

RESEARCH PAPER

Cannabinoids and bone: endocannabinoids modulate human osteoclast function *in vitro*

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Keywords

cannabinoid; CB₁; CB₂; endocannabinoid; 2-AG; AEA; bone; osteoclast; LPS

Received

21 January 2011

Revised

27 April 2011

Accepted

20 May 2011

BACKGROUND AND PURPOSE

Both CB₁ and CB₂ cannabinoid receptors have been shown to play a role in bone metabolism. Crucially, previous studies have focussed on the effects of cannabinoid ligands in murine bone cells. This study aimed to investigate the effects of cannabinoids on human bone cells *in vitro*.

EXPERIMENTAL APPROACH

Quantitative RT-PCR was used to determine expression of cannabinoid receptors and liquid chromatography-electrospray ionization tandem mass spectrometry was used to determine the presence of endocannabinoids in human bone cells. The effect of cannabinoids on human osteoclast formation, polarization and resorption was determined by assessing the number of cells expressing $\alpha_v\beta_3$ or with F-actin rings, or measurement of resorption area.

KEY RESULTS

Human osteoclasts express both CB₁ and CB₂ receptors. CB₂ expression was significantly higher in human monocytes compared to differentiated osteoclasts. Furthermore, the differentiation of human osteoclasts from monocytes was associated with a reduction in 2-AG levels and an increase in anandamide (AEA) levels. Treatment of osteoclasts with LPS significantly increased levels of AEA. Nanomolar concentrations of AEA and the synthetic agonists CP 55 940 and JWH015 stimulated human osteoclast polarization and resorption; these effects were attenuated in the presence of CB₁ and/or CB₂ antagonists.

CONCLUSIONS AND IMPLICATIONS

Low concentrations of cannabinoids activate human osteoclasts *in vitro*. There is a dynamic regulation of the expression of the CB₂ receptor and the production of the endocannabinoids during the differentiation of human bone cells. These data suggest that small molecules modulating the endocannabinoid system could be important therapeutics in human bone disease.

LINKED ARTICLES

This article is part of a themed section on Cannabinoids in Biology and Medicine. To view the other articles in this section visit <http://dx.doi.org/10.1111/bph.2012.165.issue-8>. To view Part I of Cannabinoids in Biology and Medicine visit <http://dx.doi.org/10.1111/bph.2011.163.issue-7>

Abbreviations

AEA, anandamide; 2-AG, 2-arachidonoyl glycerol; $\alpha_v\beta_3$, $\alpha_v\beta_3$ integrin (vitronectin receptor); BCA, bicinchoninic acid; CB₁, cannabinoid receptor 1; CB₂, cannabinoid receptor 2; DMSO, dimethyl sulphoxide; FAAH, fatty acid amide hydrolase; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; M-CSF, macrophage colony stimulating factor; NAPE-PLD, N-acyl phosphatidylethanolamine phospholipase D; PBMC, peripheral blood mononuclear cell; RANKL, receptor activator of NF- κ B ligand; TBS, Tris buffered saline; TBST, Tris buffered saline + 0.1% (v/v) Tween; TRAP, tartrate resistant acid phosphatase; TRPV1, transient receptor potential cation channel subfamily V member 1; VNR, vitronectin receptor

Introduction

Mammalian tissues express at least two cannabinoid receptors, CB₁ and CB₂, both of which are G-protein coupled (Howlett

et al., 2002; Alexander *et al.*, 2011). Endogenous ligands (endocannabinoids) for these receptors also exist, prominent examples include arachidonoyl ethanolamide (anandamide or AEA) and 2-arachidonoyl glycerol (2-AG). Both AEA and

2-AG are synthesized on demand, removed from their sites of action by tissue uptake processes and metabolized by intracellular enzymes (Pertwee and Ross, 2002). Together these lipids, enzymes and receptors constitute the endocannabinoid system. A clear role for the endocannabinoid system has been demonstrated in a variety of physiological processes including cardiovascular regulation, appetite control, pain processing, learning and memory, with evidence that the levels of endocannabinoids are altered in many pathophysiological situations (Di Marzo and Petrosino, 2007).

The endocannabinoid system is a recently identified therapeutic target in the control of bone mass. Recent studies have demonstrated abnormal bone phenotypes in mice lacking either CB₁ or CB₂ receptors; these phenotypes vary with age, gender or genetic background (for reviews see Bab *et al.*, 2009; Idris and Ralston, 2010). Mouse bone cells and sympathetic nerve fibres that lie in close proximity to bone express cannabinoid receptors (Idris *et al.*, 2005; Ofek *et al.*, 2006; Tam *et al.*, 2006; Bab *et al.*, 2008). Importantly, the endocannabinoids 2-AG and AEA are produced in murine trabecular bone (Tam *et al.*, 2008) and human osteoclasts (Rossi *et al.*, 2009). Synthetic cannabinoid receptor agonists and inverse agonists have also been shown to reduce bone loss in mice following ovariectomy and to have direct effects on mouse bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) *in vitro* (Idris *et al.*, 2005; Ofek *et al.*, 2006; Tam *et al.*, 2006; Idris, 2008; Idris *et al.*, 2009; Sophocleous *et al.*, 2011).

In this study we further investigated the role of cannabinoids in bone physiology by addressing a key question that has been little investigated; what effect do cannabinoids have on the function of human bone cells *in vitro*? In the light of recent reports demonstrating significant associations between CB₂ receptor genotypes and osteoporosis in humans (Karsak *et al.*, 2005), it is crucial to understand the pharmacology of the cannabinoids in human bone cells. Previous *in vitro* studies have primarily focussed on the effects of cannabinoid ligands in murine bone cells (Bab *et al.*, 2009). There is a possibility that the effects of cannabinoid ligands may be subject to species differences and the compounds may have distinct profiles in mouse and human bone cells. It is well known that GPCR agonists can display divergent effects on bone cells obtained from mouse compared to human cells, this is exemplified in the effects of PGE₂ (Take *et al.*, 2005). Only one study (Rossi *et al.*, 2009) has examined the effect of only one CB₂ receptor antagonist on human osteoclast formation; there are no data in the current literature on the effects of cannabinoids on human osteoclast function.

Our results provide important new insights into the dynamic regulation of the endocannabinoid system in human osteoclast differentiation and demonstrate effects of cannabinoid agonists on human bone resorbing cells *in vitro*. This study therefore adds to the evidence, primarily obtained from murine cells *in vitro*, that modulation of CB₁/CB₂ may be an approach to the treatment of diseases caused by excessive osteoclast activity such as osteoporosis.

Methods

Cell culture reagents and chemicals were purchased from Sigma Aldrich Company Ltd (UK) unless otherwise

stated. Cannabinoid compounds 2-AG, AEA, CP 55,940 [- (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol], JWH 015 [- (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenylmethanone], AM251 [- N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], AM630 [- 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone] were from Tocris Cookson Ltd (Bristol, UK), and SR141716A [- N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] and SR144528 [- N-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide] were from Sanofi-Aventis (Montpellier, France). URB-597 (cyclohexylcarbamic acid 3'-carbamoyl-biphenyl-3-yl ester) was purchased from Biomol International (USA). All compounds were dissolved in dimethyl sulphoxide (DMSO) such that the final concentration of DMSO did not exceed 0.1%.

Cell culture

All cells, unless otherwise stated, were cultured at 37°C/5% CO₂ in α -modified minimal essential media (α -MEM) containing 10% (v/v) fetal calf serum (FCS), 2% antibiotic mixture (PS; 100 IU·mL⁻¹ penicillin, 100 μ g·mL⁻¹ streptomycin) and 2 mM L-glutamine. CB₂ stably transfected CHO cells (Ross *et al.*, 1999) and mouse brain membranes (Price *et al.*, 2005) were prepared as previously described.

Isolation of osteoblasts

Mouse calvarial osteoblasts were isolated from the calvarial bones of 2-day-old C57BL/6 mice by sequential collagenase digestion and grown to confluence as previously described (Armour *et al.*, 2001).

Peripheral blood mononuclear cell isolation and osteoclast generation

Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) donated with informed consent from healthy volunteers (with approval from the North of Scotland Research Ethics Committee) as previously described (Whyte *et al.*, 2009). In brief, PBMCs were isolated by centrifugation over Lymphoprep (Axis-Shield Diagnostics Ltd) and seeded into 75 cm² flasks in culture medium supplemented with 20 ng·mL⁻¹ macrophage colony stimulating factor (M-CSF) (R&D Systems Europe Ltd) to allow adherence and expansion of M-CSF-dependent monocytes (approximately 7 days). To generate osteoclasts, the highly enriched M-CSF-dependent monocytes were harvested by trypsinization and gentle scraping, and then re-plated at a seeding density of 200 000 cells·mL⁻¹ in medium containing 20 ng·mL⁻¹ M-CSF and 100 ng·mL⁻¹ receptor activator of NF- κ B ligand (RANKL, Peprotech EC Ltd). Medium was refreshed every 2–3 days and after approximately 5–7 days in RANKL more than 80% of cells were α _v β ₃-positive with numerous multinucleated osteoclasts.

Analysis of human osteoclast formation

To study the effect of cannabinoids on osteoclast formation, osteoclasts were generated on plastic 96-well plates as described above, in the presence of test compounds or vehicle

(0.1% v/v DMSO). Compounds were added to highly enriched M-CSF-dependent monocytes at the same time as the RANKL and were present throughout the duration of the culture. Osteoclasts generated in the presence of 2-AG, AEA or vehicle were cultured in medium containing 2% FCS rather than 10% FCS in order to reduce the binding of these lipophilic compounds to serum. This reduction in serum levels had no detrimental effect on cell viability or osteoclast formation. Once formed, total cell viability was determined using the Alamar Blue assay as described by Taylor *et al.* (2007). Cells were then washed thoroughly in HBSS before being fixed with 4% paraformaldehyde (v/v). Osteoclasts were stained with 23c6 anti- $\alpha_v\beta_3$ (Serotec) and Alexa Fluor 488 goat anti-mouse antibodies to quantify total $\alpha_v\beta_3$ fluorescence as a measure of osteoclast formation using a BioTek FL600 plate reader. Total $\alpha_v\beta_3$ immunofluorescence showed a significant correlation with the total number of $\alpha_v\beta_3$ -positive multinucleated cells (Figure S1).

Analysis of human osteoclast polarization and resorption

Human osteoclasts were generated on 5 mm diameter elephant ivory dentine discs in 96-well plates and treated with test compounds at the first sign of osteoclast resorption (approximately day 7 of RANKL treatment). Upon addition of AEA or vehicle at the first signs of resorption, cultures were switched from 10% FCS to 2% FCS for reasons described above; cultures treated with CP 55 940 or JWH015 were maintained at 10% FCS. Cultures were terminated 3–5 days later by fixing in 4% paraformaldehyde. Intracellular F-actin was visualized by staining with 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ tetramethylrhodamine isothiocyanate (TRITC)-phalloidin as previously described (Coxon *et al.*, 2001) and the number of F-actin rings per disc was counted. To assess osteoclast-mediated resorption, cells were removed from the discs and pits in the mineral surface were visualized by reflected light microscopy. The area of resorbed dentine was examined using a Zeiss AxioLab reflective light microscope and quantified using software developed in-house based on Aphelion (ADCIS, France) ActiveX components.

Quantitative PCR

RNA extraction and cDNA synthesis were carried out as described previously (Idris *et al.*, 2005). RNA was extracted from M-CSF-dependent monocytes and mature osteoclasts (after 7 days in RANKL). The expression of the human CB₁ and CB₂ receptors and the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real time PCR using FAM- and VIC-labelled TaqMan probes (Applied Biosystems, UK, CB₁- Hs00275634_m1 FAM and CB₂- Hs00275635_m1, VIC-GAPDH) and using a DNA Engine Opticon 2 Real Time Cycler (MJ Research). Control reactions with no template and no reverse transcriptase were run on each occasion. Reactions were carried out at 95°C for 15 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min.

Immunocytochemistry

Human osteoclasts and mouse osteoblasts were fixed with 4% paraformaldehyde in PBS, blocked using 10% FCS for 10 min and stained using an antibody to CB₁ (Abcam) or CB₂ (Affinity

Bioreagents) followed by Alex Fluor goat anti-rabbit 488 (Invitrogen). Nuclei were counterstained with 0.5 μM TO-PRO-3 iodide (Invitrogen). Control cells were stained with normal rabbit IgG (Santa Cruz). Cells were examined on a Zeiss LSM510 Meta Confocal microscope and images captured using LSM image capture software.

Western blotting

Mouse osteoblasts, mouse brain homogenates, CHO cells stably transfected with CB₂, human monocytes and human osteoclasts (isolated at three time points during the course of differentiation), were lysed in RIPA buffer [PBS, 0% (w/v) sodium dodecyl sulphate, 0.5% (w/v) sodium deoxycholate and 1% (v/v) Igepal CA-630] containing Protease Inhibitor cocktail (Sigma). The protein concentration of the lysates was determined (BCA assay, Pierce) and equal quantities (50 μg) of denatured samples were electrophoresed on pre-cast Bis-Tris 12% criterion gels (Bio-Rad, USA) and transferred to polyvinylidene difluoride (PVDF) membranes by semi-dry transfer [Bio-Rad Trans-blot SD Semi-Dry Transfer cell plates (Bio-Rad, USA)]. Membranes were blocked for 1 h by using Odyssey blocking buffer (LI-COR, USA) diluted 1:1 in Tris buffered saline (TBS), then incubated with primary antibodies for CB₁ or CB₂ overnight at 4°C in blocking buffer, prepared as outline above, containing 0.1% Tween (TBST). The following day membranes were washed with TBST and then incubated with infrared-labelled secondary antibodies for 1 h in the dark followed by further wash steps, the final of which was in TBS. Blots were visualized using a LI-COR Odyssey Infrared Imager.

Analysis of Rho activation in human osteoclasts

Human osteoclasts were deprived of FCS, RANKL and M-CSF overnight and then treated for 2.5 or 5 min with α -MEM alone (control), α -MEM containing 10% FCS or 1 $\text{mg}\cdot\text{mL}^{-1}$ BSA, vh (0.1% DMSO) or 1 μM CP 55 940. Quantification of Rho-GTP in 0.5 mL of cleared cell extract (harvested from two wells of a six-well plate) was performed using a Rhotekin pull-down assay according to the manufactures instructions (Upstate) as previously described (Whyte *et al.*, 2009). Treatments with GTP γ S (positive control) and GDP (negative control) ensured that the pull-down procedures were working properly. Total Rho was determined from cell extracts not subject to pull-down in order to ensure equal protein quantities between samples.

LC-MS/MS analysis

M-CSF-dependent monocytes, mature osteoclasts (after 7 days in RANKL) (9.5 cm^2 wells) and cell lines at 70–90% confluence (75 cm^2 flasks) were washed and scraped into PBS and collected by centrifugation at 548 $\times g$ for 10 min at 4°C. The pellet was resuspended in 50/50 methanol/acetonitrile containing 6 pmol d4-AEA (internal standard) (QMX Laboratories Ltd) and homogenized by sonication. The sonicated mixture was made up to 70% water and centrifuged to remove cell debris. The supernatant was applied to a preconditioned Strata-X SPE cartridge (Phenomenex), washed twice, eluted in 100% methanol and evaporated to dryness under nitrogen. The residue was resuspended in 50 μL mobile phase. Standards containing AEA (0.01–2 pmol

50 μL^{-1}) and 2-AG (25–2000 pmol 50 μL^{-1}) were prepared in methanol/acetonitrile containing 6 pmol d4-AEA and processed as above. The extraction efficiency was >95% and the limit of quantification was determined to be 0.01 pmol for AEA and 25 pmol for 2-AG. Samples were stored at 4°C and 10 μL of extract analysed by LC-ESI-MS-MS using a Thermo Surveyor – TSQ Quantum system. Isocratic reversed phase separation was carried out on an ACE 5 μ C8 (150 mm \times 2.1 mm) column (Hichrom Ltd), temperature controlled at 30°C with a mobile phase consisting of 15% water and 85% methanol (both containing 0.5% formic acid) at a flow rate of 0.2 mL \cdot min $^{-1}$. MS-MS analysis was carried out using electrospray ionization (ESI) in positive ion mode with spray voltage 3500 V, sheath gas pressure 40, auxiliary gas 10, capillary temperature 375°C and skimmer offset 10 V. Single reaction monitoring was used for detection and quantification, utilizing the following parent – product ion pair transitions: 348.2–62.2 (AEA, retention time 8 min) and 379.0–287.0 (2-AG, retention time 8.5 and 9 min) and 352.2–66.2 (d4-AEA, retention time 8 min). The collision gas pressure was 1.4 and the collision energy was 13 V. Usually, 2-AG was observed as a doublet because of isomerization to 1(3)-AG and therefore the area of both peaks were combined to yield total 2-AG. The levels of both 2-AG and AEA were analysed in each sample. Endocannabinoid levels were normalized to total protein content.

Statistical analysis

Data analysis and statistical comparisons were made by use of Student's *t*-test or one-way ANOVA. Following ANOVA, a Dunnett's or Bonferroni post-test was applied to identify significant differences unless otherwise stated.

Results

Cannabinoid receptor expression in human monocytes and osteoclasts

To confirm and build upon previous reports that bone cells express cannabinoid receptors, human osteoclasts derived from highly enriched M-CSF-dependent monocytes and mouse calvarial osteoblasts were analysed for CB₁ and CB₂ expression by quantitative PCR and/or Western blotting. Quantitative real time PCR detected the presence of both CB₁ and CB₂ receptors in human monocytes and mature osteoclasts. The expression of CB₁ mRNA remained constant during differentiation (Figure 1A), whereas the level of CB₂ mRNA decreased significantly by 76% during osteoclast differentiation (Figure 1B). Detection of CB₁ and CB₂ at the protein level in human monocytes and osteoclasts was not associated with changes in receptor expression during differentiation (Figure 1C and D). Expression of CB₁ and CB₂ in human osteoclasts and mouse osteoblasts was confirmed by immunocytochemical staining as shown in Figure 1E and F, respectively. Immunocytochemistry revealed CB₁ and CB₂ cannabinoid immunoreactivity both intracellularly and at the cell surface. This is in line with cannabinoid receptor localization as seen in other cell types (McIntosh *et al.*, 1998; Rayman *et al.*, 2004).

Endocannabinoids are produced by human osteoclasts, osteoblast-like cells and mouse osteocytes

Basal levels of 2-AG and AEA were measured in primary human monocytes and osteoclasts. 2-AG was detected in both human monocytes and osteoclasts (Figure 2A), with significantly lower levels in osteoclasts compared with monocytes (monocytes -0.43 ± 0.14 nmol \cdot mg $^{-1}$ protein; osteoclasts 0.11 ± 0.02 nmol \cdot mg $^{-1}$ protein). 2-AG was also detected in the mouse osteocyte-like cell line MLO-Y4 (0.83 ± 0.11 nmol \cdot mg $^{-1}$ protein) and two human osteoblast-like cell lines, MG-63 (0.11 ± 0.02 nmol \cdot mg $^{-1}$ protein) and HOS TE85 (0.28 ± 0.03 nmol \cdot mg $^{-1}$ protein). AEA was not detected in human monocytes but present in osteoclasts (0.13 ± 0.02 nmol \cdot mg $^{-1}$ protein) (Figure 2B). AEA was also detected in MG-63 cells (0.12 ± 0.01 nmol \cdot mg $^{-1}$ protein), MLO-Y4 cells (0.10 ± 0.01 nmol \cdot mg $^{-1}$ protein) but not in HOS TE85 cells.

Treatment with LPS did not alter the levels of 2-AG detected in human osteoclasts from three separate donors (0.06 ± 0.01 to 0.09 ± 0.01 nmol \cdot mg $^{-1}$ protein, 0.24 ± 0.08 to 0.20 ± 0.02 nmol \cdot mg $^{-1}$ protein, 0.19 ± 0.04 to 0.12 ± 0.04 nmol \cdot mg $^{-1}$ protein, Figure 2C). However, treatment of human osteoclasts with LPS did increase the amount of AEA detected (0.06 ± 0.01 to 0.14 ± 0.03 nmol \cdot mg $^{-1}$ protein, not detected to 0.15 ± 0.09 nmol \cdot mg $^{-1}$ protein or 0.22 ± 0.07 nmol \cdot mg $^{-1}$ protein, Figure 2D).

Endocannabinoids inhibit human osteoclast formation at micromolar concentrations

The detection of both cannabinoid receptor subtypes together with the presence of endocannabinoids in human monocytes and fully differentiated osteoclasts suggests that cannabinoid receptor ligands may have direct effects on the differentiation of osteoclasts. To determine the effect of endocannabinoids on human osteoclastogenesis, highly enriched M-CSF-dependent human mononuclear cells were stimulated to differentiate into multinucleated osteoclasts, capable of functional resorption, by the addition of M-CSF and RANKL in the continual presence of 1 nM–1 μM AEA or 2-AG. Osteoclast formation was significantly reduced in the presence of 10 μM AEA (Figure 3A) or 10 μM 2-AG (Figure 3B) to $36 \pm 3\%$ and $42 \pm 4\%$ of control, respectively. Cell viability was not altered in the presence of AEA or 2-AG; therefore, the inhibition of osteoclastogenesis was caused by an attenuation of differentiation rather than a decrease in cell number caused by toxicity (Figure S2A and B).

In order to determine the receptor responsible for the endocannabinoid-induced inhibition of osteoclastogenesis, selective receptor antagonists were utilized with the purpose of selectively blocking the CB₁ (100 nM AM251), CB₂ (100 nM AM630) or TRPV1 (1 μM capsazepine) receptors. At the concentration tested, none of the antagonists significantly affected osteoclast formation alone, and when added to cultures containing 10 μM AEA or 2-AG the antagonists were incapable of reversing the inhibitory effects of the endocannabinoids (Figure 3C). These results suggest that the endocannabinoid-mediated inhibition of osteoclast differentiation is independent of CB₁, CB₂ or TRPV1 receptor activa-

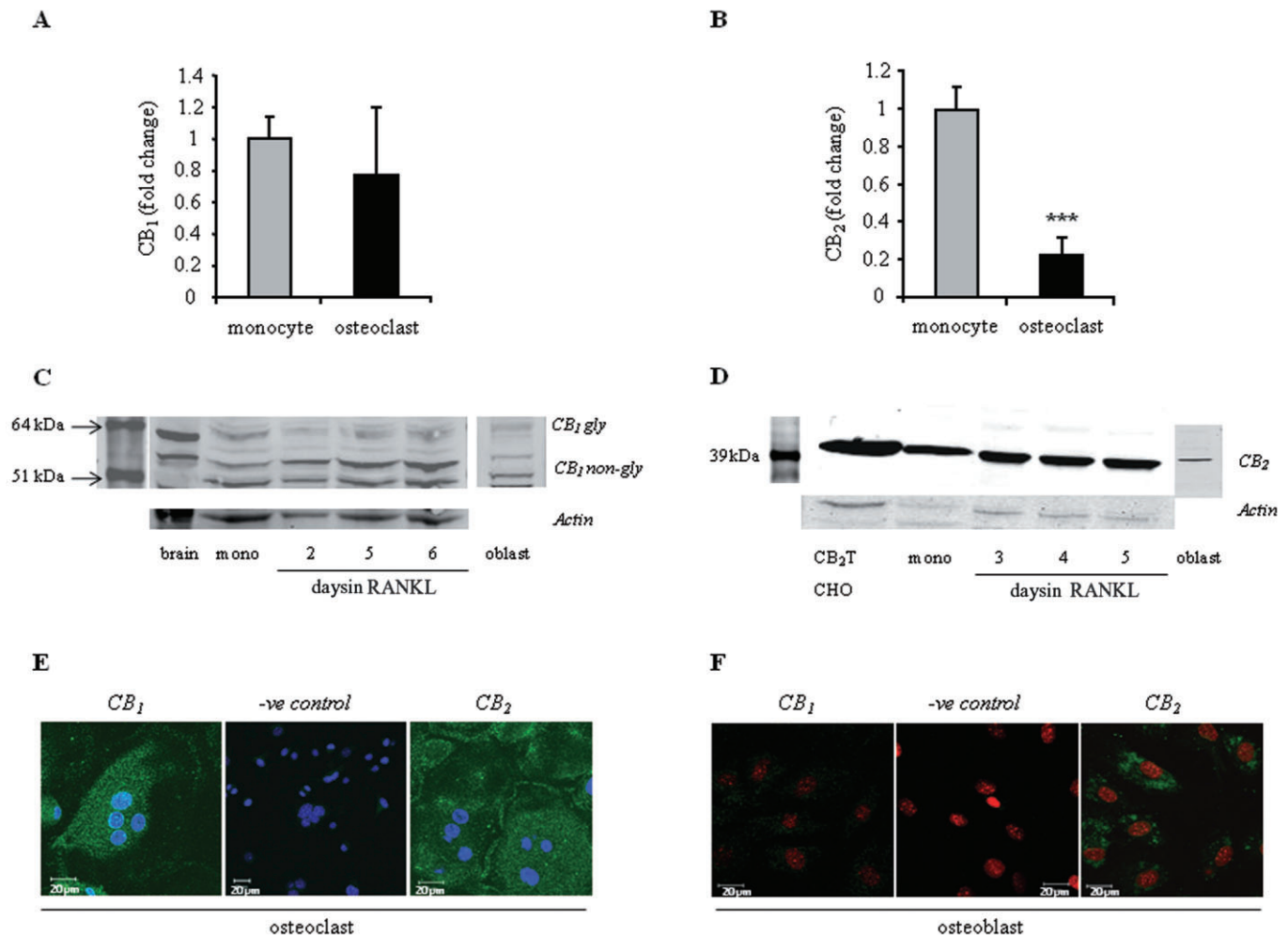


Figure 1

Cannabinoid receptor expression in human monocytes, osteoclasts and mouse osteoblasts. Quantitative real time PCR was performed using a primer Taqman probe set specific for CB₁ (A) or CB₂ (B) on isolated RNA from human M-CSF-dependent monocytes and differentiated osteoclasts formed in the presence of RANKL and M-CSF. CB₁ and CB₂ mRNA levels were normalized to GAPDH (housekeeping gene – levels remained constant during osteoclast differentiation). Results reported as fold change in gene expression relative to untreated cells after normalization against GAPDH. Mean \pm SEM; $n = 4$ experiments – levels measured in triplicate for each donor. *** $P < 0.001$ Student's t -test. CB₁ and CB₂ protein expression in human monocytes, osteoclasts and mouse osteoblasts was detected by Western blotting. Cell lysates were prepared and equal amounts of protein were electrophoresed on polyacrylamide-SDS gels and immobilized to PVDF membranes by Western blotting. (C) CB₁ expression is shown in mouse brain homogenates (brain), M-CSF-dependent monocytes (mono) and osteoclasts formed in the presence of RANKL isolated at days 2, 5 and 6 (note the glycosylated form of CB₁ is 64 kDa and the non-glycosylated form is 54 kDa) and mouse calvarial osteoblasts (oblast). (D) CB₂ expression is shown in CB₂ transfected CHO cells (CB₂TCHO), monocytes, osteoclasts formed in the presence of RANKL isolated at days 3, 4 and 5 and mouse osteoblasts. β -Actin served as a loading control. Results shown are representative of three experiments. Immunocytochemical detection of CB₁ and CB₂ in human osteoclasts (E) and mouse osteoblasts (F) by immunofluorescence staining – CB₁ or CB₂ in green with a nuclear counterstain in either blue (osteoclasts) or red (osteoblasts). Cells were visualized by confocal microscopy. Negative controls were stained with normal rabbit IgG (middle panel). Bar = 20 μ m.

tion and is mediated by another receptor, such as the putative cannabinoid receptor GPR55, or is a non-receptor mediated effect.

CP 55 940 inhibits osteoclast formation – CB₂ mediated

Similar to the effects of endogenous cannabinoids on human osteoclast formation, the synthetic non-selective CB₁/CB₂ agonist CP 55 940 did not significantly affect osteoclast for-

mation at concentrations from 1 nM to 100 nM; however, 1 μ M CP 55 940 significantly reduced osteoclast formation as assessed by measurement of VNR fluorescence (Figure 4A). This decrease in osteoclast formation was not associated with any significant reduction in cell viability (Figure S2C). Human osteoclast formation was not significantly affected in the presence of the CB₂-selective agonist JWH015 (Figure 4B); however, although not significant, JWH015 showed a trend towards an inhibition of osteoclast formation whereby at

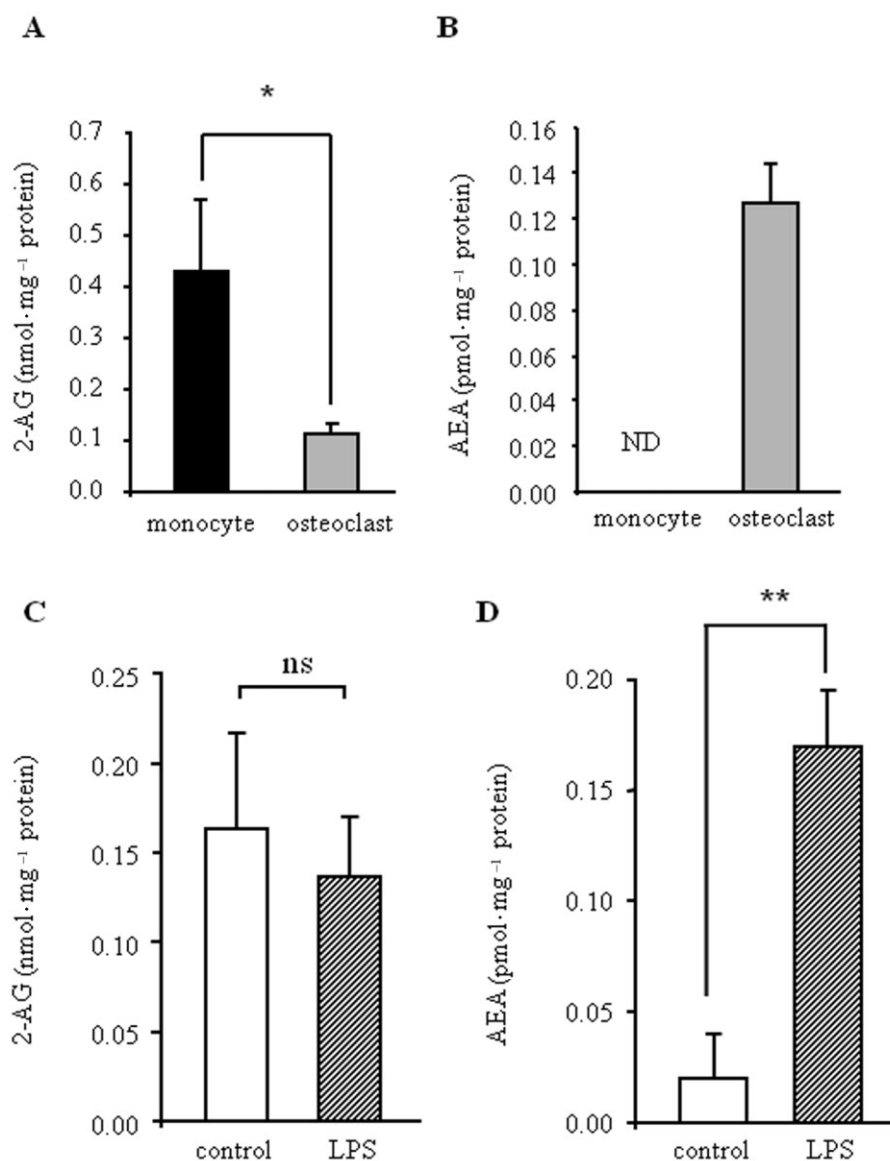


Figure 2

Endocannabinoids are produced by human osteoclasts – levels change during osteoclast differentiation and are influenced by LPS. (A) Detection of 2-AG in human monocytes ($n = 15$ from six separate donors) and osteoclasts ($n = 16$ from six separate donors). Levels in cell extracts were normalized to mg⁻¹ of protein. (B) Detection of AEA in the same samples as in (A). Values represent the mean \pm SEM * $P < 0.05$, Student's unpaired t -test. ND, not detected. Levels of 2-AG (C) and AEA (D) in human osteoclasts following LPS treatment. Cells were washed in PBS and treated for 90 min with 200 μ g·mL⁻¹ LPS. Values represent the mean \pm SEM from three separate donors. Values represent the mean \pm SEM ** $P < 0.01$, Student's unpaired t -test. ns, not significant.

1 μ M VNR fluorescence was reduced to 76.9% of control, again this was not associated with a reduction in cell viability. This may have reached statistical significance at higher concentrations but in order to retain CB₂ receptor selectivity higher concentrations were not used.

To determine the receptor responsible for the modest inhibitory effect of CP 55 940 on human osteoclast formation, M-CSF-dependent mouse bone marrow macrophages from CB₁^{-/-}, CB₂^{-/-} and GPR55^{-/-} mice were stimulated to differentiate into TRAP-positive multinucleated osteoclasts by the addition of M-CSF and RANKL in the presence of either

vehicle or 1 nM–1 μ M CP 55 940. In wild-type cultures 100 nM–1 μ M CP 55 940 significantly decreased mouse osteoclast formation relative to control (Figure 4C). Likewise, in CB₁^{-/-} and GPR55^{-/-} cultures, CP 55 940 significantly decreased mouse osteoclast formation relative to control as determined by a reduction in the number of TRAP-positive osteoclasts with three or more nuclei. The inhibitory effect of CP 55 940 on mouse osteoclast formation was in fact significantly augmented in CB₁^{-/-} cultures, further ruling out the possibility that CB₁ mediates the inhibitory response on osteoclast formation induced by CP 55 940. Notably, in wild-

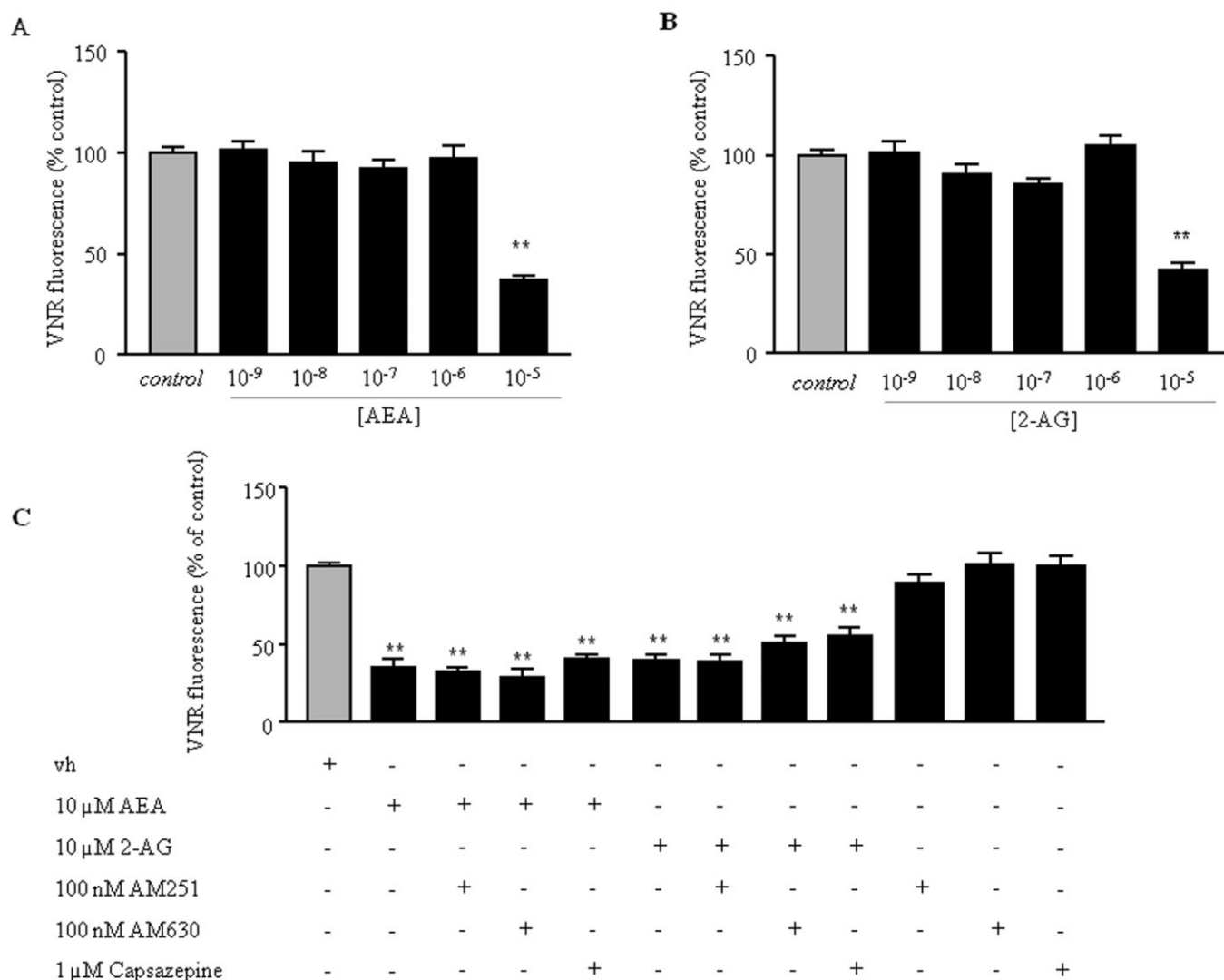


Figure 3

The inhibition of human osteoclast differentiation induced by 10 μM AEA or 2-AG is not mediated by CB₁, CB₂ or TRPV1 receptors. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of RANKL and 1 nM–10 μM AEA (A) or 2-AG (B) for 7 days. (C) Osteoclasts were cultured, as described above, in the presence of 10 μM AEA or 2-AG together with 100 nM AM251, 100 nM AM630 or 1 μM capsazepine. Cells were fixed and stained for the vitronectin receptor and immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean ± SEM; *n* = 3–4 experiments – 4–5 replicates each. ***P* < 0.01 compared with control; one-way ANOVA with Dunnett's post-test.

type, CB₁^{-/-} and GPR55^{-/-} cultures monocytes still appeared to differentiate into TRAP-positive mononuclear cells in the presence of CP 55 940. In CB₂^{-/-} cultures the inhibitory effect of CP 55 940 was absent, implicating CB₂ as the receptor mediating the inhibition of mouse osteoclast formation induced by CP 55 940 *in vitro* – representative images shown in Figure 4D.

In light of the inhibitory effect of CP 55 940 on osteoclast formation, we also examined the effect of cannabinoid receptor antagonists on human osteoclast formation. The CB₂ cannabinoid receptor antagonists SR144528 and AM630 together with the CB₁ receptor antagonist SR141716A did not significantly affect human osteoclast formation; however, the CB₁

antagonist AM251 at 1 μM significantly increased human osteoclast formation relative to control, albeit a modest increase to 134% of control (Figure S3).

AEA stimulates human osteoclast polarization and resorption – CB₂ mediated

The effect of the endocannabinoids on the polarization and resorptive activity of mature human osteoclasts was investigated by the addition of AEA to cultures after osteoclasts had formed on dentine discs in the absence of drugs, in order to mitigate any potential effects on formation. Cultures were terminated approximately 5 days later by fixing with 4% paraformaldehyde after which F-actin ring number, osteo-

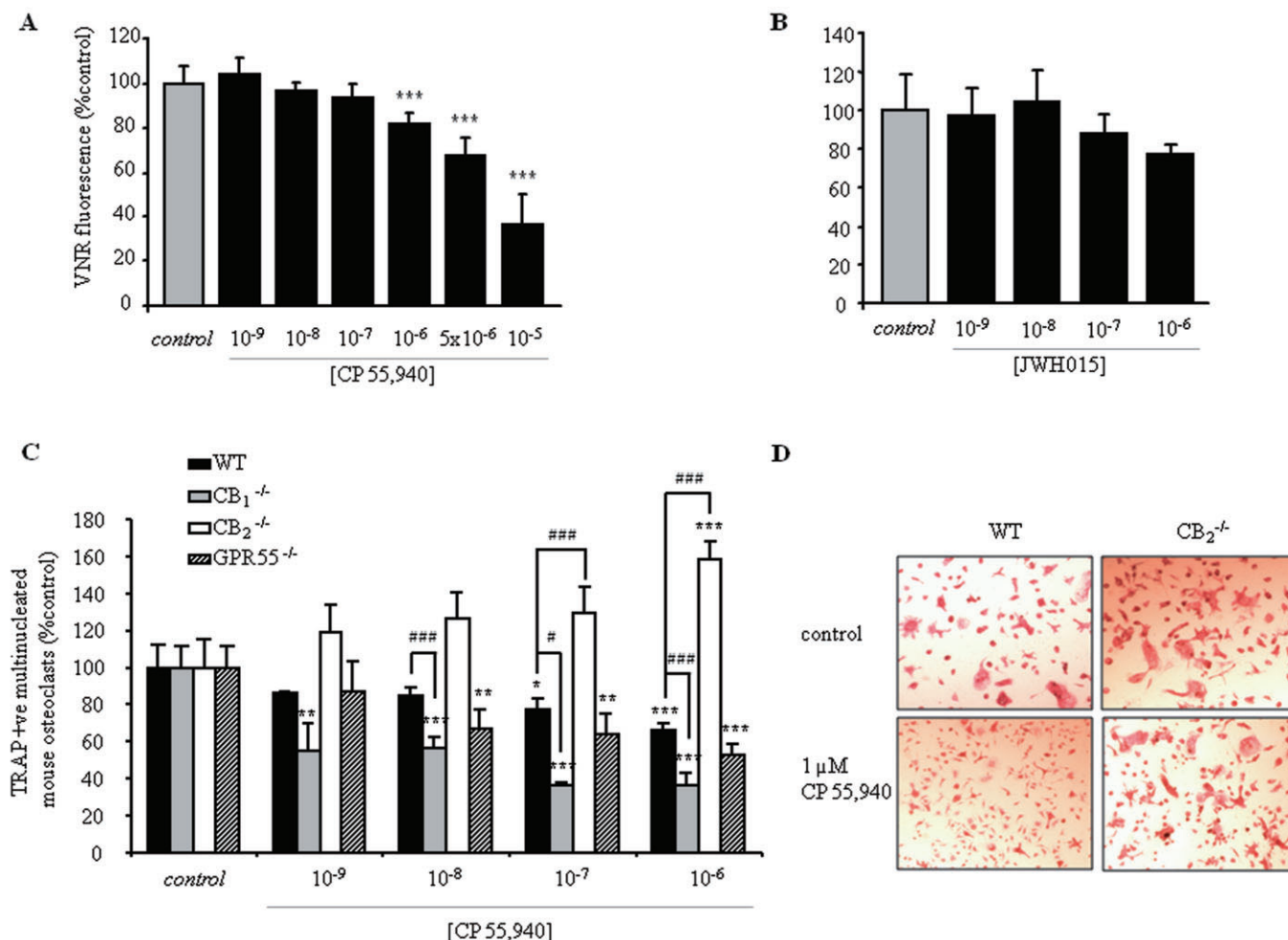


Figure 4

CP 55 940 inhibition of human and mouse osteoclast differentiation is mediated by CB₂ receptors. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of 1 nM–10 μM CP 55 940 (A) or JWH015 (B) for 7 days, assayed for cell viability and then fixed and stained for VNR to quantify osteoclast number. Immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean ± SEM; *n* = 6–7 experiments (CP 55 940) and 4 experiments (JWH015) – 5 replicates each. ****P* < 0.001 one-way ANOVA with Bonferroni post-test. (C) M-CSF-dependent bone marrow macrophages from wild-type (WT), CB₁^{-/-}, CB₂^{-/-} or GPR55^{-/-} mice were cultured in M-CSF and RANKL in the presence of vehicle or 1 nM–1 μM CP 55 940 for 5 days and then fixed and stained for TRAP. The number of TRAP-positive multinucleated osteoclasts were counted and expressed as a percentage of control. Mean ± SEM; *n* = 3–4 experiments – 5 replicates each. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 CP 55 940 compared to control and #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 compared with WT – one-way ANOVA with Bonferroni multiple comparison post-test. (D) Representative images of TRAP positive, multinucleated mouse osteoclasts – note the lack of inhibition of osteoclast formation in the presence of CP 55 940 in CB₂^{-/-} cultures.

clast number and resorption pit area were quantified. F-actin rings are a characteristic cytoskeletal feature of polarized, actively resorbing osteoclasts. The formation of an F-actin ring is essential for osteoclast function and is a quantifiable measure of osteoclast activity. Treatment of human osteoclasts with 100 nM AEA resulted in an increase in the number of F-actin rings to 168 ± 22% of control (Figure 5A) and resorption pit area to 390 ± 100% of control (Figure 5B) – representative images of resorption pits in dentine shown in Figure 5C. Collectively, these data show that the endocannabinoid AEA significantly enhances osteoclast function. Interestingly, treatment of human osteoclasts with AEA in the presence of an inhibitor (URB-597) of FAAH, the major

enzyme for AEA catabolism, did not significantly augment the stimulatory effect on osteoclast polarization and resorption seen in the presence of AEA alone (Figure 5D). To determine the receptor implicated in the stimulating effects of AEA in human osteoclasts, similar experiments were performed in the presence of the CB₁ and CB₂ antagonists SR141716A and SR144528, respectively. A significant attenuation of the stimulating effect of AEA in the presence of SR144528 compared to treatment with 100 nM AEA alone (Figure 5E) suggests that the effect of AEA on human osteoclast function was mediated by CB₂ receptors.

The effect of 2-AG on human osteoclast activity was also investigated. However, there was a high level of inter-donor

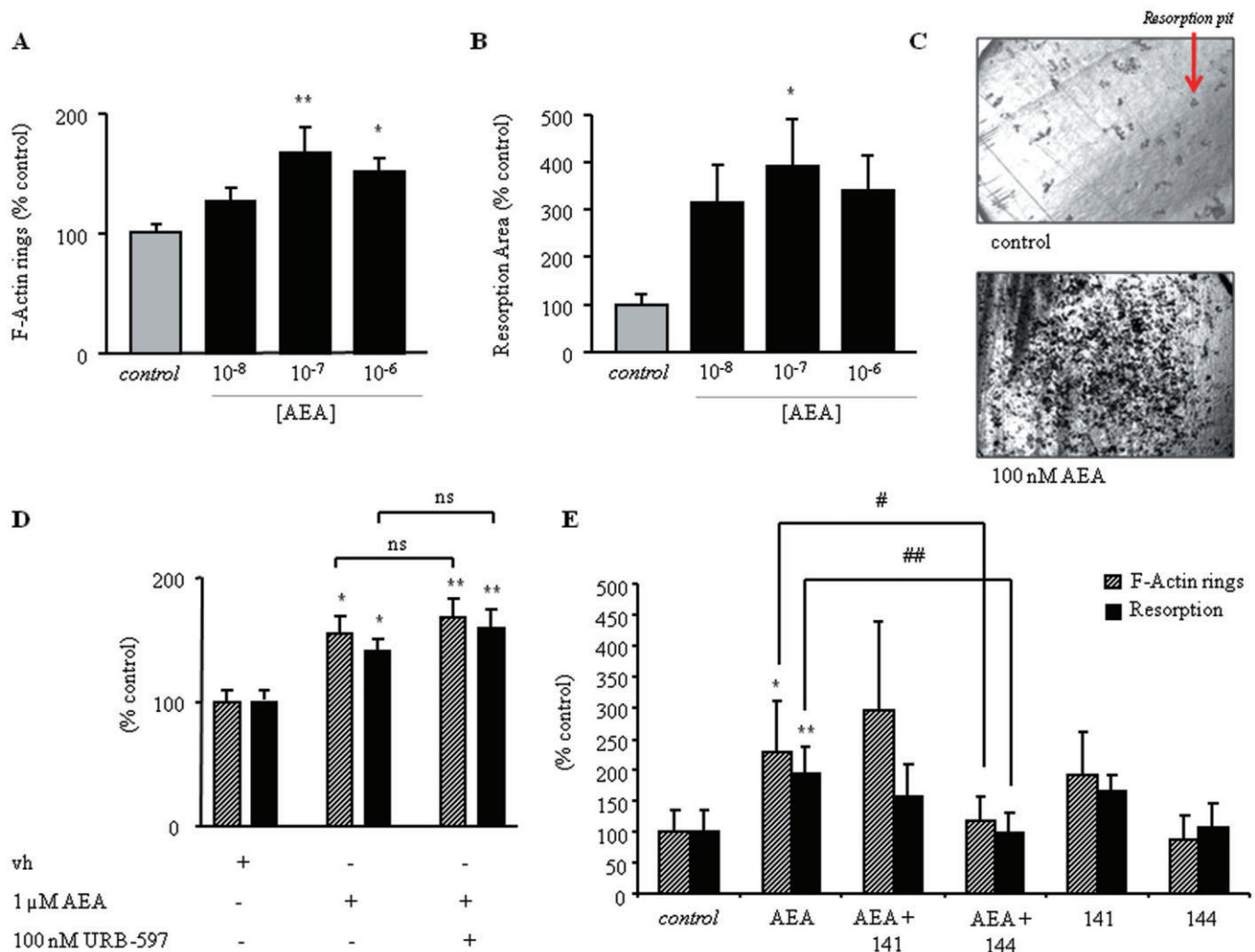


Figure 5

AEA stimulation of the polarization and activity of human osteoclasts *in vitro* is mediated by CB₂ receptors. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured on dentine discs for 7 days and then treated with AEA for a further 3–5 days. (A) Cells were fixed and stained for F-actin and the number of actin rings per disc counted and expressed as a percentage of vehicle (0.1% DMSO) control \pm SEM ($n = 4$ experiments – 5 replicates each). (B) Discs were then cleared and resorption pit area quantified using reflective light microscopy and expressed as a percentage of vehicle control \pm SEM ($n = 3$ experiments – 5 replicates each). * $P < 0.05$, ** $P < 0.01$ compared with control, one-way ANOVA with Dunnett's post-test. (C) Representative images of resorbed dentine discs are shown with areas of resorption shown by black pits (red arrow). Human osteoclasts were cultured as above and treated for a further 3–5 days in the presence of vehicle or 1 μ M AEA in the presence or absence of (D) URB-597 (FAAH inhibitor), (E) the CB₁ antagonist SR141716A (141) or the CB₂ antagonist SR144528 (144). Cells were fixed and the number of F-actin rings per disc quantified. Cells were removed from discs and resorption pit area measured by reflective light microscopy. Results are mean \pm SD (mean of 2 experiments with 4 replicates per experiment) * $P < 0.05$, ** $P < 0.01$ compared with the corresponding vehicle; # $P < 0.05$, ## $P < 0.01$ 100 nM AEA alone compared with 100 nM AEA plus 100 nM 144 – one way ANOVA with Dunnett's multiple comparison post-test. ns, not significant.

variability in the results, preventing accurate analysis of the effect of this endocannabinoid.

The synthetic cannabinoid CP 55 940 stimulates human osteoclast polarization and resorption – mediated by CB₁/CB₂

Consistent with the effects seen with the endogenous cannabinoid AEA, treatment of human osteoclasts with the synthetic agonist CP 55 940 resulted in a significant increase in

the number of F-actin rings (with 100 nM and 1 μ M CP 55 940) (Figure 6A) and resorption area (with 1 nM–1 μ M CP 55 940) relative to control (Figure 6B) – representative images of actin rings and resorption pits in dentine shown in Figure 6C. Treatment of cultures with 1 μ M CP 55 940 in the presence of 100 nM SR141716A or SR144528 resulted in a significant reversal of the increases in F-actin ring number (79% and 82% reduction, respectively) and resorption area (80% and 78% reduction, respectively) seen after treatment with 1 μ M CP 55 940 alone (Figure 6D).

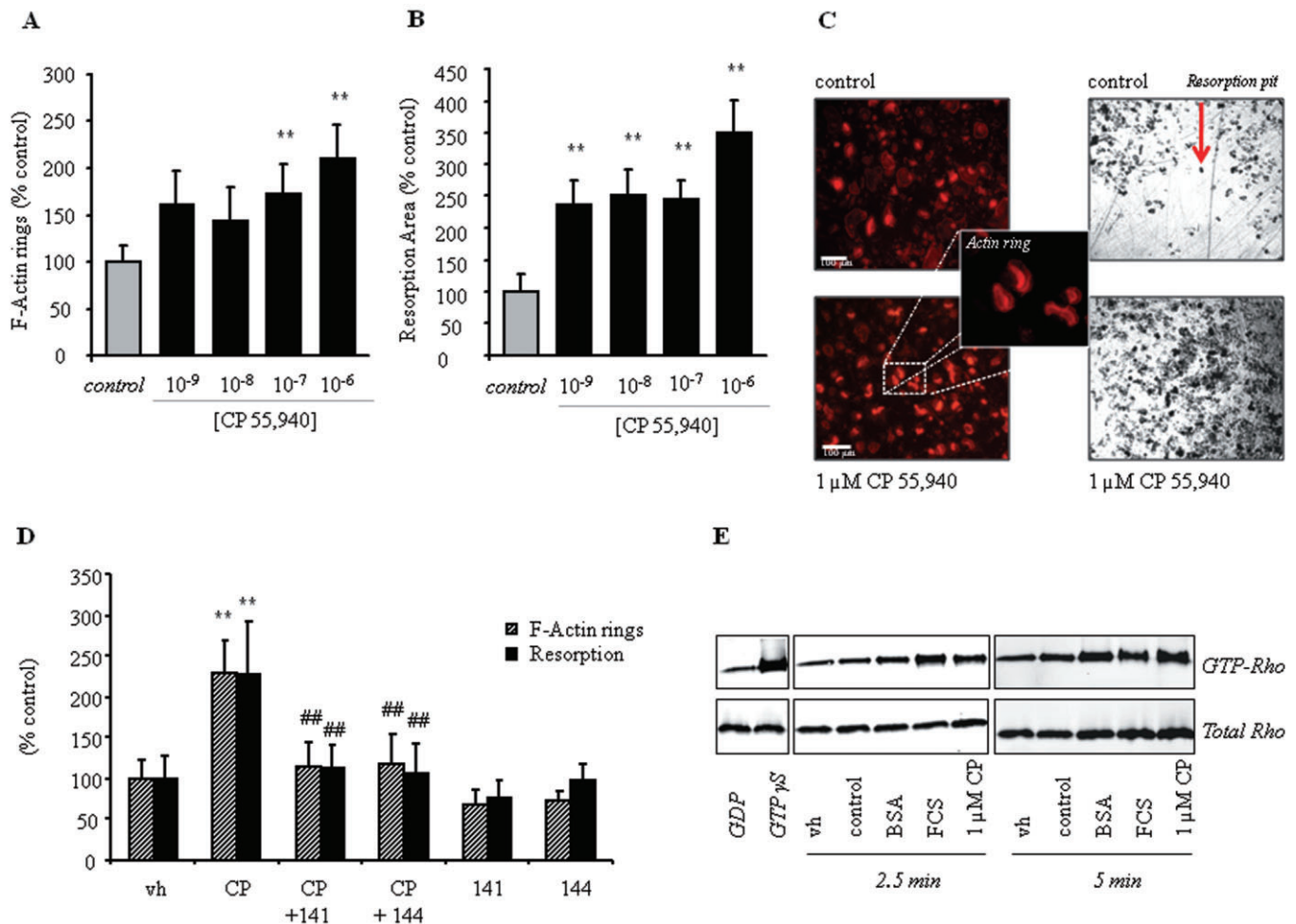


Figure 6

The synthetic agonist CP 55 940 stimulates the polarization and activity of human osteoclasts *in vitro*. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured on dentine discs for 7 days and then treated with CP 55 940 \pm SR141716A (141) or SR144528 (144) for a further 3–5 days. (A) Cells were fixed and stained for F-actin and the number of actin rings per disc counted and expressed as a percentage of vehicle (0.1% DMSO) control \pm SEM ($n = 6$ –7 experiments – 5 replicates each). (B) Discs were then cleaned to remove cells and resorption pit area quantified using reflective light microscopy and expressed as a percentage of vehicle control \pm SEM ($n = 6$ –7 experiments – 5 replicates each). $^{**}P < 0.01$ compared with control, ANOVA with Dunnett's multiple comparison test. (C) Representative images of osteoclast cultures stained to visualize polarized cells with F-actin rings (left and inset) and of dentine discs with resorption pits (right), shown as black pits (red arrow), after treatment with vh or 1 μ M CP 55 940. (D) Human osteoclasts were cultured as above and treated for a further 3–5 days in the presence of CP 55 940 (CP) \pm 141 or 144. Cells were fixed and the number of F-actin rings per disc quantified. Cells were removed from discs and resorption pit area measured by reflective light microscopy. Results are mean \pm SEM ($n = 4$ experiments – 5 replicates each) $^{**}P < 0.01$, 1 μ M CP 55 940 compared with control and $^{##}P < 0.01$, 1 μ M CP 55 940 in the presence of antagonist compared with CP 55 940 alone, one-way ANOVA with Dunnett's multiple comparison post-test. (E) Human osteoclasts were deprived of FCS and cytokines for 18 h before treatment with vehicle (0.1% DMSO), 1 mg·mL⁻¹ BSA, 10% FCS or 1 μ M CP 55 940 for 2.5 or 5 min; FCS is a positive control for Rho activation. Cells were lysed, total fractions removed and GTP samples generated by subjecting the remaining lysates to a pull-down assay using Rho assay reagent containing Rhotekin RBD bound agarose. Total and GTP-bound samples were subject to SDS-PAGE under reducing conditions, transferred to PVDF membranes and probed for Rho before visualization using a LI-COR Infra-red Imager. Blot shown is representative of 2 independent experiments.

The synthetic cannabinoid CP 55 940 stimulates Rho activation in human osteoclasts

Having established an increase in osteoclast polarization and activity with CP 55 940, we next investigated potential signalling responses that may be involved in eliciting these

responses. The small GTPase Rho is known to play a role in cytoskeletal arrangement and osteoclast resorption (Chelliah, 2005). Using a pull-down assay, we showed that treatment of human osteoclasts with CP 55 940 for 2.5 or 5 min caused an increase in levels of active GTP-bound Rho relative to control (Figure 6E).

The CB₂-selective agonist JWH015 stimulates human osteoclast polarization and resorption

Consistent with the effects seen with CP 55 940 and AEA, treatment of human osteoclasts with the CB₂-selective agonist JWH015 increased osteoclast actin ring formation and resorption. Treatment with 100 nM JWH015 caused a highly significant increase in the number of actin rings to $214.5 \pm 42\%$ of control (Figure 7A) and resorption area to $279 \pm 75\%$ of control (Figure 7B) further supporting a role for CB₂ receptor activation stimulating osteoclast function.

Discussion and conclusions

We and others have recently shown that the CB₁ and/or CB₂ receptors are expressed on mouse osteoclasts and osteoblasts (Idris *et al.*, 2005; Ofek *et al.*, 2006; Tam *et al.*, 2006). In this study, both receptors were detected at the mRNA level in human monocytes and fully differentiated osteoclasts, as previously described by Galieue *et al.* (1995) and Rossi *et al.* (2009), respectively. Here we report for the first time a significant decrease in CB₂ mRNA levels during osteoclast differentiation; no such changes were seen in CB₁ mRNA expression during osteoclast differentiation. In line with our findings in human osteoclasts, previous studies have demonstrated that down-regulation of CB₂ is necessary for successful differentiation of B cells (Carayon *et al.*, 1998) and of myeloid precursors into neutrophils (Jorda *et al.*, 2003). It would appear that the down-regulation of CB₂ is also necessary for the initiation of osteoclast differentiation.

N-linked glycosylation is the most common post-translational modification of GPCRs that correlates to the localization and function of receptors. Studies in the rat brain have shown that the CB₁ receptor contains three glycosylation sites, two of which are actually glycosylated. The majority of CB₁ receptors found in the brain are in the mature glycosylated form (65–80%). The molecular weight of mature glycosylated CB₁ is 64 kDa (Song and Howlett, 1995), this is in line with the band detected in both osteoblasts and osteoclasts by Western blotting in this study. The predicted molecular weight of the non-glycosylated CB₁ receptor is 53 kDa (Pettit *et al.*, 1998). The non-glycosylated form is thought to be produced as a result of the receptor either escaping co-translational glycosylation or being trimmed of carbohydrate chains by exo- and endoglycosidases, thus different cells with differences in their carbohydrate processing may give rise to different molecular weight species producing a shift in size on a Western blot and this may account for the different molecular weight bands consistently produced when probing for CB₁ by Western blotting in bone cells in this study. Here we have shown that osteoclasts and osteoblasts appear to express CB₁ mainly in the non-glycosylated form (stronger 53 kDa band compared with 63 kDa band), whereas the brain, as has been shown by others, contains high levels of the glycosylated 64 kDa form and lower expression of the non-glycosylated/partly glycosylated forms (Song and Howlett, 1995). The role of CB₁ receptor glycosylation in ligand binding, activation and trafficking is not known (Song and Howlett, 1995) and as such the relevance of this finding in bone cells remains to be determined.

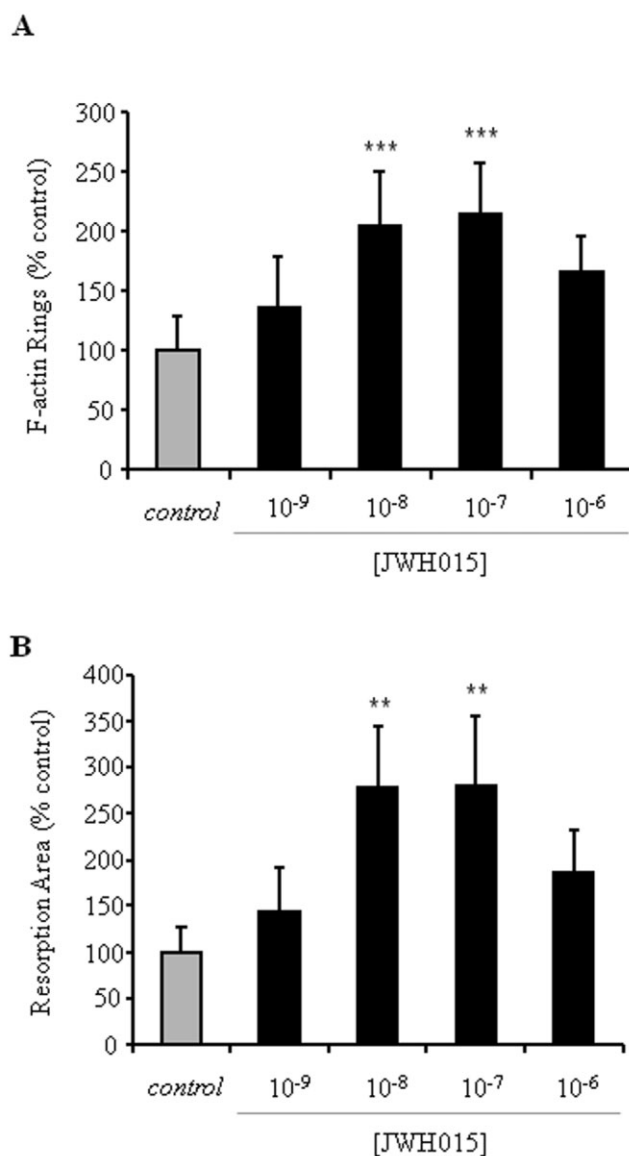


Figure 7

The CB₂-selective agonist JWH015 stimulates the polarization and activity of human osteoclasts *in vitro*. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured on dentine discs for 7 days and then treated with JWH015 for a further 3–5 days. (A) Cells were fixed and stained for F-actin and the number of actin rings per disc counted and expressed as a percentage of vehicle (0.1% DMSO) control. (B) Discs were then cleaned to remove cells and resorption pit area quantified using reflective light microscopy and expressed as a percentage of vehicle control. Mean \pm SEM ($n = 4$ experiments – 5 replicates each). *** $P < 0.01$ compared with control, ANOVA with Dunnett's post-test.

Previous studies have detected AEA and 2-AG in human osteoclasts (Rossi *et al.*, 2009), whole bone (Tam *et al.*, 2008) and mouse osteoblast-like cells (Bab *et al.*, 2008). Using LC-MS/MS, we demonstrate the basal production of 2-AG and AEA in human monocytes and osteoclasts, with 2-AG levels decreasing, and AEA levels increasing during the differentia-

tion of M-CSF-dependent mononuclear cells into multinucleated osteoclasts. The present study is the first to show that the levels of these endogenous ligands are altered during the differentiation of osteoclasts from monocyte precursors. The significance of this decrease in 2-AG levels and the increase in AEA levels during osteoclast differentiation, together with the decrease in CB₂ receptor expression remains to be determined but suggests a dynamic regulation of the endocannabinoid system in osteoclastogenesis. However, in the present study, exogenously added cannabinoids had little or no effect on human osteoclast formation at concentrations up to 1 μ M and the inhibition of osteoclast differentiation observed at 10 μ M of the cannabinoids appears not to be mediated by CB₁, CB₂ or TRPV1. Indeed one would expect AEA to be active at CB₁ and CB₂ receptors in the nM range. There is a possibility that the compounds act via GPR55 (Whyte *et al.*, 2009); however, the ability of the endocannabinoids to activate GPR55 remains controversial (Ross, 2009; Sharir and Abood, 2010). The apparent lack of effect of exogenously added cannabinoids on human osteoclast formation may reflect complex temporal and spatial regulation of the endocannabinoid system controlling human osteoclast differentiation that is not readily modulated by exogenously added agonists. Indeed, the high levels of endogenous AEA present in mature osteoclasts (Figure 1) may preclude an effect of exogenously added compound. In line with these observations, human osteoclasts express both FAAH and NAPE-PLD (Rossi *et al.*, 2009). Interestingly, the CB₂ receptor antagonist, AM630 at 10 μ M has previously been shown to increase human osteoclast formation (Rossi *et al.*, 2009), which is in line with a decrease in CB₂ agonism/expression, which necessarily accompanies osteoclast formation. The profile is different in mouse osteoclasts where direct agonism of CB₂ appears to modify osteoclast formation *in vitro*. In line with previous studies in murine osteoclasts (Ofek *et al.*, 2006), here we show that nM concentrations of CP 55 940 significantly inhibit mouse osteoclast formation; this effect is absent in CB₂^{-/-} mice. This is in accord with the increased osteoclast number seen in CB₂^{-/-} mice (Ofek *et al.*, 2006). Interestingly, in cultures from CB₂^{-/-} mice, CP 55 940 induces an increase in osteoclast formation and in CB₁^{-/-} mice the inhibitory effect of CP 55 940 is enhanced; these data suggest that in mouse osteoclasts CB₁ agonism may increase mouse osteoclast formation. These observations further highlight differences in the effects of exogenously added cannabinoids on human and mouse osteoclast formation *in vitro*.

To date, we are not aware of any studies demonstrating the effect of cannabinoid ligands on human osteoclast function. In order to study human osteoclast resorption independently of osteoclast formation, cells were treated with cannabinoids once osteoclasts had formed and the first signs of resorption were visible on spare discs of dentine. This method eliminates any effect on osteoclast formation that would then influence overall resorption. At low concentrations (1–100 nM), that had no effect on human osteoclast formation, CP 55 940 (non-selective CB₁/CB₂ agonist) and JWH015 (CB₂-selective agonist) stimulated human osteoclast polarization and resorption. The stimulating effect of CP 55 940 on osteoclast polarization and resorption was attenuated by both the CB₁-selective antagonist SR141716A and the CB₂-selective antagonist SR144528, possibly implicating a

role for both CB₁ and CB₂ receptors. In this study, the levels of active, GTP-bound Rho were investigated in osteoclasts by use of a Rho pull-down assay whereby CP 55 940 significantly increased the levels of GTP-bound Rho compared with vehicle control. Similarly, the endogenous cannabinoid AEA, levels of which are increased in mature osteoclasts (Figure 2), stimulates human osteoclast polarization and resorption at nM concentrations. The increase in osteoclast polarization in the presence of AEA is consistent with its known ability to stimulate pathways inducing actin polymerization (Gokoh *et al.*, 2005), involving c-src and Rho GTPase, which are also known to be essential for osteoclast function (Saltel *et al.*, 2004). The stimulating effect of AEA appears to be CB₂-receptor mediated. It is also notable that the pharmacological profile of the antagonists used is such that they are receptor subtype selective but not entirely specific; both compounds have affinity for CB₁ and CB₂ receptors and may interact with novel cannabinoid receptors (Ryberg *et al.*, 2007). Indeed, we have demonstrated a role of GPR55 in osteoclast physiology (Whyte *et al.*, 2009). While AEA had a robust, reproducible effect on human osteoclasts function, the effect of 2-AG could not be clearly established in this study; there was a high level of inter-donor variability in the results, preventing accurate analysis of the effect of this endocannabinoid. This variability may indeed reflect the observation of CB₂ single nucleotide polymorphisms known to affect bone physiology in humans and is the subject of ongoing investigations (Karsak *et al.*, 2005; 2009; Yamada *et al.*, 2007). It is also notable that the inhibition of osteoclast formation observed at 10 μ M 2-AG (Figure 3) is not subject to inter-donor variability, perhaps further evidence of the non-receptor mediated nature of this effect.

Our finding that LPS increased the production of osteoclast-stimulating AEA from osteoclasts, showing that the levels of these endogenous ligands are subject to modulation by known osteogenic factors, supports the relevance of endocannabinoid production by bone cells and its local effect on osteoclasts. LPS is a major bacterial endotoxin that stimulates the production of 2-AG and/or AEA in macrophages (Varga *et al.*, 1998; Di Marzo *et al.*, 1999; Liu *et al.*, 2003). We found that treatment of human osteoclasts with LPS caused an increase in the levels of AEA but did not cause any detectable change in 2-AG levels. LPS is a potent activator of osteoclasts and promotes osteoclast survival (Suda *et al.*, 2002). Given that LPS has been shown to cause inflammatory bone loss in diseases characterized by increased osteoclast activity, such as periodontal disease (Nair *et al.*, 1996; Abu-Amer *et al.*, 1997), it is possible that this may be due in part to the local production of AEA, causing an autocrine stimulation of osteoclastic bone resorption.

In conclusion, in this study we demonstrated a dynamic regulation of the expression of cannabinoid receptors and production of endocannabinoids during human osteoclast differentiation. Crucially we demonstrated for the first time that cannabinoid agonists have profound effects on human osteoclast function *in vitro*; both synthetic and endogenous cannabinoids significantly increased human osteoclast polarization and resorption and the stimulating effect of AEA on human osteoclast resorption was attenuated by CB₂ receptor antagonists. Furthermore, as pro-inflammatory agents such as LPS can modulate endocannabinoid production, our find-

ings suggest that these endogenous lipid mediators may exert a local influence on bone turnover by stimulating bone resorption particularly during inflammation. It is notable that CB₂^{-/-} mice display age-related bone loss. Our data suggest that, in contrast to observations in mouse, cannabinoids do not affect human osteoclast formation at physiologically relevant concentrations, but rather stimulate human osteoclast activation. Hence, there may be important species differences in the role and function of CB receptors in bone physiology. Our observations are also important in the context of the previous demonstrations that there are significant associations between CB₂ receptor genotypes and osteoporosis in humans (Karsak *et al.*, 2005; 2009; Yamada *et al.*, 2007). The consequences of these polymorphisms to either the under/over activation or expression of the CB₂ receptor have yet to be elucidated. Our findings indicate that small molecules targeting the endocannabinoid system, in particular CB₂ receptor antagonists, may act as novel anti-resorptive therapeutics in the treatment of human bone disease such as post-menopausal osteoporosis where excessive osteoclastic resorption contributes to the pathology of the disease.

Acknowledgements

This work was funded by Allergan Inc. and The Nuffield Foundation Oliver Bird Rheumatism Programme.

Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 High throughput quantification of VNR fluorescence using a plate reader as a measure of osteoclast formation. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of M-CSF, M-CSF and RANKL or M-CSF, RANKL and 10 ng·mL⁻¹ TGF β for 7 days. Cells were fixed and stained for the vitronectin receptor and immunofluorescence intensity measured as an indication of osteoclast number (A). Results are expressed as a percentage of M-CSF and RANKL treated cells – mean \pm SEM; n = 4–8 experiments – 5 replicates each. *** P < 0.001; Student's t -test. (B) Comparison of osteoclast number determined by counting versus plate reader VNR fluorescence. (C) Representative images of osteoclasts formed in the presence of M-CSF alone, M-CSF and RANKL or M-CSF, RANKL and TGF β are shown with vitronectin receptor in (green) and nuclei (blue).

Figure S2 Anandamide, 2-AG and CP 55 940 do not have a toxic effect on osteoclast precursors. M-CSF-dependent human PBMCs were cultured in the presence of RANKL and treated with anandamide (A), 2-AG (B) or CP 55 940 (C) for 7 days, after which an AlamarBlue (Invitrogen) assay was performed to assess the number of viable cells. Results are expressed as mean \pm SEM (mean of 4–7 experiments with 5 replicates per experiment).

Figure S3 Cannabinoid receptor antagonist AM251 increases human osteoclast formation. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of vh (control), 10 ng·mL⁻¹ TGF β (positive control) or cannabinoid receptor antagonists SR141716A (141), SR144528 (144), AM251 and AM630 for 7 days and then fixed and stained for VNR. Immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean \pm SEM; n = 4 experiments – 5 replicates each. ** P < 0.01, *** P < 0.001 compared with control – one way ANOVA with Bonferroni post-test.

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