, bones are built to resist and adapt to mechanical strains according to

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five different levels of organization [5]. First of all, bones have a dual composition as the bone matrix is a complex nanocomposite material made of mineral and organic phases. The organic phase is mostly composed of type I collagen (~90% of total bone proteins) and non-collagenous proteins (~10% bone proteins). The mineral phase is made of poorly crystalline hydroxyapatite tablets with hydrogen phosphate and carbonate groups substituting for phosphate ions [6]. Bone texture represents a second degree of organization. In lamellar bone, collagen is oriented in a precise way with angular changes between each lamella, giving the characteristic appearance of bone texture in polarization microscopy. Woven bone, also called non-lamellar bone, can be found in zones where osteoblast activity is very high (fracture callus, microfractures, metaplastic bone in bone metastasis, Paget’s disease…) and is characterized by an anarthric texture in which collagen microfibers have random directions. Biomechanical properties of woven bone are reduced compared to those of lamellar bone. The third degree of organization is represented by the presence of osteons and arch-like bone structure units. In the cortices, the bone structural units consist of osteons with a cylindrical shape centred on a canal. Typically a complete osteon is 200–300 μm in diameter with a central canal of ~50 μm in diameter. Inside the canal, blood vessel and sympathetic nerve fibres may be observed. Canals are intercommunicating and branched to ensure the communication between periosteal and endosteal spaces. Between complete osteons are incomplete remnants of old osteons, partially eroded that constitute the interstitial bone. In trabecular bone, structural units have an arch-like appearance. These arch-like units are ~40–45 μm in thickness and represent a stack of lamellae. Trabecular bone (or cancellous bone) is sometimes improperly termed “spongy bone”; this term is now considered as improper since it underlies a biomechanical property that bone does not have [7]. New structural units are laid over the trabecular surfaces that have been previously eroded by osteoclasts. Between the newly apposed structural units, remnants of partially eroded units persist and constitute the interstitial trabecular bone. The fourth degree is represented by the bone microarchitecture [5,7]. In the cortices, osteons are compacted so that the axes of the central canal run parallel with the resulting stress line exerted on bone. Trabecular bone tissue is composed of structural units constituting two different types of trabeculae: large plates (arranged along the stress line) connected laterally by pillars or rods, which ensure the cohesion of the network [8]. The role of trabecular bone is to resist to compression loads and transfer the strains to the cortices. Finally the fifth and last level of organization is represented by the bone macroarchitecture. Bones have special angulations and curvatures that are genetically and epigenetically determined and enable them to resist to mechanical strains, including compression, tension or shear stress loads [9,10]. As such, any modification of one of the organization level would affect the quality of the matrix, i.e. an umbrella term representing microarchitectures, microcrack propagation and tissue material properties.

To adapt to its mechanical and metabolic functions, bone is remodeled permanently by a coupling between osteoclasts, the bone-resorbing cells, and osteoblasts, the bone-forming cells responsible for the synthesis of new structural units. Osteocytes (‘‘the third bone cell’’) are derived from osteoblasts and are found embedded in the bone matrix where their main role is to serve as a mechanosensor/mechanotransducer and to inform osteoclasts and osteoblasts about bone areas that are damaged and should be remodeled. Bone remodeling is traditionally considered to be regulated by hormones (parathyroid hormone, calcitonin, estrogen…), autocrine/paracrine signals from the bone microenvironment (receptor activator of nuclear factor kappa-B ligand, tumor necrosis factor-alpha, cell-to-cell contact, etc.), mechanical loading and the central and sympathetic nervous systems.

In the quest of better understanding the different endocrine factors that may regulate bone remodeling, the role of several products from the gastrointestinal tract has been suspected. Indeed, metabolic bone disease associated with long-term parenteral feeding was first described in the early 1980s. Klein et al. reported that, in patients receiving long-term parenteral nutrition, bone physiology was altered with evidence of bone pain, hypercalcemia, elevated serum alkaline phosphatase despite normal ranges of serum calcium, phosphorus and 25-hydroxyvitamin D [11]. These findings have then been confirmed by several bone groups and are reviewed in [12]. Although these effects may be related to the composition of parenteral nutrition itself (low calcium and phosphorus, aluminum, fluoride, etc.), a role for the gastrointestinal tract can also be suspected. Further evidences are brought by a reduction in bone resorption after nutrient ingestion [13]. Indeed the elegant study of Henriksen et al. highlighted reductions of 52%, 39% and 52% after oral intake of glucose, triglycerides, and protein, respectively, in healthy individuals aged between 30 and 40 years old and with a body mass index of 22.7 kg/m² [13]. Furthermore, the experimental use of food fractionation results in higher bone mineral density as compared with a matched nutrient load given once a day [14].

The gastrointestinal (GI) tract is one of the largest endocrine organs with more than 12 different endocrine cells [15]. Among the plethora of bioactive peptides that the GI tract secretes, a class of peptides called incretins has emerged as important modulators of energy metabolism. The term “incretin” was initially proposed by Creutzfeld in 1979 and represents hormones that are secreted from the intestine in response to glucose and stimulate insulin release in a glucose-dependent manner [16]. Although several hormones with insulinotropic action are secreted by the gut, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) are the only two physiological incretins identified so far [17]. GIP and GLP-1 are produced by the K- and L-enteroendocrine cells, respectively, that are localized sparsely in the intestinal epithelium (Fig. 1A). Once released to the blood stream, these two hormones are rapidly degraded by an endopeptidase, the dipeptidylpeptidase-4 (DPP-4) found in the vicinity of capillaries in the intestinal mucosa or liver. DPP-4 is expressed widely as indicated in Table 1. However, due to the wide list of DPP-4 substrates, the role of DPP-4 inhibitors in bone physiology is out of the scope of this review and will not be discussed further.
Figure 1  GIP expression and schematic representation of GIP-producing enteroendocrine K-cells. A. GIP detection was made on a sample from a 68-year old woman using the HPA021612 antibody (Sigma Aldrich). GIP-positive cell localizations are indicated by arrows. This immunostaining is part of the human protein atlas [80] and full dataset can be found at http://www.proteinatlas.org. B. GIP-containing secretory granules are found at the basal pole of the open-type K-cells in close proximity with nerve endings and capillaries in the lamina propria. Entry of nutrients at the apical pole of the K-cells results in a cascade of activation of intracellular pathways leading to augmentations of protein kinase A and C activities (PKA and PKC, respectively) and ultimately rise in intracellular calcium responsible for GIP secretion. Several paracrine peptide (somatostatin) and neuromediators may also modulate GIP secretion.

Expression du GIP et représentation schématique des cellules entéroendocrines K produisant le GIP. A. La détection du GIP a été effectuée dans un prélèvement chez une femme de 68 ans en utilisant l’anticorps HPA021612 (Sigma Aldrich). La localisation des cellules positives au GIP est indiquée par des flèches. Cette détection immunologique fait partie de l’atlas des protéines humaines [80] et l’intégralité des données est disponible à l’adresse http://www.proteinatlas.org. B. Les granules contenant du GIP sont retrouvées au pôle basal des cellules K en étroite association avec des terminaisons nerveuses et des capillaires sanguins du chorion. L’entrée de nutriments au pôle apical des cellules K provoque une cascade d’événements intracellulaires conduisant à l’activation des protéines kinases A et C (PKA et PKC, respectivement) et finalement à l’augmentation du calcium intracellulaire qui est responsable de la sécrétion de GIP. D’autres peptides agissant par voie paracrine (somatostatine) et des neuromédiateurs peuvent également moduler la sécrétion de GIP.

The aim of the current review is to provide the reader with a comprehensive overview of the effects of incretin hormones on bone physiology.

Glucose-dependent insulinotropic polypeptide (GIP)

GIP is produced and secreted mostly by intestinal K-cells, located primarily in proximal region of the small intestine. The K-cell is highly polarized with the GIP-containing secretory granules concentrated at the basal pole of the cell, ready to be released through the basolateral membrane [18,19] (Fig. 1B). Based on the morphological features, GIP secretion from K-cells is regulated by neural stimuli, hormones and intraluminal contents [20]. K-cells are found in close association with the capillary network running through the lamina propria allowing GIP to enter into the blood stream rapidly after secretion. Furthermore, intraluminal contents do not only affect GIP secretion but also GIP expression. Indeed, glucose and lipids are potent stimulator of GIP gene transcription [21,22].

The human proGIP is a 153-amino acid polypeptide that is encoded by six exons representing a 459-bp open reading frame and whose gene is localized in humans on chromosome 17q [23,24]. The mature 42-amino acid bioactive form of GIP (GIP1-42), mainly encoded by exons 3 and 4, is released from its precursor via prohormone convertase 1/3-dependent post-translational cleavage at flanking single arginine residues [25]. The peptides encoded within the remaining fragments of the proGIP have no known biological functions [26].

To exert its biological actions, GIP binds to its receptor, the GIPr. The human GIPr gene comprises 14 exons that span approximately 14.2 kb and is localized on chromosome 19q13.3 [27]. The GIPr belongs to the class B 7-transmembrane-spanning G-protein coupled receptor (GPCR) superfamily [28] and is composed of 466 amino acids. The GIPr is expressed in the endocrine pancreas, gastrointestinal tract, adipose tissue, adrenal cortex, pituitary gland, vascular endothelium and several regions in the CNS [26]. The principal physiological role of the GIP/GIPr is to increase insulin secretion from the pancreatic beta-cells in a glucose-dependent manner. However, extrapancreatic actions of this pathway have been reported. GIP acts on lipid metabolism including augmentation of plasma triglyceride clearance, increased lipoprotein lipase activity and promotion of fat storage in adipocytes [29–31]. In animal
model of GIP deficiency or chemically-induced GIPr antagonism, interrupting GIP signaling appears to be beneficial in reducing high fat diet induced obesity [30,32—34]. GIP has also been reported to play a role in neural progenitor cell proliferation and behavior [35].

The GIP/GIPr pathway is also an important regulator of bone physiology. First of all, the GIPr is physiologically expressed at the mRNA and protein level in osteoblasts and osteoclasts [36—39]. In osteoblasts, GIP administration results in increases in intracellular calcium and CAMP that lead to augmentations in type I collagen expression, alkaline phosphatase and lysyl oxidase activities, and higher enzymatic collagen cross-linking [37,40]. GIP is also capable of reducing the extent of bone resorption by fully mature osteoclasts although the exact molecular mechanisms remain to be elucidated [39,41]. At the organ level, much of our understanding of the actions of the GIP/GIPr pathway arises from genetically-modified mice. Indeed, to date, two animal models of GIPr deletion exists with either deletion of exons 4—5 or exons 1—6. These portions of the Gipr gene either code for several amino acids of the extracellular domains, involved in GIP binding (exons 4—5) or for the totality of the extracellular domain and the first transmembrane segment (exons 1—6). These animals, although with opposite trabecular bone phenotype, suggest that trabecular and cortical bone are dramatically compromised in the absence of a functional GIPr as presented in Fig. 2 [41—44]. Furthermore, these animal models also present with alterations of tissue material properties represented by reduced mineralization degree (Fig. 3) and collagen maturity in the bone matrix [43]. Overall these modifications of bone mass, microarchitecture and tissue material properties result in skeletal fragility.

However, lack of GIPr action leads to increased GLP-1 sensitivity [45]. As such, to answer whether the observed above alterations observed in GIPr KO mice resulted from the lack of a functional incretin receptor or by a compensatory mechanism induced by elevated sensitivity to the other incretin hormone, the bone phenotype of double incretin receptor knock-out (DIRKO) animals, lacking both the GIPr and GLP-1r, was investigated [46]. DIRKO animals also exhibit profound reductions in bone mass as well as alterations of trabecular and cortical microarchitectures and tissue material properties demonstrating the important role of incretins in bone physiology.
In healthy humans, although initially GIP had been shown ineffective in reducing bone resorption [13], recent evidences suggested that exogenous administration of GIP was effective in reducing circulating markers of bone resorption [49]. However, whether these results reflect direct actions of GIP on osteoclast-mediated resorption or indirect actions of other GIP-targeted tissues remain to be determined in the future. Furthermore, the link between GIP/GIPr pathway and bone mass/strength is definitely established and represented by low mineral bone density at the femoral neck and total hip, as well as higher incidence of non-vertebral fractures, in a cohort of perimenopausal women with a single-nucleotide polymorphism (rs1800437), that results in decreased GIPr activity [50,51].

**Glucagon-like peptide-1 (GLP-1)**

Glucagon-like peptide 1 is produced by post-translational processing of the glucagon gene (Fig. 4). Indeed, in intestinal L-cells, the glucagon gene (160-amino acids) gives rise to glicentin (amino acids 1–69), glicentin-related polypeptide (amino acids 1–30), oxyntomodulin (amino acids 33–69), glucagon (amino acids 33–61), peptide 1 (amino acids 64–69), GLP-1 (amino acids 72–107), peptide 2 (amino acids 111–123) and GLP-2 (amino acids 126–158) [52] [53]. Thus, processing of proglucagon in intestinal cells generates equimolar concentration of GLP-1 and GLP-2. Until now, little is known about the biological actions of glicentin and peptide 2.

Two forms of GLP-1 are produced in the intestine, GLP-17-36NH2 and GLP-17-37, although the major circulating form is GLP-17-36NH2 [54]. L-cells are also an open-type endocrine cells highly polarized with secretory granules at their basolateral pole. GLP-1 secretion from L-cells is regulated by intraluminal contents, neural stimuli and hormones [26]. L-cells are found in close association with the capillary network running through the lamina propria. GLP-1 has also been suspected to act via the autonomous nerve system and vagal afference on specific hypothalamic and brainstem nuclei to exert its action [55].

To act, GLP-1 engages its receptor, the GLP-1r that is coded by the human GLP1R gene comprises 13 exons that span approximately 13.8 kb [56] and is localized on chromosome 6p21 [27]. The GLP-1r is expressed in the endocrine pancreas, gastrointestinal tract, lung, heart, kidney and several regions of the brain [26]. Recent evidences also suggest that GLP-1 can bind in specific circumstances to the glucagon receptor [57]. The principal physiological role of GLP-1 is to potentiate glucose-dependent insulin secretion [58]. Extrapancreatic actions of GLP-1 result in reduction of food intake through the CNS, inhibition of gastric emptying, positive actions on the cardiovascular system and a role in energy expenditure [58].

Presence of the known GLP-1r in bone cells was controversial until recently. Indeed, this confusion was due to the use of poorly-characterized cell lines [36], selection of wrong primer pairs [59], or the use of non-selective anti-GLP-1r antibodies [60] that were not very specific of the mouse Glp1r gene. Recent evidences by Pereira et al. seem suggest the presence of the known GLP-1r in skeletal
**Figure 4** Post-translational processing of proglucagon. The proglucagon gene spans over 12.4 kb located in human on chromosome 2q36-q37. The proglucagon gene comprised 6 exons that encode several peptides: a signal peptide (SP, in blue), glicentin-related polypeptide (GRPP, in green), glucagon (GLG, in orange), peptide-1 (P1, in black), glicentin, oxyntomodulin (Oxm), glucagon-like peptide-1 (GLP-1, in pink), peptide-2 (P2, in violet), glucagon-like peptide-2 (GLP-2, in yellow) and the major proglucagon fragment (MPGF). Depending on the tissue, post-translational processing by prohormone convertase 1 and 2 may give rise to several combinations of these peptides.

**Figure 5** Skeletal phenotype observed in GLP-1r-deficient animals. Bone microarchitectures were assessed by X-ray microcomputed tomography. Three-dimensional models of tibia proximal metaphysis observed in wild-type (WT) and GLP-1r-KO mice.

Nuche-Berenguer et al. revealed the presence of this second GLP-1 receptor in the MC3T3-E1 murine osteoblastic cell line [62]. However, as recent evidences pointed out that GLP-1 could bind to the glucagon receptor in certain circumstances [57], it would be compulsory to ascertain whether this second GLP-1r correspond to a modulation of ligand specificity at the glucagon receptor.

Here again, our understanding of GLP-1 actions in skeletal physiology arises from GLP-1r-KO mouse. At 10 weeks of age, GLP-1r-KO animals exhibited a mild reduction in trabecular bone volume at the tibia, although not significant, associated with increased number of osteoclasts and eroded surfaces [65]. Unpublished observation from our laboratory made in the same KO model at 16 weeks of age corroborated these findings (Fig. 5). On the other hand, the mineral apposition and bone formation rates appeared unaffected by GLP-1r inactivation [65]. Taken together these results suggested a control of bone resorption (osteoclast differentiation and/or action) by the GLP-1r. However, GLP-1 was unable to directly control osteoclast formation and resorption in osteoclast cultures [65], suggesting that the control of bone resorption was indirect. Indeed, these authors found a reduction in calcitonin gene expression in GLP-1r-deficient animals. Now, we know that in rodents, but not in non-human primate or humans, GLP-1r is expressed in C-cells of the thyroid gland, and responsible for a rise in calcitonin secretion [66]. However, in humans, administration of exogenous GLP-1 does not result into lower CTx levels [13].

The effect of GLP-1r deficiency in cortical bone has been investigated in 16 week-old GLP-1r-deficient mice. These animals presented with reduction in bone strength observed by 3-point bending [42]. In these animals, modification of cortical microarchitecture was evidenced with reduction in
a GLP-1r agonist, results in a rapid augmentation of osteocalcin gene expression and reductions in sclerostin expression and in the balance RANKL/OPG (receptor activator of nuclear factor kappa-B ligand/osteoprotegerin) in the bones of normal, type 2 diabetic or insulino-resistant rats [59,67,68].

In type-1 diabetes mellitus, administration of liraglutide for 21 days contributed to significant improvement in bone strength at the tissue levels and reduction in collagen degradation in the bone matrix [48]. However, no improvements in neither trabecular nor cortical bone microarchitecture were observed. Nevertheless, these mild ameliorations occurred in the absence of insulin secretion. Due to the marketing of GLP-1 mimetic for type 2 diabetes, the efficacy of GLP-1 analogues has been performed in type 2 diabetic rodent models. In this condition, the administration of liraglutide ameliorated trabecular and cortical bone microarchitectures [69]. It is however worth noting that the administration of liraglutide has been performed at a dose regimen of 0.4 mg/kg/day as compared with the 0.02 mg/kg/day used in human clinical trials [70,71]. The effects of GLP-1 mimetic have also been conducted in osteoporotic rodent models. Indeed, 16 weeks administration of exendin-4 (dose regimen of 10 μg/kg/day similar to the dose used in humans [72]) in OVX rats, is capable of improving trabecular bone mass and microarchitecture at the femur and lumbar vertebrae, bone strength and revert hyper-resorption observed after ovariectomy [61,73]. It is also noteworthy that at this dose regimen, exendin-4 was incapable of reversing the observed deterioration of cortical microarchitecture. Pereira et al., also reported positive effects of liraglutide (0.3 mg/kg/day) in improving trabecular but not cortical microarchitecture [61]. However, administration of liraglutide at a dose of 1.8 mg/kg/day for 8 weeks, significantly improved trabecular and cortical microarchitecture [74]. This dose regimen is 90 times more elevated than the dose given to humans. Moreover, recently, evidences have been provided that undercarboxylated osteocalcin was capable of inducing GLP-1 release from the L-cells and as such this complementary mechanism reinforced the role of osteocalcin in energy expenditure [75,76].

Now several GLP-1 analogues, enzymatically resistant to degradation by DPP-4, have been approved for the treatment of type-2 diabetes mellitus. Regarding the skeletal alterations observed in animals deficient in GLP-1r and the rapid and favorable effects of GLP-1 or GLP-1r agonists on bone gene expression, one could expect an improvement in bone quality in type-2 diabetic patients. However, in a recent meta-analysis conducted by our group on the incidence of bone fractures in type-2 diabetic patients taking GLP-1 mimetic, we failed to evidence any beneficial effects of GLP-1 mimetic [77]. This was further confirmed by investigation of the British clinical practice research datalink, where 216,816 diabetic patients were screened and dichotomized depending on their anti-diabetic medications [78,79]. No significant reduction in the occurrence of bone fractures have been evidenced under the use of GLP-1 mimetic. Nevertheless, it is also important to bear in mind that no increase of fracture risk was noted with these medications and that GLP-1 mimetics in humans are neutral in term of bone safety.
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