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REVIEW

Skeletal lipidomics: regulation of bone metabolism by fatty acid amide family

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There is increasing evidence demonstrating that fatty acid derivatives play a key regulatory role in a variety of tissues. However, the study of skeletal lipidomics is just emerging and global strategies, such as targeted lipidomics, have not been applied to bone tissue. Such strategies hold great promises as in the case of genomics and proteomics. A partial profile of endocannabinoids and endocannabinoid-like compounds has demonstrated the presence of several long-chain fatty acid amides (FAAs), some of which displaying potent effects on osteoblasts, the bone forming cells and osteoclasts, the bone resorbing cells. In the skeleton, the FAAs activate the CB_1 cannabinoid receptor present in sympathetic nerve terminals as well as CB_2 cannabinoid receptor, the Gi-protein coupled receptor GPR55, and the transient receptor potential vanilloid type ion channel expressed by osteoblasts and/or osteoclasts. This review on the skeletal FAA system focuses on the production of FAAs in the skeleton and their net bone anabolic and anti-catabolic activity resulting from the stimulation of bone formation and inhibition of bone resorption. As the FAA family holds great promise as a basis for the treatment of osteoporosis and other diseases involving bone, further studies should aim towards the complete profiling of these lipids and their receptors in bone tissue, followed by elucidation of their function and mechanism of action.

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Abbreviations
BMD, bone mineral density; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; CFU-Ob, colony forming unit osteoblastic; EC, endocannabinoid; FAA, fatty acid amide; FAAH, fatty acid amide hydrolase; GPR55, Gi-protein coupled receptor 55; LCPUFA, long-chain polyunsaturated fatty acid; NE, norepinephrine; OPG, osteoprotegerin; OS, oleoyl serine; OVX, ovariectomy; PTH, parathyroid hormone; RANKL, receptor activator of NFκB ligand; TRPV1, transient receptor potential vanilloid type 1

Introduction

In vertebrates, skeletal metabolism is reflected as a continuous process of bone renewal, known better as bone remodelling, which consists of the concerted and balanced action of osteoclasts, the bone resorbing cells, and osteoblasts, the bone forming cells. Osteoporosis, the most prevalent degenerative disease in developed countries, results from the impairment of this balance, leading to bone loss and increased fracture risk. Bone remodelling is regulated by a

complex convergence of circulating hormones including sex steroids, parathyroid hormone, neurotransmitters, neuropeptides and pituitary-derived thyroid and follicle-stimulating hormones, on one hand, and local regulators of bone cell activity such as bone morphogenetic proteins, receptor activators of nuclear factor κB ligand (RANKL) and a number of cytokines, on the other hand (Rosen, 1997; Takeda *et al.*, 2002; Abe *et al.*, 2003; Robling *et al.*, 2006; Sun *et al.*, 2006; Lee and Herzog, 2009).

The super-family of lipids includes several families of naturally occurring compounds such as fats; cholesterol; fat-soluble vitamins; phospholipids; mono-, di- and triacylglycerols; as well as fatty acids and their derivatives. The biological function of lipids is not restricted to energy storage or as structural determinants of the cell membrane. It is well established now that lipids also serve as signalling molecules important in several physiological and pathological processes (Connor *et al.*, 2010). Yet the structural diversity of lipid compounds, their complex purification procedures and the chemical instability of many lipid entities decelerated their reliable identification and quantification. Recently, however, considerable progress in lipid analytical techniques has been made using advanced liquid chromatography combined with various MS systems, which made possible lipid analysis even in extremely complex mixtures. Based on these new technologies, the term 'lipidomics' was coined to define 'the full characterization of lipid molecular species and of their biological roles with respect to the expression of proteins involved in lipid metabolism and function, including gene regulation' (Spener *et al.*, 2003). As part of lipidomics, a remarkably growing attention has been channelled to the endocannabinoid system, as a novel paradigm to enhance the understanding of a particular family of lipids as signalling molecules (Astarita *et al.*, 2009). This family of fatty acid derivatives includes compounds consisting of saturated and unsaturated (ω-3, ω-6, ω-7, ω-9) long-chain fatty acids amides (FAAs). The identification of members of this subclass has been carried out concurrently using advances in MS techniques. Lipidomic approaches have been successfully applied to the endocannabinoid system (Piomelli *et al.*, 2007) and related FAAs, mainly in the central nervous system.

In spite of a growing interest in the circumstantial and functional inter-relationship between bone and adiposity, so far the study of lipid signalling in bone has focused primarily on prostaglandin E_2 (Uppal *et al.*, 2008). In addition, there is increasing evidence that various long-chain polyunsaturated fatty acids (LCPUFAs), as well as non-prostanoid LCPUFA metabolites, also regulate bone metabolism and may have therapeutic potential in the management of osteoporosis. Modification of dietary LCPUFA content, particularly increasing the intake of n-3 LCPUFAs, has been shown to minimize the decline in bone mass caused by menopause in women and by ovariectomy in animal models (Jee and Ma, 1997).

Since the initial publications of the regulation of skeletal metabolism by the endocannabinoid (EC) system half a decade ago (Bab, 2005; Idris *et al.*, 2005; Karsak *et al.*, 2005; Ofek *et al.*, 2006), a substantial body of information has been accumulated on the role of the FAA family in the control of bone remodelling and bone mass. The present article reviews this information, including recent publications on the presence of FAAs in the skeleton and role of a few of them in bone renewal.

Presence of FAAs in bone tissue

Several members of the FAA family have already been identified in bone tissue (Table 1). *N*-oleoyl serine (OS), *N*-oleoyl ethanolamide, *N*-stearoyl ethanolamide and the EC arachidonoyl ethanolamide (anandamide) are present in bone in pmol/g concentrations (Smoum *et al.*, 2010, Table 1). A structurally and functionally related compound, the EC 2-arachidonoylglycerol (2-AG), is present in bone at nmol/g levels. Notably, the EC bone levels are similar to those found in the brain (Bab *et al.*, 2008), but the blood EC levels are several orders of magnitude lower. Hence, it is very likely that

Table 1

Skeletal FAAs and their functional features

FAA	pmol/g wet bone	Effect on cell number		Receptor	Receptor expressing cell(s)	Skeletal status of receptor null mice
		Osteoblast	Osteoclast			
Anandamide	0.25 ± 0.01	↑	↓	CB1	Sympathetic terminals	Low bone mass
				CB2	Osteoblasts Osteoclasts	High turnover, age-related low bone mass
				TRPV1	Osteoclasts	?
Oleoyl serine	3.20 ± 0.25	↑	↓	?	Osteoblast? Osteoclast?	?
Oleoyl ethanolamide	13.7 ± 0.95	↑	?	GPR119	?	?
Stearoyl ethanolamide	18.3 ± 1.43	No effect	?	?	?	?
?	?	?	↑	GPR55	Osteoblasts Osteoclasts	High bone mass

these compounds, and other FAAs alike, are synthesized locally in the skeleton (Bab *et al.*, 2008). Indeed, at least the ECs and OS are produced by bone marrow stromal cells (that include osteoblast progenitors), osteoblasts and osteoclasts in culture (Jiang *et al.*, 2011; and our unpubl. results). A multitude of fatty acids is present in bone marrow (Griffith *et al.*, 2009) and it has been shown that brain *N*-acyl ethanolamide levels are correlated with dietary 20:4n-6 and 22:6n-3 fatty acids (Berger *et al.*, 2001). Hence, long-chain fatty acids may serve as precursors of skeletal FAAs. In addition, diacylglycerol lipases α and β, enzymes critically involved in 2-AG biosynthesis, are expressed in osteoblasts, osteocytes, bone-lining cells and osteoclasts (Tam *et al.*, 2008). The FAA biosynthetic and degrading enzymes, *N*-acyl phosphatidylethanolamine phospholipase D and fatty acid amide hydrolase (FAAH) (Ueda *et al.*, 2010), are also expressed in bone cells (Rossi *et al.*, 2009; our unpubl. results). A recent targeted lipidomics profiling of FAAs in brain revealed the presence of many more such compounds (Tan *et al.*, 2009). Therefore, given the similarity in FAA content so far found in bone and brain, the occurrence of multiple FAAs is anticipated also in bone.

FAA receptors and bone metabolism

The known FAA skeletal targets are shown in Table 1. The initial findings pointing to a role for FAAs in bone metabolism include the expression of the CB_1 and CB_2 cannabinoid receptors in the skeleton and their activity in regulating bone formation by osteoblasts and bone resorption by osteoclasts (Bab, 2005; Idris *et al.*, 2005; Ofek *et al.*, 2006). More recently, another G-protein coupled receptor, GPR55, has also been demonstrated in these cells (Whyte *et al.*, 2009). In addition, the membrane ion channel TRPV1 has been reported in osteoclasts (Rossi *et al.*, 2009).

Work carried out so far has focused mainly on CB_1 and CB_2. In the osteogenic cell lineage, undifferentiated osteoprogenitor cells exhibit very low levels, if any, of CB_1. CB_2 mRNA expression in these cells is also very low (Bab, 2005; Ofek *et al.*, 2006). However, when these cells are allowed to differentiate for 2–4 weeks in 'osteogenic medium' (Bellows *et al.*, 1986), CB_2 mRNA expression increases progressively together with the expression of osteoblastic marker genes, e.g. tissue non-specific alkaline phosphatase (Zhou *et al.*, 1994), parathyroid hormone receptor 1 (Zhang *et al.*, 1995), and the osteoblastic master regulatory gene, *RUNX2* (Araujo *et al.*, 2004). CB_1 is expressed at low levels in monocytic cells undergoing osteoclastogenesis induced by RANKL and macrophage colony-stimulating factor (Zou *et al.*, 2002). By contrast, CB_2 mRNA transcripts in these cells are present in high abundance (Bab, 2005; Idris *et al.*, 2005; Ofek *et al.*, 2006; Scutt and Williamson, 2007). CB_2 is also expressed by periodontal ligament cells, which may undergo osteoblastic differentiation (Qian *et al.*, 2010). *In vivo*, CB_2 was identified in trabecular osteoblasts and their descendants, the osteocytes, as well as in osteoclasts (Ofek *et al.*, 2006). CB_1 protein is abundant in skeletal sympathetic nerve terminals in close proximity to osteoblasts (Tam *et al.*, 2006).

Several mouse lines of FAA receptor null mice have been used to assess the physiologic role of CB_1, CB_2 and GPR55 in the control of bone metabolism. The skeletal phenotype of *cnr1* (the gene encoding mouse CB_1) mutated mice depends on the animal strain and/or the construct used for gene mutation. In one CB_1-deficient line, backcrossed to CD1 mice ($CD1^{cnr1-/-}$), the N-terminal 233 codons of *cnr1* were ablated (Ledent *et al.*, 1999). The skeletal phenotype of young, sexually mature $CD1^{cnr1-/-}$ mice shows a sex disparity. Females have normal trabecular bone with a slight cortical expansion, whereas males exhibit a high bone mass phenotype (Idris *et al.*, 2005; Tam *et al.*, 2006). Sexually mature young mice of either sex display normal bone formation and resorption parameters, suggesting that the male phenotype is acquired early in life. In the second line, backcrossed to C57Bl/6J mice ($C57^{cnr1-/-}$), almost the entire protein-encoding sequence was removed (Zimmer *et al.*, 1999). Both male and female $C57^{cnr1-/-}$ have a low bone mass phenotype accompanied by increased osteoclast counts and decreased bone formation rate (Tam *et al.*, 2006). More recently, an aging-related low bone mass phenotype has also been reported in a $CD1^{cnr1-/-}$-derived mouse line (Idris *et al.*, 2009).

CB_2-deficient animals have a skeletal phenotype that is gender independent. Both male and female $cnr2^{-/-}$ mice accrue a normal peak trabecular bone mass, but later display a markedly enhanced age-related bone loss (Ofek *et al.*, 2006). Reminiscent of human postmenopausal osteoporosis (Brown *et al.*, 1984), the $cnr2^{-/-}$ mice have a high bone turnover characterized by increases in both bone resorption and formation, which are at a net negative balance (Ofek *et al.*, 2006). Because healthy *cnr2* null mice are otherwise normal, it appears that the main physiologic role of CB_2 is in maintaining bone remodelling at balance.

Studies in humans have confirmed that CB_2 is an important determinant of bone metabolism. The locus of *CNR2* (the gene encoding human CB_2) is located on chromosome 1p36. This genomic region and its mouse ortholog on chromosome 4 have been linked to bone mineral density (BMD) and osteoporosis in several association analyses (Devoto *et al.*, 1998; 2001; 2005). Several genetic association studies have consistently shown that a common variant of *CNR2* contributes to the aetiology of low BMD and osteoporosis in humans (Karsak *et al.*, 2005; 2009; Yamada *et al.*, 2007; Huang *et al.*, 2009). So far, similar analyses of *CNR1* (the gene encoding human CB_1) have failed to demonstrate such an association with osteoporosis.

GPR55 expression has been reported in osteoblasts and osteoclasts. While the skeleton of female GPR55 null nice is normal, male $GPR55^{-/-}$ animals have a high bone mass phenotype, resulting from reduced bone resorption, secondary to an osteoclast malfunction. On the other hand, GPR55 agonists also restrain bone resorption, consequent to the inhibition of osteoclast formation (Whyte *et al.*, 2009).

Signalling pathways targeted by FAAs in bone have been so far reported for CB_1, CB_2 and the putative OS receptor. It appears that CB_1 controls osteoblast function by negatively regulating norepinephrine (NE) release from sympathetic nerve terminals in the immediate proximity of these cells. NE suppresses osteoblast function by binding to osteoblastic β2-adrenergic receptors (Takeda *et al.*, 2002), which is alleviated by activation of sympathetic CB_1 (Tam *et al.*, 2008). Although expressed at a low level in osteoblasts and osteoclasts, the relative contribution of bone cell versus sympa-

thetic CB_1 to bone metabolism is still an open issue. Given that *cnr1*$^{flox/flox}$ mice are already available (Quarta *et al.*, 2010), this question should be addressed by phenotypic characterization of mice with conditional *cnr1* deletion in osteoblasts, osteocytes, osteoclasts and sympathetic nerves.

Activation of CB_2 in osteoblasts targets a mitogenic Gi protein – Erk1/2 – Mapkapk2 – CREB – cyclin D1 pathway (Ofek *et al.*, 2011). CB_2 agonists also stimulate mineralized matrix formation (Ofek *et al.*, 2006). In addition, CB_2 activation leads to decreased osteoclastogenesis resulting from decreases in osteoclast progenitor cell proliferation and in the RANKL/OPG expression ratio in bone marrow stromal cells (Ofek *et al.*, 2006).

Although the putative OS receptor has not been identified yet, it is likely coupled to a Gi protein, as its actions are inhibitable by pertussis toxin. It also activates Erk1/2, but unlike the case of CB_2, its signalling downstream of Erk1/2 does not involve Mapkapk2 and CREB (Smoum *et al.*, 2010).

The presence of FAA receptors in bone does not derogate from the potential role of free fatty acids (FFAs) in skeletal metabolism. Indeed, receptors known to bind FFAs were found in osteoblastic (GPR120) and osteoclastic (GPR40, 41, 43, 120) cells (Cornish *et al.*, 2008). However, the relationship between the FFA and FAA activity in bone remains an open, very fascinating issue. Additional possible FAA targets, such as ion-channels and peroxisome proliferator-activated receptors, have been identified and should be looked for in the skeleton (Hansen, 2010; Pertwee *et al.*, 2007).

Therapeutic potential of FAAs and related agonists

Of the FAAs so far identified, only the skeletal effects of OS administration have been studied *in vivo*. OS increases bone mass in normal and osteoporotic mice by a dual action consisting of stimulating osteoblast proliferation/bone formation and inhibiting bone resorption by enhancing osteoclast apoptosis (Smoum *et al.*, 2010). These features suggest that OS can be used as a bone antiresorptive, as well as anabolic agent.

Because of its instability, anandamide administration has not been tested *in vivo*. However, like OS, synthetic CB_2 agonists have been shown to be both anabolic and antiosteoporotic by stimulating bone formation and restraining bone resorption (Ofek *et al.*, 2006; Bab *et al.*, 2008).

TRPV1 is targeted by many FAAs (Movahed *et al.*, 2005). A recent study in osteoclasts derived from osteoporotic patients suggests that its desensitization by FAAs, or its enhanced trafficking, together with TRPV1 agonist-induced CB_2 receptor overexpression, might be critical to minimize calcium entry into osteoclasts, which could be responsible in turn for cell over-activation and increased bone resorption and bone loss, advocating the use of TRPV1 agonists together with CB_2 agonists in osteoporosis (Rossi *et al.*, 2011).

If indeed the dual potent bone anabolic-antiresorptive action is shared by many skeletal FAAs, inhibition of FAAH, the FAA degrading enzyme, may prove as a useful therapeutic strategy to combat osteoporosis and perhaps other skeletal deficits. Notably, CB_1 or CB_2 agonists reduce bone cancer pain in animal models (Curto-Reyes *et al.*, 2010; Kawamata *et al.*, 2010). Therefore, this approach may be applied also for analgetic treatment in patients with bone metastases.

Conclusions

A handful of studies suggest that FFAs are involved in the regulation of bone remodelling, either by themselves or through their conversion to other molecules such as prostaglandins and FAAs. Recent studies demonstrate several FAAs and FAA receptors in bone tissue. Most of the FAAs identified in the skeleton stimulate osteoblast proliferation. Findings in mice and humans suggest that they also inhibit osteoclastogenesis. Of these FAAs, the occurrence and activity of OS have been characterized in more details. These data, together with skeletal phenotyping of FAA receptor (CB_1, CB_2, GPR55)-deficient mice, suggest an important role for the FAAs in the regulation of skeletal remodelling and the consequent implications on bone mass and biomechanical function as well as the alleviation of bone pain.

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Conflict of interest

We do not have any conflict of interest.

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