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Pituitary-bone connection in skeletal regulation

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Abstract: Pituitary hormones have traditionally been thought to exert specific, but limited function on target tissues. More recently, the discovery of these hormones and their receptors in organs such as the skeleton suggests that pituitary hormones have more ubiquitous functions. Here, we discuss the interaction of growth hormone (GH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), prolactin, oxytocin and arginine vasopressin (AVP) with bone. The direct skeletal action of pituitary hormones therefore provides new insights and therapeutic opportunities for metabolic bone diseases, prominently osteoporosis.

Keywords: glycoprotein hormone coupled receptor (GPCR); osteoblast; osteoclast; pituitary-bone axis.

Introduction

It has become clear over the past decade that pituitary hormones and their receptors have more ubiquitous functions in integrative physiology than was previously proposed. Organs, such as the skeleton, are regulated by and respond to pituitary hormones, particularly when circulating levels are perturbed in disease. Additionally, certain pituitary hormones are also expressed in bone cells, underscoring paracrine regulation. Growth hormone (GH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH), prolactin, oxytocin and vasopressin all affect bone, and in genetically modified mice, the haploinsufficiency of either the ligand and/or receptor yields a skeletal phenotype with the primary target organ remaining unaffected. Recognition of novel mechanisms of action of each pituitary hormone has opened new therapeutic opportunities. Here we discuss the interaction of each pituitary hormone with bone and the potential it holds in understanding and treating osteoporosis.

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Growth hormone and insulin-like growth factor-1

GH plays key functions in skeletal growth, modeling and remodeling. It directly acts through a glycoprotein coupled receptor (GPCR), but its primary action occurs *via* the release of insulin-like growth factors (IGFs). IGF-1 is synthesized mainly in the liver and ~80% circulates bound to IGF-binding protein-3 (IGFBP3) and the acid labile subunit (ALS). In growth hormone receptor (GHR)-deficient mice, both growth retardation and osteoporosis are rescued by IGF-1 over-expression [1], attesting to the relative importance of IGF-1 over GH. Additionally, despite elevated GH levels, mice lacking both liver IGF-1 [liver-specific IGF1-deficient (LID)] and ALS, with depleted serum IGF-1, show reduced bone growth and strength [2]. These results suggest that the skeletal effects of GH require IGF-1. In addition, GH-induced osteoclastic activity appears to require IGF-1 released from bone, which then activates bone resorption by acting on osteoclastic receptors, as well as by altering receptor activator of nuclear factor kappa-B ligand (RANK-L) expression [3–5]. However, evidence to suggest that GH can act independently of IGF is limited. GH replacement reverses the increased adiposity in hypophysectomized rats, while IGF-1 replacement does not [6]. Furthermore, GH reverses osteopenia in ovariectomized LID mice [7]. These results suggest that GH also directly acts on bone.

Follicle stimulating hormone

We discovered that FSH directly stimulates bone resorption by osteoclasts [8, 9]. Several studies have confirmed direct effects of FSH on the skeleton in rodents and humans. For example, amenorrheic women with a higher mean serum FSH (~35 IU/L) have greater bone loss than those with lower levels (~8 IU/L) in the face of near-equal estrogen

levels [10]. Patients with functional hypothalamic amenorrhea, in whom both FSH and estrogen were low, show slight to moderate skeletal defects [11]. Women harboring an activating FSHR-N680S polymorphism, rs6166, have lower bone mass and high resorption markers [12]; this attests to a role for FSHRs (follicle stimulating hormone receptors) in human physiology. The exogenous administration of FSH to rats augments ovariectomy-induced bone loss, and a FSH antagonist reduces bone loss after ovariectomy or FSH injection [13, 14].

Mechanism of action

FSH increases osteoclast formation, function and survival through a distinct FSHR isoform [8, 15–17]. Two groups have, however, failed to identify FSHRs on osteoclasts, having likely used primers targeted to the ovarian isoform [18, 19]. We very consistently find FSHR in human $CD14^+$ cells and osteoclasts using nested primers and sequencing to verify the specificity of the reaction, and amplifying regions that contain an intron to avoid the pitfall of genomic DNA contamination [15].

Wu et al. showed that the osteoclastogenic response to FSH was abolished in mice lacking tyrosine-based activation motif (ITAM) adapter signaling molecules [17]. This suggests an interaction between FSH and immune receptor complexes, although the significance of such an interaction remains unclear. In a separate study, FSHR activation was shown to enhance RANK receptor expression [20]. In addition, FSH indirectly stimulates osteoclast formation by releasing osteoclastogenic cytokines, namely interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in proportion to the surface expression of FSHRs [9, 21]. In a study of 36 women between the ages of 20 and 50 years, serum FSH concentrations correlated with circulating cytokine concentrations [21, 22].

Human studies

Correlations between bone mineral density (BMD) declines and serum FSH levels have been documented extensively. The Study of Women's Health Across the Nation (SWAN), a longitudinal cohort of 2375 peri-menopausal women, showed a strong correlation between serum FSH levels and markers of bone resorption, as well as an equally strong correlation between changes in FSH levels over 4 years and decrements in BMD [23]. Analyses of data from Chinese women showed similar trends: a significant association between bone loss and high serum FSH [24, 25]. In a group of southern Chinese women aged between 45 and 55 years, those in the highest quartile of serum FSH lost bone at a 1.3- to 2.3-fold higher rate than those in the lowest quartile [26]. Furthermore, analysis of a National Health and Nutrition Examination Survey (NHANES) III cohort of women between the ages of 42 and 60 years showed a strong correlation between serum FSH and femoral neck BMD [27]. Likewise, a cross-sectional analysis of 92 post-menopausal women found that serum osteocalcin and C-telopeptide levels were both positively correlated with FSH, but not with estradiol [28].

The bone turnover range of normality (BONTURNO) study group showed that women with serum FSH levels of >30 IU/mL had significantly higher bone turnover markers than age-matched women, despite having normal menses [29]. In contrast, Gourlay et al. failed to show a strong relationship between bone mass and FSH or indeed estrogen [30]. Interestingly, however, the same authors show an independent correlation between FSH and lean mass [31]. This latter association makes biological sense inasmuch as FSHRs are present on mesenchymal stem cells [8], known to have the propensity for adipocyte and myocyte differentiation. Notwithstanding whether there is a cause-effect relationship between a rising FSH and BMD changes, there is clear evidence favoring the use of FSH as a serum marker for identifying "fast bone losers" during the early phases of the menopausal transition, most notably the late peri-menopause [32].

Relative actions of FSH and estrogen

It has been difficult to tease out the action of FSH from that of estrogen in vivo as FSH releases estrogen and the actions of FSH and estrogen on the osteoclast are opposed. The injection of FSH into mice with intact ovaries [19], or its transgenic over-expression [18], even in *hpg* mice, is unlikely to reveal pro-resorptive actions of FSH. This is because direct effects of FSH on the osteoclast will invariably be masked by the anti-resorptive and anabolic actions of the ovarian estrogen so released in response to FSH.

Clinically, there is evidence that women with low FSH levels undergo less bone loss than women with higher FSH levels, estrogen levels being nearly equal [10], and importantly, that the effectiveness of estrogen therapy is related to the degree of FSH suppression [33]. With that said, patients with pituitary hypogonadism do lose bone. Luperide treatment, and hence the lowering of FSH, has not been shown to prevent hypogonadal hyper-resorption [34]. While this proves that low estrogen is a *cause of* acute hypogonadal bone loss, it does not exclude a role

for FSH in human skeletal homeostasis [34]. Rather than blocking FSH in acute hypogonadism, where the effect of low estrogen is likely to be overwhelming, FSH inhibition during the late peri-menopause, particularly when estrogen levels are normal and FSH is high, could potentially be of therapeutic significance.

FSH antibody rescues hypogonadal bone loss

We have recently developed a polyclonal antibody against the 13-amino-acid-long conserved peptide sequence of the computationally defined FSHR-binding domain of FSHβ [35, 36]. We found that this antibody not only attenuated osteoclastogenesis induced by FSH in vitro, but also prevented the loss of bone post-ovariectomy [35, 36]. Most importantly, we noted through a detailed histomorphometric analysis that while bone resorption was reduced, bone formation was surprisingly elevated significantly [35]. This decoupling likely underscored the osteoprotective action of the antibody. Further studies documented FSHRs on mesenchymal stem cells. Additionally, bone marrow stromal cells from $FSHR^{-/-}$ mice showed significantly greater colony-forming (Cfu-f) potential, suggesting a profound increase in osteoblastogenesis in the absence of the FSHR. Importantly, antibody injections did not affect serum estrogen levels in wild type mice [35]. Collectively, our studies provide clear evidence that lowering FSH in a hypogonadal state prevents bone loss.

Thyroid stimulating hormone

TSH directly inhibits osteoclasts [37]. Thyroid stimulating hormone receptor (TSHR) haploinsufficiency in heterozygotic $TSHR^{+/-}$ mice results in osteoporosis, while thyroid hormone levels remain unaffected [37]. Moreover, $TSHR^{-/-}$ mice are osteoporotic, a phenotype that cannot be explained by the known pro-osteoclastic action of thyroid hormones, particularly as $TSHR^{-/-}$ mice are hypothyroid [38]. Furthermore, thyroid hormone replacement that renders $TSHR^{-/-}$ mice euthyroid reverses skeletal runting but not the osteoporotic phenotype [37]. Thus, TSH acts on bone independently of thyroid hormones. These findings also suggest that the osteoporosis of hyperthyroidism may, in part, be due to low TSH [39, 40]. At least 20 clinical studies have since documented tight and highly reproducible correlations between low TSH levels, bone loss, bone geometry, and fracture risk in patient cohorts across the globe [41–61]. Evidence also shows that TSH protects the skeleton by exerting anti-resorptive and anabolic actions in rodent models and in people [37, 62–71].

Mechanism of action

The osteoporosis of TSHR deficiency is of the high-turnover variety. Osteoclastic activity is increased in $TSHR^{-/-}$ mice, similar to *hyt*/*hyt* mice in which TSHR signaling is defective [72, 73]. Recombinant TSH has been shown to attenuate the genesis, function and survival of osteoclasts in ex vivo murine bone marrow [37] and in vitro embryonic stem cell cultures [74]. The latter suggested a role early in bone development [74]. In contrast, the over-expression of constitutively activated TSHR in osteoclast precursor cells [64] or transgenically, in mouse precursors [73] inhibited osteoclastogenesis. In post-menopausal women, a single subcutaneous injection of TSH drastically lowered serum C-telopeptide to premenopausal levels within 2 days, with recovery at day 7 [71]. In none of the studies with TSH replacement did thyroid hormones increase, exemplifying again that the pituitary-bone axis is more primitive than the pituitary-thyroid axis.

This anti-osteoclastogenic action of TSH is mediated by reduced NF-κB and JNK signaling, and TNFα production [37, 64]. The effect of TSH on TNFα synthesis is mediated transcriptionally by binding of two high mobility group box proteins, HMGB1 and HMGB2, to TNFα gene promoter [75]. TNFα production is expectedly upregulated in osteoporotic $TSHR^{-/-}$ mice [37], and the genetic deletion of TNFα in these mice reverses the osteoporosis, as well as the bone formation and resorption defects, proving that the $TSHR^{-/-}$ phenotype is mediated by TNFα, at least in part [64, 76]. The osteoporosis, low bone formation, and hyperresorption that accompany TSH deficiency were fully rescued in compound mouse mutants in which TNFα is genetically deleted on a homozygote $TSHR^{-/-}$ or heterozygote $TSHR^{+/-}$ background [76]. Studies using ex vivo bone marrow cell cultures show that TSH inhibits and stimulates TNFα production from macrophages and osteoblasts, respectively [76]. TNFα, in turn, not only stimulates osteoclastogenesis but also enhances the production in bone marrow of a variant TSHβ [76, 77]. This locally produced TSH suppresses osteoclast formation in a negative feedback loop.

The role of TSH in osteoblast regulation is less defined. While it inhibits osteoblastogenesis in bone marrow-derived cell cultures, TSH stimulates differentiation and mineralization in murine cell cultures through a Wnt5a-dependent mechanism [67]. Likewise, in vivo,

intermittently administered TSH is anabolic in both rats and mice [69, 73]. Thus, the effect of TSH may be differentiation stage-dependent. However, in vivo, in rats, TSH, injected up to once every 2 weeks, inhibits ovariectomy-induced bone loss 28 weeks following ovariectomy [73]. Calcein-labeling provides evidence for a direct anabolic action of intermittent TSH [69]. In humans, Martini et al. [70] showed an increase in procollagen type I N propeptide (PINP), a marker of bone formation, validating the conclusion that bolus doses of TSH are indeed anabolic.

Clinical studies

Consistent with our work, and that from other labs, epidemiologic studies show a 4.5-fold increase in the risk of vertebral fractures and a 3.2-fold increase in the risk of non-vertebral fractures is seen at TSH levels <0.1 IU/L [78]. There is also a strong negative correlation between low serum TSH and high C-telopeptide levels, without an association with thyroid hormone [79]. In patients on L-thyroxine, greater bone loss has been noted in those with a suppressed TSH than those without suppression [57, 80, 81]. The Tromso study supports this: participants with serum TSH below 2 SD had a significantly lower BMD, those with TSH above 2 SD had a significantly increased BMD, whereas there was no association between TSH and BMD at normal TSH levels [44]. In patients taking suppressive doses of thyroxine for thyroid cancer, the serum level of cathepsin K, a surrogate resorption marker, was elevated [60], and the Nord-Trøndelag Health Study 2 (HUNT 2) study found a positive correlation between TSH and BMD at the distal forearm [58]. Furthermore, evaluation of the NHANES data has shown that the odds ratio for correlations between TSH and bone mass ranged between 2 and 3.4 [43]. Euthyroid women with serum TSH in the lower tertile of normal displayed a higher incidence of vertebral fractures, independent of age, BMD and thyroid hormones [41]. Finally, patients harboring the TSHR-D727E polymorphism had high bone mass [59]; similar allelic associations have been reported from the United Kingdom and in the Rotterdam study [82, 83].

Physiologically, therefore, TSH uncouples bone remodeling by inhibiting osteoclastic bone resorption and stimulating osteoblastic bone formation, particularly when given intermittently. Furthermore, absent TSH signaling stimulates bone remodeling directly, and through TNFα production, causing net bone loss. We compared the effect of inducing hyperthyroidism through the implantation of T_4 pellets in wild type and $TSHR^{-/-}$ mice to determine whether mice with absent TSHR signaling lost more bone [40]. Whereas wild type hyperthyroid mice lost bone, expectedly from the pro-resorptive actions of thyroid hormones, the loss was greater in hyperthyroid $TSHR^{-/-}$ mice – clearly demonstrating a direct action of TSH signaling on bone that was independent of thyroid hormone levels [40]. Low TSH levels may thus contribute to the pathophysiology of osteoporosis of hyperthyroidism, which has traditionally been attributed to high thyroid hormone levels alone.

Adrenocorticotrophic hormone

Glucocorticoids, under natural regulation mainly by ACTH, are important co-regulators of many processes including vascular tone, central metabolism, and immune response. At higher pharmacological levels, they become anti-inflammatory and immunosuppressant drugs, with incident complications including diabetes, osteoporosis, and osteonecrosis. Osteonecrosis, in particular, is a painful debilitating condition that affects metabolically active bone, typically the femoral head [84], and invariably requires surgical treatment. The underlying mechanisms of glucocorticoid-induced osteonecrosis are poorly understood, although a key finding is that osteonecrosis occurs prior to macroscopic vascular changes [85].

Isales et al. [86] discovered that bone-forming units strongly express melanocortin-2 receptors (MC2Rs). We showed that as with the adrenal cortex, ACTH induces Vascular endothelial growth factor (VEGF) production in osteoblasts through its action on MC2Rs [87]. This likely translates into the protection by ACTH of glucocorticoid-induced osteonecrosis in a rabbit model [87]. An independent report with consistent findings was recently published [88]. We speculate that VEGF suppression secondary to ACTH suppression may contribute to the bone damage with long-term glucocorticoid therapy. Much needs to be done to validate this idea towards a therapeutic advantage, considering that ACTH analogs are already approved for human use.

Prolactin

Prolactin (PRL), a peptide hormone secreted by the anterior pituitary, acts to induce and maintain lactation, as well as to suppress folliculogenesis and libido. During pregnancy, it increases the calcium bioavailability for milk production and fetal skeletalogenesis by promoting intestinal calcium absorption and skeletal mobilization [89]. Accelerated bone turnover and bone loss is noted

in hyperprolactinemic adults [90]. Antagonism of PRL by bromocriptine, a dopamine agonist, reverses the bone loss [91]. This osteoclastic action of PRL is traditionally thought to arise from the accompanying hypoestrogenemia [92]. However, it has been shown that osteoblasts express prolactin (PRLRs) [93], suggesting a direct interaction between PRL and the osteoblast. In fact, the pattern of bone loss is distinct in PRL-exposed and ovariectomized rats [94]. Ex vivo, PRL decreases osteoblast differentiation markers [94], in part, through the PI3K signaling pathway [95]. PRL injected into adult mice accelerates bone resorption [94], notably by increasing the RANK/OPG (osteoprotegerin) ratio [94]. Osteoclasts themselves do not possess PRLRs [93]. In contrast, in infant rats, PRL causes net bone gain [96], increased osteocalcin expression. Likewise, in human fetal osteoblast cells, PRL decreases the RANKL/OPG ratio [95]. It appears therefore that the net effect of PLR on bone depends on the stage of development. In the fetus, it promotes bone growth and mineralization, while accelerating bone resorption in the mother to make nutrients available.

Oxytocin

Oxytocin (OXT) is a nonapeptide synthesized in the hypothalamus and released into circulation *via* the posterior pituitary. Its primary function is to mediate the milk ejection reflex in nursing mammals. It also stimulates uterine contraction during parturition; however OXT is not a requirement for this function. Thus, OXT null mice can deliver normally, but are unable to nurse. Subcutaneous OXT injection completely rescues the milk ejection phenotype, attesting to this being a peripheral, as opposed to a central action [97]. Central actions of OXT include the regulation of social behavior, including sexual and maternal behavior, affiliation, social memory, as well as penile erection and ejaculation [98–101]. It also controls food, predominantly carbohydrate, intake centrally [102]. Thus, the social amnesia, aggressive behavior and overfeeding observed in $OXT^{-/-}$ and $OXTR^{-/-}$ (oxytocin receptor) mice are reversed on intracerebroventricular OXT injection [103].

Mechanism of action

OXT acts on a GPCR, present in abundance in osteoblasts [104], osteoclasts, and their precursors [105]. In line with the ubiquitous distribution of OXTRs, cells of bone marrow also synthesize OXT, suggesting the existence of autocrine and paracrine interactions [106]. In vitro, OXT stimulates osteoblast differentiation and bone formation. Thus, $OXT^{-/-}$ and $OXTR^{-/-}$ mice, including the haploinsufficient heterozygotes with normal lactation, display severe osteoporosis due to a bone-forming defect [107]. This not only indicates that the osteoblast is the target for OXT, but also that bone is more sensitive to OXT than the breast, *hitherto* considered its primary target. Once again, the finding emphasizes a relatively primitive pituitary-bone axis. Effects of OXT on bone resorption in vivo appear minimal, as OXT stimulates osteoclastogenesis, but inhibits the activity of mature osteoclasts, with a net zero effect on resorption.

In vivo gain-of-function studies document a direct effect of OXT on bone. Intra-peritoneal OXT injections result in increased BMD and ex vivo osteoblast formation [107]. In contrast, short-term intracerebroventricular OXT does not affect bone turnover markers. OXT injections in wild type rats alter the RANKL/OPG ratio in favor on bone formation, again attesting to an anabolic action [108].

Skeletal effects of pregnancy and lactation

Although unproven, OXT may have a critical role in bone anabolism during pregnancy and lactation. Both are characterized by excessive bone resorption in favor of fetal and post-partum bone growth, respectively [109]. This bone loss is, however, completely reversed upon weaning by a yet unidentified mechanism [110]. OXT peaks in blood during late pregnancy and lactation, and while its pro-osteoclastogeneic action may contribute to intergenerational calcium transfer, its anabolic action could enable the restoration of the maternal skeleton. That $OXT^{-/-}$ pups show hypomineralized skeletons, and $OXT^{-/-}$ moms display reduced bone formation markers are suggestive of such actions. The question whether estrogen, *via* its positive regulation of osteoblastic OXT production, can synergize this action through a local feed-forward loop, remains to be determined. These studies nonetheless pave the way for a greater understanding of pregnancy and lactation-associated osteoporosis, as well as new potential therapeutic options.

Vasopressin

Arginine vasopressin (AVP), another posterior pituitary hormone, is a nonapeptide that differs from OXT only by two amino acids. Its primary functions are to retain water

in the body by increasing water reabsorption in the kidney, and to constrict blood vessels, thereby increasing arterial blood pressure. AVP exerts its actions through GPCRs. AVPR1A is present in the kidney, liver, peripheral vasculature, and brain. AVPR2 is expressed predominantly in the kidney. Expression of AVPR1A and AVPR2 are also found in osteoblasts and osteoclasts [111]. AVPR1B, also known as vasopressin 3 receptor, is expressed in the anterior pituitary and the brain, but not in the skeleton.

AVP can affect the skeleton directly and is yet another component of the pituitary-bone axis. For example, mice injected with AVP display reduced osteoblast formation and increased osteoclast formation [111]. Conversely, mice injected with the AVPR1A antagonist SR49059 had enhanced bone mass due to increased osteoblastogenesis and reduced osteoclast formation and bone resorption [111]. This high bone mass phenotype was also observed in AVPR1A-deficient mice [111]. In contrast, AVPR2 does not have a significant role in bone remodeling, since the specific AVPR2 inhibitor tolvaptan does not affect bone formation or bone mass [112]. Collectively, the data establish a primary role for AVP signaling in bone mass regulation. Of note is that hyponatremic patients have elevated circulating AVP levels and a high fracture risk, prompting for further studies on the skeletal actions of AVPR inhibitors used commonly in these patients.

The highly homologous AVP and OXT can share their receptors in the regulation of bone formation by osteoblasts. AVPR1A and OXTR have opposing effects on bone mass. Notably, the deletion of OXTR in $OXTR^{-/-}$:$AVPR1A^{-/-}$ double-mutant mice reversed the high bone mass phenotype in $AVPR1A^{-/-}$ mice [112]. While OXTR deletion in $AVPR1A^{-/-}$ cells inhibits AVP-stimulated gene expression, OXTR is dispensable for the anti-osteoblastic action of AVP [112]. Furthermore, OXT does not interact with AVPRs in vivo in a model of lactation-induced bone loss in which OXT levels are high [112].

Conclusions

The direct regulation of bone by glycoprotein hormones, since their discovery, helps explain some of the inconsistencies of older models that assumed that pituitary signaling was mediated entirely *via* endocrine organs through steroid-family signals. They also offer new sets of therapeutic opportunities. Important direct responses include actions of TSH, FSH, ACTH, oxytocin and vasopressin in bone. It is important, in evaluating these new signaling mechanisms, to consider that the skeletal responses may or may not have similar mechanisms to the responses of the traditional endocrine targets, and that the signals may vary in importance due to secondary endocrine and paracrine control.

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