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Role of cannabinoids in the regulation of bone remodeling

Aymen I. Idris and Stuart H. Ralston

INTRODUCTION

The endocannabinoid system (endocannabinoid) is a complex network of receptors, a variety of ligands, and a series of enzymes that are responsible for ligand synthesis and breakdown. Endocannabinoids and their receptors are involved in the regulation of numerous physiological processes including neurotransmission, pain perception, learning, memory, cardiovascular homeostasis, appetite, motor function, and the immune response (reviewed in Klein et al., 2000; Grant and Cahn, 2005; Di Marzo, 2005). There is accumulating evidence to suggest that endocannabinoids and their receptors play important roles in bone metabolism by regulating bone mass, bone loss, and bone cell function. This review summarizes in vitro and in vivo findings relating to the action of cannabinoid ligands in the skeleton.

THE SKELETAL ENDOCANNABINOID SYSTEM

CANNABINOID RECEPTOR LIGANDS IN BONE

Cannabinoid receptor ligands can be classified into three groups based on their source of production: endogenous cannabinoids (endocannabinoids), phytocannabinoids, and synthetic cannabinoids. Two of the best characterized endocannabinoids are N-arachidonoyl ethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG). The endocannabinoids anandamide and 2-AG are responsible for most pharmacological actions associated with cannabinoid receptors in mammalian cells (reviewed in Pertwee, 2005). Anandamide and 2-AG are both derivatives of arachidonic acid and are produced from breakdown of glycerophospholipids in the cell membrane. Anandamide is synthesized from N-arachidonoyl phosphatidylethanolamine (NAPE) by the enzyme NAPE-phospholipase D (NAPE-PLD), whereas synthesis of 2-AG occurs through the action of phospholipase C (PLC) and the diacylglycerol lipases alpha and beta (DGALa and DGALB) on membrane phospholipids (Di Marzo et al., 1996; Maejima et al., 2001; Isbey; Simon and Cravatt, 2006). Once formed, endocannabinoids are transported across cell membranes by passive diffusion or endocytosis (for extensive review, refer to Fowler, 2012; Hermann et al., 2006). Anandamide and 2-AG are highly expressed in the brain and are also detected in a number of peripheral tissues including heart, liver, kidney, testis, and blood (Felder et al., 1993, 1996; Stella et al., 1997; Kondo et al., 1998; Di Marzo et al., 2002; Ross, 2003; van der Stelt and Di Marzo, 2005; Tam et al., 2008). Endocannabinoids have a short half-life due to the fact that they are rapidly degraded by a variety of enzymes including fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL; Elsh et al., 2002). It should be noted, however, that FAAH is not specific for cannabinoids and degrades many other lipid containing molecules (Bauvarapappu, 2007).

There is evidence that the endocannabinoids 2-AG and anandamide are produced endogenously in the bone marrow and within the metabolically active trabecular compartment (Bab et al., 2008; Tam et al., 2008). A number of studies have shown that osteoblasts and osteoclasts are capable of producing anandamide and 2-AG in culture. Ridge et al. (2007) reported in abstract form that cultured osteoblast-like cells MC3T3-E1 and mouse osteoclasts produced 2-AG in vitro and that anandamide was produced by cultured osteoblasts but not osteoclasts. A recent study by the same group reported that the differentiation of human osteoclasts from monocytes is associated with a reduction in 2-AG levels and an increase in anandamide levels (Whyte et al., 2012). Rossi et al. (2009) reported that cultured human osteoclasts produced 2-AG and detectable quantities of anandamide; levels of both cannabinoids increased when the cultures were treated with the FAAH inhibitor URB597. Taken together, these observations indicate that 2-AG and anandamide are probably produced locally within bone and by bone cells in culture. In a study by Richardson et al. (2008), neither 2-AG nor anandamide were detected in synovial fluid from normal subjects, but both endocannabinoids...
were detected in synovial fluid from patients with osteoarthritis (OA) and rheumatoid arthritis (RA). Interestingly 2-AG levels in this study were higher in patients with OA as compared with RA. Cannabinoid receptors are also activated by plant derived cannabinoids termed phytocannabinoids. The Cannabis sativa plant contains a large number of phytocannabinoids such as Δ2-tetrahydrocannabinol (THC) which is an agonist at CB1 and CB2 receptors. It should be noted, however, that many psychoactive cannabinoids such as cannabidiol bind weakly to cannabinoid receptors (reviewed by Mechoulam, 2005). A large number of synthetic cannabinoids have also been prepared some of which such as CP55,940, WIN55,238, and HU308 act as agonists thereby mimicking the action of endocannabinoids at a number of targets (Pertwee, 2005). On the other hand, a variety of synthetic compounds including SR141716A (also known as Rimonabant), AM251, and AM630 are described as inverse agonists/antagonists due to their ability to down-regulate the activity of cannabinoid receptors in the presence and absence of agonist binding (Gasley et al., 1996, 1997; Bouaboula et al., 1997; Hosohata et al., 1997a,b; Landsman et al., 1998; Liu et al., 1999; Ross et al., 1999; Mechler et al., 2000). For a comprehensive list of pharmacological properties of some of the most important cannabinoid receptor ligands refer to (Pertwee, 2005, 2010).

CANNABINOID RECEPTORS IN BONE Endocannabinoids and their synthetic analogs bind to and activate cannabinoid receptors (CB1 and CB2) (Maccarrone and Finazzi-Agro, 2002; Pertwee and Ross, 2002). Recent studies suggest that the “orphan” G protein coupled receptor GPR55 might represent a third cannabinoid receptor (Begg et al., 2005; Ryberg et al., 2007). The CB1 receptor, encoded by the CNR1 gene was the first cannabinoid receptor to be identified and it’s mainly expressed in the brain (Matsuda et al., 1990). In the skeleton, CB2 receptors are expressed on nerve fibers intervening bone (Tam et al., 2006, 2010) and on cells of the immune system within the BM compartment (Klein et al., 2000, 2003). We and others reported that CB2 receptors are also present on osteoblasts, osteoclasts, and BM derived adipocytes at both cellular and mRNA levels (Idris et al., 2009; Rossi et al., 2009). The CB2 receptor encoded by the CNR2 gene was originally identified in macrophages in the marginal zone of the spleen (Munro et al., 1993) but is now known to be expressed in many other tissues including bone and synovial joints as well as some regions of the central nervous system (Bouaboula et al., 1995; Gallegue et al., 1995; Pertwee, 1997; Noirot et al., 2001; Klein et al., 2003; Idris et al., 2005; Ofek et al., 2006; Scutt and Williamson, 2007; Palacios et al., 2008). CB2 receptors are also expressed by osteoblasts, osteoclasts, and osteocytes at significantly higher levels than that reported for CB1 (Idris et al., 2005; Ofek et al., 2006; Rossi et al., 2009; Whyte et al., 2012). Recent studies reported that bone cells including osteoblasts and osteoclasts also express GPR55 which is known to be targeted by endocannabinoids and synthetic cannabinoid ligands (Smart et al., 2000; Saunders et al., 2007; Abed et al., 2009; Rossi et al., 2009; Whyte et al., 2009). The GPR55 receptor encoded by the GPR55 gene is widely expressed but the highest levels are detected in the adrenals, the gastrointestinal tract, and the brain (Ryberg et al., 2007). It should be noted that cannabinoids such as anandamide can bind to other receptors such as nicotinic acetylcholine receptors, calcium channels, voltage-gated potassium channels, and transient receptor potential vanilloid receptors (TRPVs; Di Marzo et al., 1996, 1997; Bouaboula et al., 1997; Hosohata et al., 1997a,b; Landsman et al., 1998; Liu et al., 1999; Ross et al., 1999; Mechler et al., 2000). For a comprehensive list of pharmacological properties of some of the most important cannabinoid receptor ligands refer to (Pertwee, 2005, 2010).
REGULATION OF OSTEOCLASTIC BONE RESORPTION BY CANNABINOIDs

The classical cannabinoid receptors CB1 and CB2, and the orphan receptor GPR55 all play significant roles in the regulation of osteoclast function and bone resorption. Idris et al. (2005) were first to report that genetic inactivation of the CB1 receptor results in high peak bone mass in young mice. In this study, a detailed micro-computed tomography scanning of bone and histomorphometric analysis of bone formation and resorption revealed that mice deficient in CB1 receptor have less osteoclasts and reduced bone resorption (Idris et al., 2005). These findings have led to the realization that cannabinoid receptors play a significant role in the regulation of peak bone mass. In keeping with these observations, the CB1 selective antagonist/inverse agonists AM251 and SR141716A have been reported to cause osteoclast apoptosis and inhibit osteoclast formation in vitro and osteoclasts cultured from CB2 deficient mice were found to be resistant to the effects of AM251 indicating that the mechanism of osteoclast inhibition was mediated at least in part, by the CB2 receptor (Idris et al., 2005). Further studies have shown that the CB2 selective antagonist/inverse agonist AM630 inhibited osteoclast formation and these effects were significantly inhibitory of osteoclast differentiation and bone resorption (Idris et al., 2005).

There is evidence that the defect in osteoclast formation in CB1 deficient mice is caused both by a reduction in the sensitivity of osteoclast precursors to RANKL and a reduction in the ability of CB2 deficient osteoclasts to support osteoclast formation due to reduced RANKL expression (Idris et al., 2009).

The CB2 receptor also regulates osteoclast activity and bone resorption but research in this area to date has yielded rather contradictory results. Ofek et al. (2016) reported that CB2 deficient mice developed osteoporosis with increasing age due to increased bone turnover. In keeping with these findings, it was reported in the same study that the CB2 selective agonist HU308 inhibited RANKL-induced osteoclast formation in bone marrow and RAW 264.7 cultures in vitro. In complete contrast to these findings, Idris and colleagues reported, that anandamide and 2-AG, HU308 and JWH133 enhanced M-CSF- and RANKL-induced osteoclast formation over the concentration range 1–1000 nM (Idris et al., 2005), whereas the CB2 selective agonist/inverse agonist AM630 was inhibitory (Idris et al., 2005, 2008). Further studies by Idris et al. (2008) showed that bone marrow cells isolated from CB1 deficient mice produce fewer osteoclasts in response to RANKL than wild type controls and that CB2 deficient mice were partially protected from ovariectomy-induced bone loss as compared with wild type littermates. In agreement with these observations others have reported that the endocannabinoids 2-AG and anandamide were found to stimulate bone resorption by human osteoclasts in vitro (Ridge et al., 2007; Whyte et al., 2012). Schuehly et al. (2011) have recently introduced a new class of highly CB2 selective ligands that strongly inhibited RANKL stimulated osteoclastogenesis in murine and human cultures. In the same study, the authors went to demonstrate that endocannabinoids stimulate osteoclast formation and these effects were significantly inhibited by natural biphenyl neolignan derivatives (Schuehly et al., 2011). In vivo, Idris et al. (2008) showed that the CB2 selective antagonist/inverse agonist AM630 prevents from ovariectomy-induced bone loss in a CB2 dependent and independent manner depending on administered dose. In broad agreement with this, Geng et al. (2010) showed that AM630 protected against the development of titanium particle induced osteolytic bone loss by reducing osteoclastogenesis. Furthermore, Lunn et al. (2007) reported that the novel CB2 selective antagonist Sch.036 prevented bone damage in arthritic mice. Taken together these studies indicate that pharmacological inhibition of the CB2 receptor inhibits osteoclast formation and reduces bone loss in adult mice. However, Rossi et al. (2009) reported that the CB2 selective antagonist/inverse agonist AM630 at high concentrations (10 μM) stimulated human osteoclast formation. The stimulatory effects of AM630 on osteoclast formation in human cultures reported by Rossi and colleagues are exactly opposite to the inhibitory effects if AM630 on osteoclast formation in mouse cultures reported by Idris et al. (2005, 2008). The reasons for this are unclear but possibilities include; species differences in responsiveness to AM630, off-target effects of AM630 both inhibited osteoclast formation from bone marrow macrophages in vitro, whereas the GPR55 antagonist cannabidiol increased osteoclast formation. Although GPR55 agonists were found in this study to inhibit osteoclast formation, they actually stimulated the resorptive activity of osteoclasts. Conversely, the GPR55 antagonist cannabidiol enhanced osteoclast formation and inhibited resorptive activity. In keeping with these observations male mice with targeted inactivation of GPR55 were found to have increased numbers of osteoclasts in vivo, but these appeared unable to resorb bone effectively since trabecular bone mass was increased and cartilage remnants at the growth plate were not resorbed efficiently. Rather surprisingly, female mice deficient in GPR55 were found to have reduced numbers of osteoclasts and increased amounts of unresorbed growth plate cartilage, but had normal bone mass (Whyte et al., 2009). Further studies in wild type mice revealed that cannabidiol reduced levels of CTX – a biochemical marker of bone resorption – consistent with the in vitro observations. Taken together these observations indicate that activation of GPR55 inhibits osteoclast formation, but increases the ability of osteoclasts to resorb bone. Conversely, inhibition of GPR55 appears to increase osteoclast formation but reduces the ability of osteoclasts to resorb bone. The cannabinoid derivative ajulemic acid (AJA) which is structurally similar to both CBD and THC has been found to inhibit formation of multinucleated TRAP positive cells in RANKL treated RAW264.7 cultures and in M-CSF- and RANKL treated bone marrow macrophages (George et al., 2008). The mechanism by which AJA regulates osteoclast formation is unclear since it has weak affinity for CB1, and presumably does not activate CB2 in view of the fact that it lacks psychoactive properties. Possibilities would include an effect on...
PPAR\(\gamma\), which has recently been implicated in the regulation of osteoblast differentiation (Wan et al., 2007) or on the effect on the GPR55 receptor.

In summary, there is evidence that both cannabinoid “classical” receptors CB\(_1\) and CB\(_2\) and the “orphan” receptor GPR55 regulate osteoblast activity and bone resorption. Conflicting results have been reported with regard to the role of CB\(_2\) in regulating osteoblast differentiation in vitro with stimulatory effects reported by some workers and inhibitory effects by others. The reasons for these differences remain unclear at present but one explanation may be the fact that many cannabinoid receptor ligands have complex pharmacology and are not entirely specific for the cannabinoid receptors they were originally designed to target. For example, AM251, which was classically considered to be specific CB\(_2\) selective antagonist/inverse agonist has recently been reported to act as a GPR55 agonist (Lauckner et al., 2008, Ross, 2009). Perplexingly SR141716A has been reported to act as a GPR55 antagonist (Kapur et al., 2009) or agonist (Lauckner et al., 2008), depending on the assay system used. Similarly, the synthetic cannabinoid agonist CP55,940 has been shown to be act as a GPR55 agonist/partial agonist and JWH015, formerly considered a specific CB\(_2\) agonist has been shown to be a GPR55 agonist (Kapur et al., 2009). These data indicate that the interpretation of some ligand activities previously attributed to effects on the CB\(_1\) or CB\(_2\) receptors may have been confounded by effects on the GPR55 receptor. Further research will clearly be required to investigate these issues and to explore the relative roles that CB\(_1\), CB\(_2\), and GPR55 play in regulating osteoblast differentiation and function.

**REGULATION OF OSTEOBLAST FUNCTION AND BONE FORMATION BY CANNABINOIDs**

Endocannabinoids and their receptors are involved in the regulation of osteoblast differentiation and bone formation. Mice with targeted deletion of the CB\(_1\) receptor have been found to develop osteoporosis with increasing age due to reduced bone formation and accumulation of adipocytes in the bone marrow space (Idris et al., 2009). In these studies, bone marrow stromal cells from CB\(_1\) deficient mice had an increased capacity for adipocyte differentiation and a reduced capacity for osteoblast differentiation and these effects were reproduced by treatment of wild type cultures with the CB\(_1\) selective antagonist/inverse agonist AM251 (Idris et al., 2009). Furthermore, the CB\(_1\) selective antagonist/inverse agonist AM251 was found to block the stimulatory effect of CP55,490 on bone nodule formation in vitro (Idris et al., 2009). Signaling studies have shown that blockade of CB\(_1\) receptors with AM251 in osteoblast-like cells and preadipocytes up-regulated cAMP, stimulated CREB phosphorylation, inhibited expression of the osteoblast-specific transcription factor RUNX2 and increased expression of the adipocyte-specific transcription factor PPAR\(\gamma\) (Idris et al., 2009). Other research has shown that the CB\(_2\) receptor plays a key role in regulating the increased bone formation that accompanies traumatic brain injury (TBI). This was investigated by Tam et al. (2008) who reported that the expected increase in bone formation following TBI was absent in CB\(_2\) deficient mice, but was present in wild type and CB\(_1\) deficient mice. In this study, Tam et al. (2008) also reported that TBI induced bone formation in wild type mice was abolished by the beta adrenergic receptor agonist isoproterenol. This led the authors to speculate that CB\(_2\) receptors present on presynaptic nerve endings in bone might enhance bone formation by suppressing catecholamine release (Tam et al., 2008). Although this is a plausible hypothesis, the experiments conducted did not determine whether the effect of the CB\(_2\) receptor on bone formation was truly mediated by this sequence of events or not. Other studies by the same authors (Tam et al., 2006) showed that bone formation rate and mineral apposition rate were reduced in young (9–12-week old) CB\(_2\) deficient mice on a CD1 background, again confirming that CB\(_2\) appears to play a key role in regulating bone formation. Recent findings have shown that the CB\(_2\) receptor plays a role in glucocorticoid-induced bone loss (Wu et al., 2011; Ko et al., 2012). CB\(_2\) blockage attenuated the deleterious actions of glucocorticoid treatment on osteoblast activity and bone formation, significantly reduced bone loss and abrogated marrow adiposity (Wu et al., 2011; Ko et al., 2012). Mechanistic studies revealed that CB\(_2\) regulates glucocorticoid-induced dysfunction in osteoblasts via activation of a number of pathways including PI3/AKT, MAPK and runt-related transcription factor 2 and peroxisome proliferator-activated receptor 2 (Wu et al., 2011; Ko et al., 2012).

The CB\(_2\) receptor also plays a role in regulating bone formation. CB\(_2\) selective agonists RU308, JWH333, and JWH015 have all been shown to stimulate bone nodule formation in bone marrow stromal cell cultures in vitro, although similar effects have been observed with non-selective agonists including anandamide, 2-AG, CP55,490, and WIN 55,212 (O’Shea et al., 2006; Scott and Williamson, 2007; Idris et al., 2009). A specific role for CB\(_2\) receptors in mediating these effects is supported by the fact that bone marrow stromal cells from CB\(_2\) deficient mice have a reduced capacity to differentiate into bone nodules when compared with those of wild type littermates. Although mice with targeted inactivation of CB\(_2\) have increased bone turnover, there is a relative defect in bone formation as evidenced by the fact that CB\(_2\) deficient mice develop age-related osteoporosis (O’Shea et al., 2006). This is consistent with a model whereby CB\(_2\) is required for maintenance of normal bone formation in high bone turnover states.

The role of the GPR55 receptor on bone formation has not been extensively studied, but Whyte et al. (2009) found no significant abnormalities in histomorphometric indices of bone formation in GPR55 knockout mice and also reported that the GPR55 agonist O-1602 had little effect on bone nodule formation in mouse calvarial osteoblast cultures. In view of this it seems unlikely that GPR55 plays a major role in regulating bone formation. Several phytocannabinoids including cannabidiol, cannabinol, cannabinodivarin, THC\(_c\), and tetrahydrocanabivarin (THCV) have been reported to stimulate bone nodule formation, collagen production, and alkaline phosphatase activity in cultures of bone marrow stromal cells (Scutt and Williamson, 2007). At the present time, however, it is unclear to what extent these compounds are acting through cannabinoid receptors or other molecular targets. For example the phytocannabinoid THCV which was found in the above study to promote bone nodule formation is known to act as an antagonist of CB\(_1\) and CB\(_2\) receptors, which would be expected to reduce bone formation...
In a recent study, Sophocleous et al. (2012) have reported in an abstract form that combined deficiency of the CB1 and CB2 receptors enhances peak bone mass but increases age-related bone loss. At 3 months of age, female CD1 mice deficient in both CB1 and CB2 receptors had significantly higher peak bone mass than wild type controls due to a significant decrease in osteoclast number and activity. Interestingly, these differences in peak bone mass and bone resorption observed were quantitatively similar to those previously observed in single knockouts of CB1 (Idris et al., 2005, 2009) and CB2 (Sophocleous et al., 2012) in the same background. By 12 months of age female deficient in both CB1 and CB2 receptors had significantly lower trabecular bone mass and histomorphometric analysis showed that this was associated with a dramatic increase in bone marrow fat accumulation and a reduction in osteoblast numbers and bone formation rate compared to wild type controls of similar age. The differences in bone mass and bone cell activity that the authors observed in female deficient in both CB1 and CB2 receptors were quantitatively similar to those previously observed in single knockouts of CB1 but not CB2 (Idris et al., 2009). Altogether, these data indicate that combined CB1 and CB2 deficiency enhances peak bone mass by an effect on bone resorption but predisposes to age-related osteoporosis by promoting adipocyte differentiation at the expense of osteoblast differentiation in the bone marrow compartment. This study demonstrates that CB1 and CB2 have overlapping but distinctive roles in skeletal homeostasis and show that CB2 in particular plays a key role in regulating osteoblast and adipocyte differentiation in the bone marrow compartment. Mice with targeted inactivation of the GPR55 receptor have been reported to have high peak bone mass affecting the trabecular compartment of the tibia and femur, but interestingly this was only noted in male mice (Whyte et al., 2009). The mechanism seemed to be impairment of bone resorption; although the reasons responsible for the gender difference in skeletal phenotype in these animals remains unclear at present.

FUTURE RESEARCH DIRECTIONS
There is a steadily growing body of evidence suggesting that the skeletal endocannabinoid system plays a significant role in regulating bone mass and bone turnover. Several outstanding questions remain unanswered however. One is to define the mechanisms by which endocannabinoid production in bone is regulated and specifically to determine if it is influenced by classical calcium regulating hormones, cytokines and mechanical loading. Further research is also required to fully define the signaling pathways used by these receptors to regulate bone cell activity. There is evidence to suggest that the CB1 receptor regulates osteoblast and adipocyte differentiation through a cAMP-mediated pathway but little is known about the mechanisms by which cannabinoids regulate osteoclast activity. There have been major discrepancies between different studies with regard to the effects of different cannabinoid receptor ligands on osteoclast differentiation and function. These remain to be completely resolved but some of the discrepancies between studies could be due to the fact that many cannabinoid receptor ligands which were previously thought to be specific for CB1 and/or CB2 have now been shown to have effects on GPR55 signaling (Kapur, 2009;


Conflict of Interest Statement: The authors are co-inventors on patents claiming the use of cannabinoid receptor ligands as treatments for bone disease.

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