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REVIEW

Cannabinoid receptors and the regulation of bone mass

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A functional endocannabinoid system is present in several mammalian organs and tissues. Recently, endocannabinoids and their receptors have been reported in the skeleton. Osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells, produce the endocannabinoids anandamide and 2-arachidonoylglycerol and express CB2 cannabinoid receptors. Although CB2 has been implicated in pathological processes in the central nervous system and peripheral tissues, the skeleton appears as the main system physiologically regulated by CB2. CB2-deficient mice show a markedly accelerated age-related bone loss and the *CNR2* gene (encoding CB2) in women is associated with low bone mineral density. The activation of CB2 attenuates ovariectomy-induced bone loss in mice by restraining bone resorption and enhancing bone formation. Hence synthetic CB2 ligands, which are stable and orally available, provide a basis for developing novel anti-osteoporotic therapies. Activation of CB1 in sympathetic nerve terminals in bone inhibits norepinephrine release, thus balancing the tonic sympathetic restrain of bone formation. Low levels of CB1 were also reported in osteoclasts. CB1-null mice display a skeletal phenotype that is dependent on the mouse strain, gender and specific mutation of the CB1 encoding gene, *CNR1*.
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Abbreviations: 2-AG, 2-arachidonoylglycerol; BMD, bone mineral density; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; DAGL, diacylglycerol lipase; SNP, single-nucleotide polymorphism

Introduction

In humans and other vertebrates alike, bone structure undergoes substantial temporal changes throughout life. These changes comprise: (i) a rapid skeletal growth phase accompanied by accrual of peak bone mass; (ii) a steady-state phase whereby bone mass remains constant; (iii) age-related bone loss (Segev *et al*., 2006). These changes are the consequence of a continuous process of resorption/formation of the mineralized matrix referred to as bone remodelling (Figure 1). Imbalanced bone remodelling leads to bone mass accrual (positive imbalance) or bone loss (negative imbalance) (Karsenty, 2001). The remodelling process occurs concomittantly in multiple foci, which in humans encompass approximately 5% of trabecular, endosteal and osteonal surfaces (Parfitt, 1982). The remodelling cycle in individual foci (Figure 1) consists of a relatively rapid (that is a few weeks) resorption of pre-existing mineralized matrix by a bone-specific haematopoietic cell type, the osteoclast, derived from monocytes (Roodman, 1999). It is then followed by a slower (that is a few months) stage of bone formation by another bone-specific cell type, the osteoblast (Parfitt, 1982), which belongs to the stromal cell system of bone marrow (Bab *et al*., 1986). Different foci are usually at different phases of the cycle and the net effect on bone mass reflects the overall balance between bone resorption and formation. The significance of balanced bone remodelling is demonstrated by osteoporosis, the most common degenerative disease in developed societies, which results from a net increase in bone resorption, bone loss, weakening of the skeleton and increased fracture risk, primarily in females but also in males.

The coordinated occurrence of multiple remodelling sites is suggestive of a complex hierarchical regulation consisting of local, autocrine/paracrine and systemic endocrine (Manolagas, 2000). Indeed, studies in genetically modified mice have demonstrated paracrine control of osteoclast formation and activity by factors such as receptor activator of NF-κB ligand, osteoprotegerin, macrophage colony-stimulating factor and interleukin 6, which are derived from neighbouring stromal cells, including osteoblasts and their precursors (Poli *et al*., 1994; Simonet *et al*., 1997; Bucay *et al*., 1998; Lacey *et al*., 1998; Kong *et al*., 1999; Suda *et al*., 2001).

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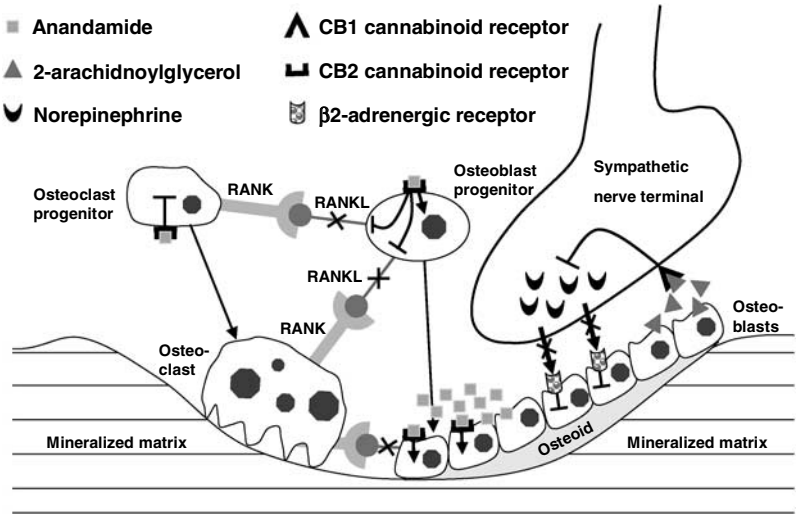

Figure 1 Model of regulation of bone remodelling by the skeletal endocannabinoid system. Shown is remodelling focus consisting of resorption lacuna carved by osteoclasts and being refilled by osteoblasts, which secrete osteoid (unmineralized bone matrix) and control its mineralization.

Locally, osteoblasts are regulated mainly by bone morphogenetic proteins (Yoshida *et al*., 2000). Systemically, it is well established that depletion of gonadal hormones in females and males favours bone loss in mammals, including humans (Most *et al*., 1997; Alexander *et al*., 2001; Gabet *et al*., 2005). In addition, parathyroid hormone (Potts and Juppner, 1998; Gunther *et al*., 2000), calcitonin (Nicholson *et al*., 1986), insulin-like growth factor I (Yakar *et al*., 2002) and the osteogenic growth peptide (Bab and Chorev, 2002) are involved in the control of bone formation. More recently, it has been reported that bone remodelling is also subject to a hierarchically superior central control by hypothalamic leptin and neuropeptide Y signalling (Ducy *et al*., 2000; Baldock *et al*., 2002) as well as downstream sympathetic signalling through osteoblastic β2 adrenergic receptors (Takeda *et al*., 2002). Lately, it has been 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 follicular-stimulating hormone and receptors expressed in bone cells (Abe *et al*., 2003; Sun *et al*., 2006).

The actions of cannabinoids and endocannabinoids are mediated mainly by G protein-coupled cannabinoid receptors type 1 (CB1) and type 2 (CB2) (Howlett, 2002). CB1 and CB2 share 44% overall identity (68% identity for the transmembrane domains). CB1 is perhaps the most abundantly expressed G protein-coupled receptor in the CNS. It is also present in peripheral neurons and the gonads and to some extent in several other peripheral tissues. CB2 is expressed in the immune system, cirrhotic liver, arteriosclerotic plaques, inflamed gastrointestinal mucosa and brain inflammation (Sugiura *et al*., 2002; Julien *et al*., 2005; Steffens *et al*., 2005; Wright *et al*., 2005). That CB1 and CB2 are not functionally identical is demonstrated by the presence of cannabinoid agonists and antagonist with distinct binding specificities to either receptor (Hanus *et al*., 1999; Shire *et al*., 1999). Both receptors signal via the G(i/o) subclass of G proteins, inhibiting stimulated adenylyl cyclase activity. Further downstream, the CBs induce the activation of p42/44 mitogen-activated protein kinase (Wartmann *et al*., 1995; Melck *et al*., 1999; Liu *et al*., 2000), p38 mitogen-activated protein kinase (Derkinderen *et al*., 2001), c-Jun N-terminal kinase (Rueda *et al*., 2000; Derkinderen *et al*., 2001), AP-1 (Liu *et al*., 2000), the neural form of focal adhesion kinase (Derkinderen *et al*., 1996), PKB (Gomez del Pulgar *et al*., 2000) and Ca^{2+} transients (Mombouli *et al*., 1999).

The main CB1 and CB2 endogenous ligands are *N*-arachidonoylethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) (Devane *et al*., 1992; Mechoulam *et al*., 1995). Anandamide is present in a variety of tissues such as the brain, kidney, liver, spleen, testis, uterus and blood in picomoles per gram concentrations, with the highest levels reported in the CNS. The low anandamide concentrations have been attributed to low substrate (arachidonic acid esterified at the 1-position) levels for this pathway (Hansen *et al*., 2000) and/or the short anandamide half-life *in vivo* ($t_{1/2} < 5$ min) (Willoughby *et al*., 1997). Anandamide is biosynthesized through *N*-acyl phosphatidylethanolamine phospholipase D-dependent and -independent pathways (Simon and Cravatt, 2006). The main anandamide-degrading enzyme is fatty acid amide hydrolase, a membrane-associated serine hydrolase enriched in the brain and liver (Cravatt *et al*., 2001). In general, the tissue distribution of 2AG is similar to that of anandamide; however, its concentration is 300–1000 higher ($ng\,g^{-1}$ range). 2AG production has been demonstrated in the CNS as well as in platelets and macrophages, especially in response to stimulation by inflammatory agents such as lipopolysaccharide (Varga *et al*., 1998; Di Marzo *et al*., 1999). 2AG is generated from arachidonic acid-enriched membrane phospholipids, such as inositol phospholipids, through the combined actions of phospholipase C and diacylglycerol lipases (DAGLα and DAGLβ) (Stella *et al*., 1997; Bisogno *et al*., 2003). It has been proposed that like other monoacylglycerols,

2AG is metabolized by a monoacylglycerol lipase (Konrad *et al*., 1994).

A couple of striking observations led us to assess the occurrence and role of a skeletal endocannabinoid system. One is that, bone formation and bone mass, as well as the central production of at least one major endocannabinoid, 2-AG, are subject to negative control by leptin (Di Marzo *et al*., 2001). The second observation is that traumatic brain injury enhances both bone formation (Orzel and Rudd, 1985; Wildburger *et al*., 1998) and central 2-AG production (Panikashvili *et al*., 2001).

Cannabinoid receptors in bone

Osteoblast progenitors, such as mouse bone marrow-derived stromal cells and MC3T3 E1 preosteoblasts (Sudo *et al*., 1983; Jorgensen *et al*., 2004), exhibit very low levels, if any, of CB1. CB2 expression in these cells is also very low (Bab, 2005; Ofek *et al*., 2006). However, when the cells are grown for 5–28 days in medium that promotes osteoblast differentiation (Bellows *et al*., 1986), CB2 mRNA expression increases progressively in parallel to the expression of osteoblastic marker genes such as tissue non-specific alkaline phosphatase (*TNSALP*) (Zhou *et al*., 1994), parathyroid hormone receptor 1 (*PTHRc1*) (Zhang *et al*., 1995) and the osteoblastic master regulatory gene, *RUNX2* (Araujo *et al*., 2004). In osteoclasts, CB1 is expressed at low levels. By contrast, CB2 mRNA transcripts in these cells are present in high abundance (Bab, 2005; Idris *et al*., 2005; Ofek *et al*., 2006; Scutt and Williamson, 2007). *In vivo*, CB2 protein is present in trabecular osteoblasts and their decedents, the osteocytes (Lian *et al*., 2004), as well as in osteoclasts (Ofek *et al*., 2006). CB1 is highly expressed in skeletal sympathetic nerve terminals (Tam *et al*., 2006).

Cannabinoid receptor activation and bone cell differentiation and activity

Activation of CB2 has different effects in early osteoblast progenitors and in more mature osteoblastic cells. In the early precursors, represented by bone marrow-derived, partially differentiated osteoblastic cells that show limited CB2 expression, the specific CB2 agonist HU-308 (Hanus *et al*., 1999) but not the specific CB1 agonist noladin ether (Hanus *et al*., 2001), triggers a G_i protein-mediated mitogenic effect and consequent expansion of the preosteoblastic pool (Bab, 2005). *Ex vivo* osteoblastic colony formation by bone marrow stromal *cb2*$^{-/-}$ cells is markedly diminished, whereas colony-forming unit osteoblastic formation by wild-type cells is stimulated by HU-308 (Ofek *et al*., 2006; Scutt and Williamson, 2007). In mature osteoblastic cells, represented by the MC3T3 E1 cell line, the same ligand stimulates osteoblast-differentiated functions such as alkaline phosphatase activity and matrix mineralization (Bab, 2005; Ofek *et al*., 2006). Thus, CB2 signalling is involved in several regulatory pro-osteogenic processes along the osteoblast lineage (Figure 1).

In bone marrow-derived osteoclastogenic cultures and in the RAW 264.7 cell line, we showed that CB2 activation inhibits osteoclast formation by restraining mitogenesis at the monocytic stage, prior to incubation with receptor activator of NF-κB ligand. It also suppresses osteoclast formation by repressing receptor activator of NF-κB ligand expression in osteoblasts and osteoblast progenitors (Figure 1; Ofek *et al*., 2006). Likewise, it has been recently shown that the cannabinoid receptor agonist ajulemic acid also suppresses osteoclastogenesis (George *et al*., 2007). By contrast, another study reported the stimulation of osteoclast formation and bone resorption by cannabinoid receptor agonists and their inhibition by antagonists (Idris *et al*., 2005). 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.

Presence and biosynthesis of endocannabinoids in the skeleton

Anandamide and 2-AG are present in bone at levels nearly as high as the brain levels of these endocannabinoids (Figure 1; Tam *et al*., 2007 and unpublished results). Both ligands are produced by osteoblastic cells in culture. In addition, DAGLα and DAGLβ, enzymes critically involved in the 2-AG biosynthesis, are expressed in osteoblasts, osteocytes and bone-lining cells. DAGLβ expression was also found in osteoclasts. Although both 2-AG and anandamide are perceived as non-selective agonists of CB1 and CB2, our findings in bone and bone cell cultures suggest that 2-AG activates CB1 in the sympathetic nerve terminals, whereas anandamide affects bone cells directly by binding to CB2 (Figure 1).

Skeletal phenotype of cannabinoid receptor-deficient mice

We used cannabinoid receptor mutant mice to assess the physiologic role of CB1 and CB2 in the control of bone mass. In the case of CB1, the skeletal phenotype depends on the mouse strain and/or the construct used for gene mutation. In one CB1-deficient line, backcrossed to CD1 mice (CD1$^{CB1-/-}$), the N-terminal 233 codons of the *CNR1* gene were ablated (Ledent *et al*., 1999). The effect of this mutation shows a clear gender disparity. Females have normal trabecular bone with a slight cortical expansion, whereas male CD1$^{CB1-/-}$ mice exhibit high bone mass (Tam *et al*., 2006). Sexually mature CD1$^{CB1-/-}$ 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 (Idris *et al*., 2005) in which these mice were further backcrossed to Biozzi ABH mice (Amor *et al*., 2005). In the second line, backcrossed to C57BL/6J mice (C57$^{CB1-/-}$), almost the entire protein-encoding sequence was removed (Zimmer *et al*., 1999). Both male and female C57$^{CB1-/-}$ have

a low bone mass phenotype accompanied by increased osteoclast counts and decreased bone formation rate (Tam *et al*., 2006). Our recent findings suggest that CB1 controls osteoblast function by negatively regulating norepinephrine release from sympathetic nerve terminals in the immediate vicinity of these cells. Norepinephrine suppresses bone formation by binding to osteoblastic β2 adrenergic receptor (Takeda *et al*., 2002); this suppression is alleviated by activation of sympathetic CB1 (Figure 1; Tam *et al*., 2007).

Cannabinoid receptor type 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 (Bab *et al*., 2007), but later display a markedly enhanced age-related bone loss; their trabecular bone volume density at 1 year of age is approximately half compared with wild-type controls (Ofek *et al*., 2006). Reminiscent of human postmenopausal osteoporosis (Brown *et al*., 1984), the *CNR2*$^{-/-}$ mice have a high bone turnover with increases in both bone resorption and formation, which are at a net negative balance (Ofek *et al*., 2006). Because healthy CB2 mutant mice are otherwise normal, it appears that the main physiologic involvement of CB2 is associated with maintaining bone remodelling at balance.

Prevention and reversal of bone loss by CB2 agonist

Unlike CB1, CB2 is not associated with the cannabinoid psychoactive effects. Therefore, CB2-specific ligands could offer an opportunity to prevent and/or rescue bone loss while avoiding the psychological side effects of cannabinoids. Indeed, the specific, non-psychoactive CB2 agonist, HU-308 (Hanus *et al*., 1999), attenuates bone loss induced by oestrogen depletion in ovariectomized (OVXed) animals using either 'preventive' (Ofek *et al*., 2006) or 'rescue' (unpublished data) protocols. In the preventive approach, HU-308 administration commenced immediately after ovariectomy. To assess reversal of bone loss, the drug was given beginning 6 weeks post-ovariectomy to allow for bone loss to occur. Treatment consisted of daily i.p. injections for 4–6 weeks. The attenuation of bone mass reflected both inhibition of bone resorption and stimulation of bone formation (Bab, 2005). Hence, CB2 agonists may become an orally available, combined antiresorptive and anabolic therapy for osteoporosis.

CB2 and osteoporosis in humans

The findings in mice prompted us to determine if cannabinoid receptors also contribute to the regulation of bone mass in humans. We therefore studied polymorphisms in the human *CNR1* locus, encoding the CB1 receptor, and the *CNR2* locus, encoding the CB2 receptor, in a case–control sample of osteoporotic patients collected by Professor de Vernejoul at the Hôpital Lariboisière in Paris (Karsak *et al*., 2005). The study comprised 68 postmenopausal osteoporotic women with an average bone mineral density (BMD, measured by dual-energy X-ray absorptiometry) T-score of -3.062 ± 0.799 at the lumbar spine and 220 age-matched healthy controls.

The *CNR1* locus is located on chromosome 5q15. It encompasses a single coding exon that is preceded by several non-coding 5′ exons, indicating a complex transcriptional regulation of this gene by different promoters (McCaw *et al*., 2004; Zhang *et al*., 2004). Analysis of four single-nucleotide polymorphisms (SNPs) spanning nearly 20 kb around the CB1-coding exon revealed no significant association with the osteoporosis phenotype, suggesting that the *CNR1* locus does not play a major role in this sample.

The *CNR2* locus is located on chromosome 1p36. This genomic region and its mouse orthologue on chromosome 4 have been previously linked to BMD and osteoporosis in several independent association analyses (Devoto *et al*., 1998, 2001, 2005). However, these analyses did not consider *CNR2* as a potential candidate gene. Like *CNR1*, the *CNR2* gene also consists of a single coding exon, which is preceded by non-coding upstream exon. We analysed a total of 26 SNPs spanning approximately 300 kb around the CNR2 locus (genomic position 23750771–24039933). Several of these SNPs showed a significant association with the disease phenotype, suggesting that *CNR2* polymorphisms are important genetic risk factors for osteoporosis. The most significant *P*-values for allele and genotype associations were observed with SNPs located within the CB2-coding region (0.0014 and 0.00073 respectively). Furthermore, when BMD at the lumbar spine was analysed as a quantitative trait, highly significant differences were found in BMD between individuals carrying different SNPs in the CB2-coding region. Hence, we sequenced the CB2-coding exon in all 388 patients and controls thus identifying two missense variants, Gln63Arg and His316Tyr, with the Arg63 variant being more common in the osteoporotic patients than in the healthy controls (Karsak *et al*., 2005). Taken together, these findings suggest that a common variant of the CB2 receptor contributes to the aetiology of osteoporosis in humans.

Recently, several candidate quantitative trait loci in BMD, including *CNR2*, were analysed in a cohort of 1110 Japanese women and 1128 Japanese men, 40–79 years of age (Yamada *et al*., 2007). This cohort was randomly recruited to a prospective study on ageing. For the *CNR2* locus, they studied a single SNP (rs2501431, A→G), which had shown the strongest association in our French sample ($P = 0.0007$). BMD, as measured by peripheral quantitative computed tomography or dual-energy X-ray absorptiometry, was always lower in women with the AA genotype compared with the AG and GG genotypes. Together, these studies strongly suggest that *CNR2* is the susceptibility gene for low BMD and osteoporosis on chromosome 1p36.

Conclusions

Our recent studies in mice and humans suggest an important role for the endocannabinoid system in the regulation of skeletal remodelling and the consequent implications on bone mass and biomechanical function. Although the CB1 cannabinoid receptor has been identified in sympathetic terminals innervating the skeleton, its role in controlling

bone turnover remains to be elucidated. The CB2 cannabinoid receptor is expressed in bone cells. Its bone anabolic action, 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*, the gene encoding CB2, as important genetic risk factors for osteoporosis. Taken together, the reports on cannabinoid receptors in mice and humans pave the way for the development of (i) cannabinoid drugs to combat osteoporosis, and (ii) diagnostic measures to identify osteoporosis-susceptible polymorphisms in *CNR2*.

Conflict of interest

The authors are inventors on patent applications related to the use of cannabinoid receptor ligands in skeletal therapy and diagnosis of osteoporosis. Rights in this intellectual property and those pertinent to HU-308 are assigned to; Yissum Research Development Company of the Hebrew University of Jerusalem.

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