Journal of Neuroendocrinology **20** (Suppl. 1), 69–74

REVIEW ARTICLE

Endocannabinoids and the Regulation of Bone Metabolism

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Journal of Neuroendocrinology

In mammals, including humans, bone metabolism is manifested as an ongoing modelling/remodelling process whereby the bone mineralised matrix is being continuously renewed. Recently, the main components of the endocannabinoid system have been reported in the skeleton. Osteoblasts, the bone forming cells, and other cells of the osteoblastic lineage, as well as osteoclasts, the bone resorbing cells, and their precursors, synthesise the endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG). CB_1 cannabinoid receptors are present in sympathetic nerve terminals in close proximity to osteoblasts. Activation of these CB_1 receptors by elevated bone 2-AG levels communicates brain-to-bone signals as exemplified by traumatic brain injury-induced stimulation of bone formation. In this process, the retrograde CB_1 signalling inhibits noradernaline release and alleviates the tonic sympathetic restrain of bone formation. CB_2 receptors are expressed by osteoblasts and osteoclasts. Their activation stimulates bone formation and suppresses bone resorption. CB_2-deficient mice display a markedly accelerated age-related bone loss. Ovariectomy-induced bone loss can be both prevented and rescued by a CB_2 specific agonist. Hence, synthetic CB_2 ligands, which are stable and orally available, provide a basis for developing novel anti-osteoporotic therapies, free of psychotropic effects. The *CNR2* gene (encoding CB_2) in women is associated with low bone mineral density, offering an assay for identifying females at risk of developing osteoporosis.

Key words: bone remodelling, endocannabinoid system, sympathtic nervous system, single nucleotide polymorphism, osteoporosis.

doi: 10.1111/j.1365-2826.2008.01675.x

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The mammalian skeleton undergoes a continuous remodelling process whereby the mineralised matrix is being continuously removed and subsequently replaced with newly-formed bone tissue. This renewal process has a key role in maintenance of the skeleton and in its physiologic function.

- It is responsible for the patterning of individual bones, including the accrual of peak bone mass, during *de novo* skeletal development and fracture healing (1, 2).
- It drives the continuous renewal of the mineralised matrix. Bones must be stiff so that they do not bend when loaded. They must also be flexible, otherwise the energy imparted to the skeleton will be dissipated only by microdamage or complete fracture. Low or no bone renewal leads to excessive mineral deposition, reduced flexibility and increased fracture risk (3).
- Remodelling is the process by which the skeleton adapts to continuously changing mechanical loading. Lack of appropriate remodelling results in the accumulation of fatigue damage and stress fractures that can be prevented by modulating bone renewal through guided exercise over time (4, 5).
- Being the largest mineral reservoir in the organism, the skeleton, through its remodelling, is a key regulator of extracellular calcium and phosphate levels. Altered remodelling is responsible for bone–blood shifts of minerals in both hypo- and hypercalcaemia (6).

Renewal of the mineralised matrix occurs in multiple foci throughout the skeleton. The remodelling cycle in individual foci consists of an initial phase of bone resorption by a specialised cell type, the osteoclast, derived from the monocyte-macrophage lineage (7), followed by bone formation by another specialised, fibroblast-like cell, the osteoblast (8). Childhood, adolescence and young adulthood are characterised by a net increase in bone formation, required for skeletal growth and the accrual of peak bone mass.

The healthy adult organism presents balanced bone remodelling. It is followed by a net increase in bone resorption and consequent bone loss in older individuals, which often results in osteoporosis, the most common degenerative disease in developed countries.

Skeletal remodelling is subject to a complex hierarchical regulation consisting of local autocrine/paracrine, systemic endocrine and central components (9). The main brain-to-bone communication route consists of hypothalamic leptin, neuropeptide Y and neuromedin U signalling and activation of the skeletal adrenergic system (8, 10–13). In addition, it has been recently suggested that imbalances in bone remodelling, previously attributed to excessive thyroid activity and oestrogen depletion, may result from the interaction between the pituitary-derived thyroid stimulating hormone and follicle-stimulating hormone and receptors expressed in bone cells (14, 15).

Two observations lead us to study the role of the endocannabinoid system in skeletal metabolism. One is that bone formation and bone mass, as well as the central production of the endocannabinoid 2-arachidonoylglycerol (2-AG), are subject to negative control by leptin (16). The other is that traumatic brain injury stimulates both bone formation at distant skeletal sites (17, 18) and central 2-AG production (19).

Evidence for the occurrence of a skeletal endocannabinoid system

Recently, several key components of the endocannabinoid system have been identified in bone. The main endocannabinoids, anandamide and 2-AG, are present in this tissue at levels similar to those found in the brain. Because the blood endocannabinoid levels are several orders of magnitude lower, it is very likely that anandamide and 2-AG are synthesised locally in bone (Table 1). Indeed, both ligands are produced by osteoblasts and osteoclasts in culture (20, 21). In addition, diacylglycerol lipases (DAGLs) α and β, enzymes critically involved in 2-AG biosynthesis, are expressed in osteoblasts, osteocytes and bone lining cells (Fig. 1) (20). Because the anandamide degrading enzyme fatty acid amide hydrolase (FAAH) is also expressed in bone cells (our unpublished findings), the expression of enzymes involved in anandamide biosynthesis, such as *N*-acyl phosphatidylethanolamine phospholipase D (22), is likewise expected in these cells.

Table 1. Comparison of Endocannabinoid Levels in Bone, Brain (Mouse) and Blood (Human).

Tissue	Anandamide (pmol/g)	2-AG (nmol/g)
Trabecular bone	35.8 ± 6.2	1.4 ± 0.1
Whole brain	35.0 ± 8.0	6.2 ± 1.8
Blood*	2.5 ± 0.7	10^{-3}

*Monteleone *et al.* (48). 2-AG, 2-arachidonoylglycerol.

In line with the occurrence of the endocannabinoid ligands in bone, both CB_1 and CB_2 cannabinoid receptors are also present in the skeleton. CB_1 expression in osteoblasts and their precursors is very low, if not absent (23–25). It is expressed, however, in tyrosine hydroxylase positive sympathetic nerve terminals in bone, in close proximity to osteoblasts and perhaps preosteoblasts (Fig. 2) (25). CB_2 has been reported in osteoblasts and committed osteoblast precursors, such as mouse bone marrow-derived stromal cells and MC3T3 E1 preosteoblasts, as well as in the osteocyte, the terminally differentiated cell in the osteoblastic lineage (Fig. 3) (23, 24, 26). The potential importance of CB_2 to bone metabolism was noted early on, when we found that its expression in undifferentiated osteoblast precursors is initially very low, but increases progressively together with the expression of osteoblastic marker genes such as tissue nonspecific alkaline phosphatase (*TNSALP*) (27), parathyroid hormone receptor 1 (*PTHRc1*) (28), and the osteoblastic master regulatory gene, *RUNX2* (29).

CB_1, CB_2, DAGLβ and FAAH expression was also found in osteoclasts (20, 24, 30). Although the osteoclastic CB_1 mRNA level is rather low (25), CB_2 mRNA is abundantly expressed in these cells and in their monocytic precursors (23, 24), in line with its expression in macrophages (31).

Physiologic role of cannabinoid receptors in skeletal metabolism

We assessed the physiologic role of CB_1 and CB_2 in skeletal metabolism by characterising the osseous phenotype of *CNR1* and *CNR2* (the respective genes encoding CB_1 and CB_2) mutated mice. In the case of CB_1, the skeletal phenotype depends on the mouse strain

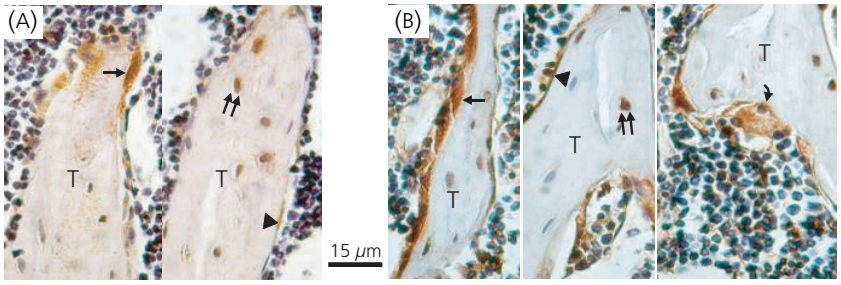

Fig. 1. Immunohistochemical localisation of diacylglycerol lipase (DAGL)α (A) and DAGLβ (B) in trabecular bone in mouse distal femoral metaphysis. T, bone trabeculae; arrows, osteoblasts; double arrows, osteocytes; arrow heads, lining cells; curved arrow, osteoclast. Copyright (2007) Federation of American Societies for Experimental Biology (20).

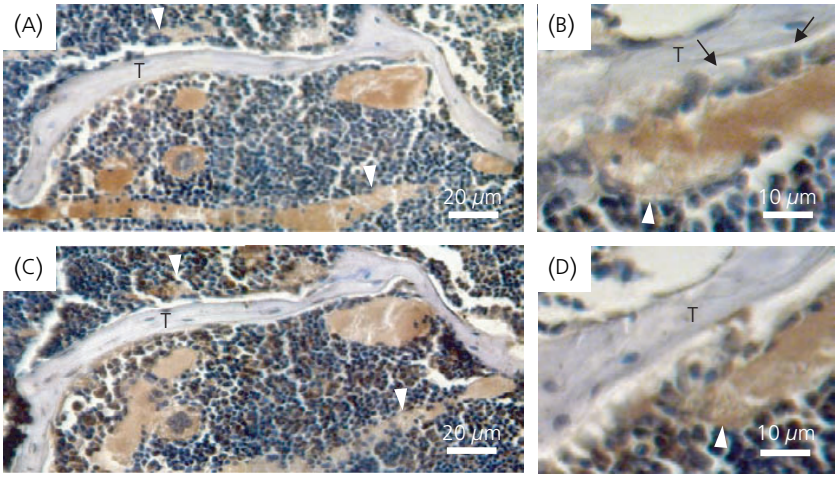

Fig. 2. Immunohistochemical colocalisation, in consecutive sections, of tyrosine hydroxylase (A, B) and CB_1 (C, D) in trabecular bone in mouse distal femoral metaphysis. T, bone trabeculae; arrows, osteoblasts; arrow heads, tyrosine hydroxylase-CB_1 positive fibres. Copyright (2006) American Society for Pharmacology and Experimental Therapeutics (25).

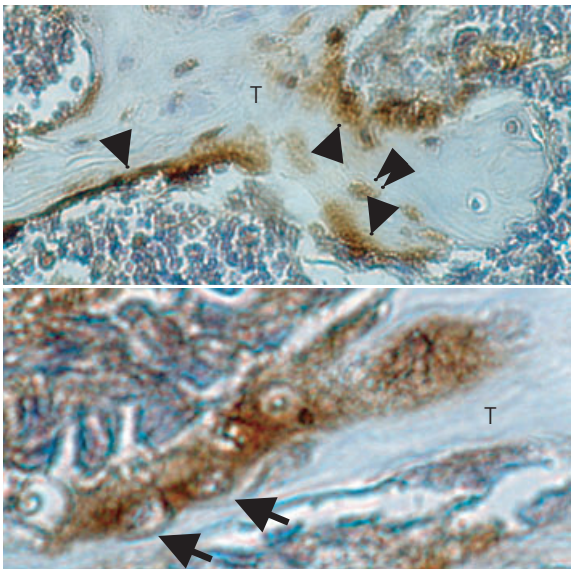

Fig. 3. Immunohistochemical localisation of CB_2-positive osteoblasts (arrowheads), osteocytes (double arrowhead) and osteoclasts (arrows) in trabecular bone (T) in mouse distal femoral metaphysis. Copyright (2006) National Academy of Sciences, USA (24).

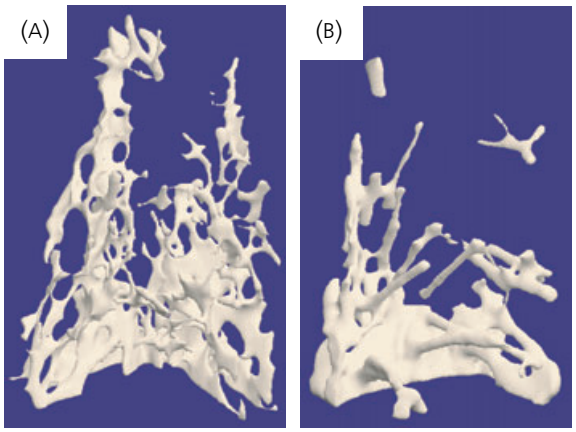

Fig. 4. Tridimensional micro-computed tomographic images of distal femoral metaphysis in 1-year-old wild-type (A) and CB_2-deficient (B) mice. The trabecular bone density and structure are markedly diminished in the absence of CB_2. Copyright (2006) National Academy of Sciences, USA (24).

and/or the construct used for gene mutation. In one *CNR1*-mutated line, backcrossed to CD1 mice ($CD1^{CNR1-/-}$), the N-terminal 233 codons of the *CNR1* gene were ablated (32). The effect of this deletion shows a clear gender disparity. Females have normal trabecular bone with a slight cortical expansion, whereas male $CD1^{CNR1-/-}$ mice exhibit high bone mass (25). Sexually mature $CD1^{CNR1-/-}$ mice of either gender display normal bone formation and resorption parameters, suggesting that the male phenotype is acquired early in life, during the developmental phase when peak bone mass is determined. A similar male phenotype was reported in an independent study (30) in which these mice were further backcrossed to Biozzi ABH mice (33). In the second line, backcrossed to C57BL/6J mice ($C57^{CNR1-/-}$), almost the entire protein-encoding sequence was removed (34). Both male and female $C57^{CNR1-/-}$ have a low bone mass phenotype accompanied by increased osteoclast counts and decreased bone formation rate (25).

CB_2 deficient animals have a gender independent skeletal phenotype. During their first 2–3 months of life, $CNR2^{-/-}$ mice accrue a normal peak trabecular bone mass (35), but later display a markedly enhanced age-related bone loss; their trabecular bone volume density at 1 year of age is approximately one-half of that in wild-type controls (Fig. 4) (24). Reminiscent of human postmenopausal osteoporosis, $CNR2^{-/-}$ mice have a high bone turnover with increases in both bone resorption and formation, which are at a net negative balance (24). Because healthy CB_2 mutant mice are otherwise normal, it appears that the main physiologic involvement of CB_2 is associated with maintaining bone remodelling at balance.

CB_1 is critically involved in brain–to-bone communication

Currently, the only direct clinical evidence for a central control of skeletal metabolism is the highly consistent observation of increased osteogenesis in patients with traumatic brain injury (TBI), which leads to heterotopic ossification and enhanced fracture healing, mainly in the appendicular skeleton (18, 36, 37). We have recently developed a mouse model to investigate the mechanism of the TBI-induced enhancement of bone formation and used it to investigate the role of the skeletal endocannabinoid system in the central control of bone metabolism (20). We showed that the TBI-induced increase in bone formation is preceded by elevation of 2-AG and a decrease in noradernaline levels in the skeleton. The TBI stimulation of bone formation is absent in both *CNR1* deficient mouse lines, but not in *CNR2*-null mice. In wild-type animals, it can be mimicked, including the suppression of noradernaline levels, by 2-AG administration. Both, the TBI- and 2-AG-induced stimulation of osteogenesis could be restrained by the β-adrenergic receptor agonist isoproterenol (20). Taken together, these findings suggest that CB_1 controls osteoblast function by negatively regulating noradernaline release from sympathetic nerve terminals in the immediate vicinity of these cells. Noradrenaline suppresses bone formation by binding to osteoblastic β2AR (11), and this suppression is apparently alleviated by activation of sympathetic CB_1 (20).

CB_2 activation regulates bone cell differentiation and activity

As expected, because osteoblasts express very few CB_1 receptors, if any, CB_1 agonists do not affect osteoblastic cells (24). By contrast, activation of CB_2 has different effects in early osteoblast progenitors, as well as in more mature cells. In the early precursors, represented by bone marrow-derived, partially differentiated osteoblastic cells with limited CB_2 expression, the specific CB_2 agonist HU-308 (38) triggers a G_i protein-mediated mitogenic effect and consequent expansion of the preosteoblastic pool (23). *Ex vivo* osteoblastic colony (CFU-Ob) formation by bone marrow stromal *CNR2*$^{-/-}$ cells is markedly diminished, whereas CFU-Ob formation by wild-type cells is stimulated by HU-308 (24, 26). In mature osteoblastic cells, represented by the MC3T3 E1 cell line, the same ligand stimulates functions typical of differentiated osteoblasts, such as alkaline phosphatase activity and matrix mineralisation (23, 24). Thus, CB_2 signalling is involved in several regulatory, pro-osteogenic processes along the osteoblast lineage.

In bone marrow-derived osteoclastogenic cultures and in the RAW 264.7 macrophage cell line, we showed that CB_2 activation inhibits osteoclast formation by restraining mitogenesis at the monocytic stage, prior to incubation with RANKL. It also suppresses osteoclast formation by repressing RANKL expression in osteoblasts and osteoblast progenitors (24). Likewise, it has been shown recently that the cannabinoid receptor agonist ajulemic acid also suppresses osteoclastogenesis (39). By contrast, another study reported the stimulation of osteoclast formation and bone resorption by cannabinoid receptor agonists and their inhibition by antagonists (30). These allegedly paradoxical results could occur because of variations in experimental conditions or, more probably, from opposite cell type-dependent specificities of some cannabinoid ligands.

Clinical relevance of the skeletal endocannabinoid system

Unlike CB_1, CB_2 is not associated with the cannabinoid psychoactive effects (38). Therefore, CB_2-specific ligands could offer an opportunity to prevent and/or rescue bone loss while avoiding the psychological adverse effects typical of cannabinoids. Indeed, preclinical assessment of the specific, nonpsychoactive CB_2 agonist, HU-308 (38), attenuates bone loss induced by oestrogen depletion in ovariectomised (OVX) animals using either 'preventive' (24) or 'rescue' (Fig. 5) protocols. In the preventive approach, HU-308 administration commenced immediately after OVX. To assess reversal of bone loss, the drug was given beginning 6 weeks post-OVX to allow for bone loss to occur. Treatment consisted of daily i.p. injections for 4–6 weeks. In the rescue protocol, the reversal of bone volumetric density by HU-308 is equivalent to that of parathyroid hormone (1–34), the only clinically approved bone anabolic agent (40). In either protocol, the attenuation of bone mass reflected both inhibition of bone resorption and stimulation of bone formation. Hence, CB_2 agonists may become an orally available, combined anti-resorptive and anabolic therapy for osteoporosis.

Involvement of the endocannabinoid system in the mouse skeletal metabolism raises the question as to what extent the endocannabinoid system contributes to the regulation of bone mass in humans. To address this issue, we studied polymorphisms in the human *CNR1* and *CNR2* loci, in a case–control sample of French Caucasian osteoporotic patients (41). The study comprised 68 post-

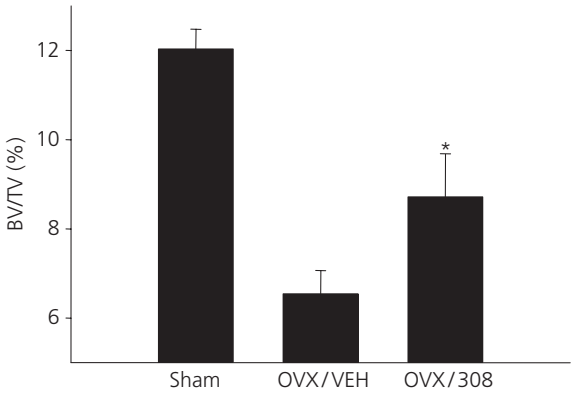

Fig. 5. Rescue of ovariectomy (OVX)-induced trabecular bone loss by CB_2 specific agonist, HU-308. Sham, sham-OVX surgery comprising exposure of ovaries; OVX/vehicle (VEH), OVX mice left untreated for 6 weeks and then given HU-308 solvent for additional 6 weeks; OVX/308, OVX mice left untreated for 6 weeks and then given HU-308, 20 mg/kg/day, for additional 6 weeks. BV/TV, trabecular bone volume density. Micro-computed tomographic analysis performed in distal femoral metaphysis 12 weeks after OVX or sham-OVX. Data are mean ± SEM obtained in six mice per condition. *Significant versus sham and OVX/VEH, ANOVA, $P < 0.05$.

menopausal osteoporotic women with an average bone mineral density (BMD) T-score of 3.062 ± 0.799 at the lumbar spine. The control group consisted of 220 age-matched healthy women.

The *CNR1* locus is located on chromosome 5q15. It includes a single coding exon, preceded by several noncoding 5′ exons, indicating a complex transcriptional regulation of this gene by several promoters (42, 43). Analysis of four single nucleotide polymorphisms (SNPs) spanning almost 20 kb around the CB_1 coding exon revealed no significant association with the osteoporosis phenotype, suggesting that the *CNR1* locus does not have a major etiologic role in osteoporosis.

The *CNR2* locus is located on chromosome 1p36. This human genomic region and its mouse orthologue on chromosome 4 have been previously linked to BMD and osteoporosis in several independent association analyses (44–46). However, these analyses did not test whether *CNR2* is a potential candidate gene. Like *CNR1*, the *CNR2* gene also consists of a single coding exon, which is preceded by a noncoding upstream exon. Here, we analysed a total of 26 SNPs spanning approximately 300 kb around the *CNR2* locus (genomic position 23750771 to 24039933). Several of these SNPs showed a significant association with the disease phenotype, portraying *CNR2* polymorphisms as important genetic risk factors for osteoporosis. The most significant allele and genotype associations were observed with SNPs located within the CB_2 coding region with respective P-values of 0.0014 and 0.00073. Moreover, when BMD was analysed as a quantitative trait, it presented highly significant differences between individuals carrying different SNPs in the CB_2 coding region. Hence, we sequenced the CB_2 coding exon in all 388 patients and controls, thus identifying two missense variants, Gln63Arg and His316Tyr, with the Arg63 variant being more common in osteoporotic patients than in controls (41). Taken together, these findings suggest that a common variant of the CB_2 receptor contributes to the aetiology of osteoporosis in humans. Recently, similar findings have been found in a Japanese cohort of 40–79-year-old women (n = 1110) and men (n = 1128), which was randomly recruited for a prospective study on aging, analysing several candidate quantitative trait loci in BMD. For the *CNR2* locus, a single SNP (rs2501431, A>G), which had shown the strongest association in our French sample (P = 0.0007), was studied. BMD was always lower in women with the AA genotype compared to the AG and GG genotypes (47). Together, these studies strongly suggest that *CNR2* is the susceptibility gene for low BMD on chromosome 1p36, and pave the way for the design of a screening assay to identify females at risk for developing osteoporosis.

Conclusions

Recent studies in mice demonstrate the occurrence of a skeletal endocannabinoid system, and suggest that it has an important role in the regulation of skeletal metabolism and the consequent implications on bone structure and function. The CB_1 cannabinoid receptor is present in sympathetic terminals innervating the skeleton. It is activated by the main endocannabinoid, 2-AG, negatively regulating skeletal noradernaline levels and alleviating the tonic adrenergic suppression of bone formation. The brain-to-bone signals in control of this process remain to be elucidated. The CB_2 cannabinoid receptor is expressed in bone cells. Its net bone anabolic activity, including some of the mechanisms involved, has been reported in some detail, and is also inferred from the human genetic studies. These studies portray polymorphisms in *CNR2*, which encodes CB_2, as important determinants in the aetiology of osteoporosis. Taken together, the reports on the endocannabinoid system in mice and humans demonstrate the potential for developing: (i) a cannabinoid-based, combined anabolic/anti-resorptive therapy for osteoporosis and (ii) tools to diagnose osteoporosis susceptible polymorphisms in *CNR2*.

Acknowledgements

All intellectual property rights related to skeletal uses of cannabinoids and cannabinoid receptor are assigned to Yissum, the Research Development Company of the Hebrew University of Jerusalem.

Conflicts of interest

The authors declare no conflicts of interest.

Received: 7 December 2007,
accepted 8 February 2008

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