Cannabinoids as Therapeutics
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The skeleton: stone bones and stoned heads?

Itai A. Bab

Bone Laboratory, The Hebrew University of Jerusalem, Jerusalem 91120, Israel

Introduction

In vertebrates, bone mass is maintained constant between the end of linear skeletal growth, when the peak bone mass is established, and gonadal failure, when accelerated bone loss begins. The bone mass is preserved by a continuous destruction/formation process termed bone remodeling [1]. This destruction/formation cycle occurs at the same time in multiple foci that in humans encompass approximately 5% of trabecular, endosteal, and Haversian system surfaces. A cycle consists initially of a relatively rapid (i.e. a few weeks) resorption of pre-existing bone by a bone-specific, bone marrow hematopoietic cell type, the osteoclast, derived from the monocyte/macrophage lineage [2]. It is then followed by a slower (i.e. a few months) step of *de novo* bone formation by another bone-specific cell type, the osteoblast [3], which belongs to the stromal cell lineage of bone marrow [4]. Although different foci present different phases of the cycle, the overall net effect is that of a balance between bone destruction and formation. The physiologic importance of bone remodeling is best illustrated in osteoporosis, the most frequent degenerative disease in developed countries, which results from impaired remodeling balance that leads to bone loss and increased fracture risk mainly in females but also in males.

The synchronized occurrence of multiple remodeling sites has long been viewed as suggestive of a complex, local, autocrine/paracrine [5] as well as endocrine regulation. Indeed, experiments in knockout (KO) and transgenic mice have demonstrated paracrine regulation of osteoclast differentiation and activity by factors such as receptor activator of NFκB (RANK) ligand, osteoprotegerin, macrophage colony-stimulating factor (M-CSF) and interleukin 6, which are derived from neighboring stromal cells, including osteoblasts and osteoblast precursors [6–11]. The most convincing evidence for local osteoblast regulation is by bone morphogenetic proteins [12]. Systemically, ablation of gonadal hormones in females and males has been repeatedly demonstrated to favor bone loss in humans, rats, and mice [13, 14]. In addition, parathyroid hormone [15, 16], calcitonin [17], insulin-like growth factor I [18], and the osteogenic growth peptide [19] have been shown to regulate bone formation. More recently, it has been demonstrated that bone remodeling is also subject to a potent central control consisting of hypothalamic leptin and neuropeptide Y

signaling [20, 21] as well as downstream noradrenergic signaling by osteobalstic β2 adrenergic receptors [22]. It thus appears that the critical systems in the control of bone remodeling are gonadal and central nervous system-derived, and apply tonic inhibition of osteocalst and osteoblast function, respectively.

A couple of recent striking findings led us to study the involvement of the endocannabinoid system in the regulation of bone remodeling. One is that, as in the case of bone formation and bone mass, the central production of at least one major endocannabinoid, 2-arachidonoyl glycerol (2-AG), is subject to negative regulation by leptin [23]. The other observation is that traumatic head injury stimulates both bone formation [24, 25] and central 2-AG production [26].

Strategy

Our approach, designed to elucidate the regulatory role of the endocannabinoid system in bone, consisted of *in vitro* experiments in bone cells followed by *in vivo* skeletal phenotyping of cannabinoid receptor (CB)-deficient mice. After demonstrating a low bone mass (LBM) in these mice we assessed the prevention of bone loss in estrogen-deficient mice by CB agonists.

The initial experiments demonstrated CB mRNA in bone cells *in vitro*, confirmed by immunostaining *in vivo*. We then further used the *in vitro* system to demonstarte the regulation of osteoblast and osteoclast differentiation and activity by cannabinoid ligands. The relevance of the *in vitro* findings to the *in vivo* scenario was established by analyzing the cortical and trabecular bone of CB-KO and ovariectomized mice using micro-computed tomography and histomorphometry.

Expression of cannabinoid receptors in bone

Undifferentiated osteoblast progenitors, such as mouse bone marrow-derived stromal and MC3T3 E1 preosteoblastic cells [27, 28], exhibit very low levels of the neuronal CB_1 and peripheral CB_2 mRNA cannabinoid receptors [29], detectable only by ultrasensitive methods. When these cells are grown for 2–4 weeks in so-called osteogenic medium, which contains vitamin C, β-glycerophosphate, and dexamethasone [30], CB_1 mRNA remains at the same levels. However, CB_2 mRNA expression increases progressively in parallel to the expression of the osteoblastic marker genes which encode tissue non-specific alkaline phosphatase (*TNSALP*) [31], parathyroid hormone receptor 1 (*PTHRc1*) [32], and particularly the osteoblastic master regulatory gene, *RUNX2* [33]. CB_2, but not CB_1 mRNA transcripts are also present in high abundance in monocytic cells undergoing osteoclast differentiation induced by RANK ligand and M-CSF [34]. *In vivo*, CB_2 protein is present in trabecular osteoblasts and their decedents, the osteocytes [33], as well as in osteoclasts.

Cannabinoid ligands regulate bone cell differentiation and activity

CB_2 activation has different effects in early preosteoblasts and in more mature osteoblastic cells. In bone marrow derived partially differentiated osteoblasts, with limited CB_2 expression, the specific CB_2 agonist HU-308 [35] but not the specific CB_1 agonist noladin ether [36], triggers a G_i protein-mediated mitogenic effect. This response leads to a dose-response expansion of the preosteoblastic pool. In more mature osteoblastic cells, represented by the MC3T3 E1 cell line, HU-308 also stimulates osteoblast-differentiated functions such as alkaline phosphatase activity and matrix mineraliztion. Thus, CB_2 signaling has multiple regulatory osteogenic anabolic functions along the osteoblast differentiation pathway. CB_2 activation has an opposite effect on osteocalstogenesis, namely, it inhibits osteoclasts differentiation.

Cannabinoid receptor signalling regulates bone mass *in vivo*

Although only the CB_2 receptor is demonstrable in bone, both CB_1- and CB_2-deficient mice have LBM. However, the underlying mechanisms of these LBMs appear to be different inasmuch as a pronounced low trabecular bone density is already found in the CB_1-KO mice at a young age. At the same age the skeleton of CB_2-KO mice is almost normal, with severe bone loss reported only in nearly 1-year-old animals [38]. This age-related difference suggests that CB_1 is mainly involved in the establishment of peak bone mass, which develops during infancy, sexual maturation, and young adulthood [39, 40]. CB_2 appears to be an important regulator of bone remodeling and maintenance of constant bone mass in later life. Further support for this notion is derived from the different pathogenic processes that lead to the LBM in the CB_1- and CB_2-deficient mouse lines. Consistent with their early LBM, CB_1-KO mice have increased bone resorption associated with decreased bone formation [38]. Reminiscent of human postmenopausal osteoporosis [41], CB_2-KO mice have a high turnover LBM characterized by increases in both bone resorption and formation which are at a net negative balance [38].

Because CB_2 is only peripherally expressed, CB_2-specific ligands could provide an opportunity to augment bone mass while avoiding the cannabinoid psychotropic activity. Indeed, HU-308 attenuates bone loss induced by estrogen depletion in ovariectomized animals with significant respective bone anti-resorptive and anabolic activities in the trabecular and cortical skeletal compartments [38].

Summary

Although the CB_2 receptor was cloned more than a decade ago, its physiological role remained elusive. Using a combined approach encompassing mole-

cular and cellular biology, pharmacology, and genetic analyses in mice, we were able to show a role for CB_2 signaling in regulating bone mass. Furthermore, attenuation of the deleterious effects of ovariectomy on bone by a peripherally selective CB_2 cannabinoid receptor agonist have major implications for osteoporosis, offering new molecular targets for the diagnosis and treatment of this disease.

CB_1 is apparently not involved in age-related bone loss commonly diagnosed in humans, but may have an important role in early skeletal development.

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