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# The Role of Type-2 Cannabinoid Receptors in Calcification of Atherosclerotic Lesions.

Kaitlyn Hinshaw
*East Tennessee State University*

# The Role of Type-2 Cannabinoid Receptors in Calcification of Atherosclerotic Lesions

Thesis submitted in fulfillment of Honors

By

Kaitlyn Hinshaw

The Honors College

University Honors Program

East Tennessee State University

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Douglas Thewke, Faculty Mentor

Antonio Rusiñol, Reader

Frank Hagelberg, Reader

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# Introduction

## Atherosclerosis

Atherosclerosis is a chronic vascular inflammatory disease characterized by the formation of lesions in the arterial wall of medium to large arteries. These raised lesions cause narrowing of the vessel and are often calcified. Atherosclerotic lesions present serious health risks and investigation into both their causes and consequences is ongoing. Lesion formation begins when monocytes, a type of white blood cell, adhere to areas of vascular injury. Vascular injury occurs when the inner endothelial layer of the vessel is damaged and broken by turbulent blood flow. The monocytes migrate into the intima, the area between the endothelium and smooth muscle cells of the vascular wall. There the monocytes proliferate and differentiate into macrophages. The primary function of these macrophages is to ingest atherogenic lipoproteins that have inadvertently entered the vessel wall. These lipoproteins enter through the same injured areas that the monocytes adhere to and include low density lipoproteins (LDL), carrier of the "bad cholesterol." LDL is oxidized when it enters the vascular wall to produce oxidized LDL (oxLDL). OxLDL causes damage to the cells in vessel walls, so the macrophages digest these molecules to remove the agents and prevent damage. Ingestion of oxLDL induces macrophage apoptosis, or programmed cell death.

The buildup of cellular debris from apoptosis and other contaminants in the vessel wall forms the atherosclerotic lesion. Despite this, macrophage apoptosis has been shown to play a protective role in the initial stages of lesion formation [1]. Clearance of apoptotic cells initially slows the growth of the plaque by removing cell debris and some of the dangerous lipoproteins. Advanced lesion macrophage apoptosis is a pro-atherosclerotic process. In later stages,

apoptosis contributes to the instability and rupture of plaques [2]. Ruptured plaques can cause a multitude of issues including causing blockages and exposing thrombogenic material within the plaque that leads to blood clotting. Very advanced atherosclerotic lesions are also often calcified for reasons that remain unclear. Calcification of coronary arteries is a well-established risk factor and a predictor of future cardiac events [3, 4], and the measure of calcified lesions is independently associated with cardiovascular disease events and total mortality [5,6]. The process of lesion calcification has been shown to be similar to mechanisms of embryonic bone formation [7-9], and is suspected to be at least partially controlled by the type-2 cannabinoid receptor (CB2).

Type-2 Cannabinoid Receptor

Two distinct types of cannabinoid receptors function in mammals. The first, the CB1 receptor, is most prevalent in the central nervous system where it controls the neurological and psychotropic effects of cannabinoids [10]. CB2, primarily expressed in immune cells, exerts the immunosuppressive effects of cannabinoids [11, 12]. The CB2 receptor has been shown to modulate several macrophage processes associated with atherosclerosis including the susceptibility to oxLDL-induced apoptosis [13]. While CB2 receptor deficiency did not affect the size of lesions in mice, it did increase macrophage accumulation and infiltration of smooth muscle cells as well as alter the extracellular matrix composition of the lesions [11]. It is possible that CB2 alters the extracellular matrix in part by controlling the formation of osteoclasts and osteoblasts in the lesion.

The osteoclast is a specialized cell type derived from a monocyte-macrophage lineage that functions to resorb and degrade bone [14]. Fibroblast-like osteoblasts are the primary

controllers of bone formation, opposing the osteoclasts [15]. Both CB1 and CB2 receptors have been found in the skeleton in proximity to these bone cells. CB1 is primarily expressed at neural junctions near osteoblasts and preosteoblasts, cells destined to become osteoblasts, [16-18] while CB2 has been found within osteoblasts and their committed precursors. CB2, as mentioned above, affects bone formation, and potentially lesion calcification, as CB2-deficient mice show bone density abnormalities and altered lesion characteristics. In the first two to three months of life CB2-null mice, grow normally and display a normal bone mass, but later they display a drastically enhanced age-related bone loss [17]. CB2-deficient mice have greatly increased osteoclast formation and a much higher rate of bone loss. Thus, CB2 probably functions as a repressor for osteoclastogenesis during bone remodeling processes.

Atherosclerotic Lesion Calcification

Lesion calcification is a cell driven process that appears to be similar to bone formation with osteoclast-like cells and osteoblast-like cells being extremely important markers in advanced or calcified atherosclerotic lesions. The differentiation of cells into osteoclasts is controlled primarily by levels of osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B ligand (RANKL) [19]. OPG acts as a decoy receptor for RANKL, preventing binding to its receptor (RANK) and differentiation of cells into osteoclasts. RANKL and its receptor are both present in osteoclasts and their precursors [19] and are expressed in the osteoclast-like cells in advanced atherosclerotic lesions. RANKL can induce osteoclastogenesis in monocyte/macrophage cell lines *in vitro* [20]. Tartrate-resistant acid phosphatase (TRAP) is produced by osteoclasts, but not macrophage lineage precursors, so TRAP expression and activity are markers of osteoclastogenesis. Osteoclasts or osteoclast-like cells have been

detected in advanced lesions using TRAP as marker [21]. High levels of TRAP have been found near RANKL-positive lesion areas [21].

RANKL is produced by smooth muscle cells in the vascular wall in response to oxidative stress from molecules like oxLDL. However, RANKL does not cause vascular smooth muscle cell (VSMC) calcification and is not required for oxidative stress-induced hardening. Rather, oxidative stress-stimulated smooth muscle cells promote migration of macrophages into a lesion in connection with RANKL. Instead of contributing directly to calcification of smooth muscle cells, RANKL recruits macrophages which then differentiate into osteoclast-like cells in the lesions based on OPG/RANKL levels [21]. Osteoclastic cells are produced in atherosclerotic lesions when RANKL from modified smooth muscle cells activates the initiation of osteoclastogenesis. The master osteogenic transcription factor Runx2 is an essential regulator in this pathway producing RANKL because it directly binds to the promoter and induces the expression of RANKL in VMSCs [21].

Oxidative stress of vascular smooth muscle cells can also cause osteoblastogenesis from VMSC precursors. *In vitro* studies have shown that hydrogen peroxide ($H_2O_2$) can induce VMSC calcification [22]. Vascular calcification of arteries reflects osteochondrogenic transformation of vascular smooth muscle cells, which is associated with increased expression of growth factors, matrix proteins and other bone-related markers [23-25]. Therefore, osteoblasts are also an important marker in advanced atherosclerotic lesions. Osteoblastic activity can be detected both by calcium deposition, since osteoblasts are calcium-depositing cells, and by alkaline phosphatase (ALP) activity. ALP can be detected using a similar assay to the one used to determine TRAP activity in osteoclasts and is a good indicator for osteoblastogenesis *in vitro* because osteoblasts and their committed precursors produce it in significant quantities while

smooth muscle cells do not. Lesion calcification occurs when osteoblasts outnumber osteoclasts causing the deposition rate to exceed the rate of calcium uptake.

Justification and Objective

The overall aim of this research is to determine CB2-dependent effects on atherosclerotic lesions. Previous studies indicate that CB2 receptors play an integral role in the activation of osteogenesis by modulating the expression of osteogenic genes during bone remodeling [20, 21] and work from our laboratory recently demonstrated that CB2 receptors modulate cellular and molecular processes within atherosclerotic lesions in a murine model of atherosclerosis [11]. This suggests that CB2 receptors may play an integral role in the calcification of atherosclerotic lesions. Though previous studies have examined the CB2-mediated effects on osteogenesis in relation to bone formation [16], there has been no investigation of the effects of CB2-signaling on lesion calcification. By increasing our understanding of the role CB2 performs in lesion calcification, it may be possible to design treatments, which effectively target CB2 and slow the calcification of atherosclerotic lesions. We hypothesize that CB2 modulates osteogenic processes within lesions to reduce osteoclastogenesis and activate osteoblastogenesis, thus promoting lesion calcification. To begin to test this hypothesis, we investigated the effects of CB2 signaling on murine osteogenic cell lines *in vitro*. The effects of CB2 agonists and antagonists on osteoclastogenesis in a murine monocyte/macrophage cell line and osteoblastogenesis in a murine smooth muscle cell line were studied.

# Materials and Methods

Cell Culture and Reagents

RAW 264.7 cells (transformed murine monocytic cell line) and MOVAS-1 cells (transformed murine vascular smooth muscle cell line) were purchased from the American type culture collection (ATCC, VA, USA). RAW 264.7 cells were cultured to confluence in 10 cm plates in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, CA, USA) containing 4.5 g/L D-Glucose, L-glutamine, and 110 mg/L sodium pyruvate, with the addition of 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (10,000 U/mL; Invitrogen, USA) [26]. MOVAS-1 cells were maintained in modified DMEM (ATCC #30-2002) containing 4.5 g/L D-Glucose, 1.5 g/L sodium bicarbonate and supplemented with 10% FBS, 0.2 mg/ml G-418, 100U/ml penicillin, and 100 μg/ml streptomycin in a 10% $CO_2$ / 95% air humidified atmosphere [26].

Agonists used in this study were Win55,212-2, a mixed CB1/CB2 agonist, and HU308, a CB2-selective agonist [27,28]. Win55,212-2 is a synthetic CB1/CB2 mixed agonist that has a slightly higher binding affinity for CB2 ($K_i$= 3.3 nM) than for CB1 ($K_i$= 62.3 nM). HU-308 is a synthetic CB2-selective agonist with a high CB2 binding affinity ($K_i$= 20 nM and > 10 μM for CB2 and CB1, respectively). Antagonists used in this experiment were SR144528, a CB2-selective antagonist, and AM251, a CB1-selective antagonist [29,30]. AM251 is a synthetic cannabinoid that acts as an antagonist of CB1 ($K_i$= 7.5 nM). SR144528 is a synthetic antagonist with a high affinity ($K_i$= 8.2 nM). Win55,212-2, AM251 and HU-308 were purchased from Cayman Chemical company (Ann Arbor MI, USA). SR144528 was a kind gift from Sanofi-Aventis R&D, Montpellier, France. Unless specified, all other reagents were obtained from

either Fisher Scientific (Pittsburg, PA) or Sigma-Aldrich (St. Louis, MO) and were of the highest quality available.

Generation of Osteoclasts from RAW264.7 Cells

RAW264.7 is a preosteoclast cell line that undergoes RANKL-induced differentiation into osteoclast-like cells [26]. Raw 264.7 cells were harvested from confluent plates by scraping and suspended in 15 mL of culture medium. The number of cells in the suspension was determined using a Bright-Line Hemocytometer (Hausser Scientific, PA, USA), and 96-well culture plates (Corning) were seeded at a concentration of $2 \times 10^3$cells/well for TRAP activity assay, or in 8-chambered slides for TRAP staining. The cells were placed in a $CO_2$ incubator overnight to allow the cells to attach to the surface. After 24 h, the culture medium was replaced with fresh media containing 50 ng/mL RANKL (PeproTech, NJ, USA) and various CB2 agonists and antagonists. All compounds were added directly to culture media from sterile stock solutions prepared in DMSO. Controls received an equivalent volume of the vehicle (DMSO) alone. Two controls were included for each experiment. The first contained media only and the second contained media supplemented with 50 ng/mL RANKL alone. The cells were then incubated for four days after which osteoclastogenesis was measured as follows.

*Method A*: TRAP Activity Assay

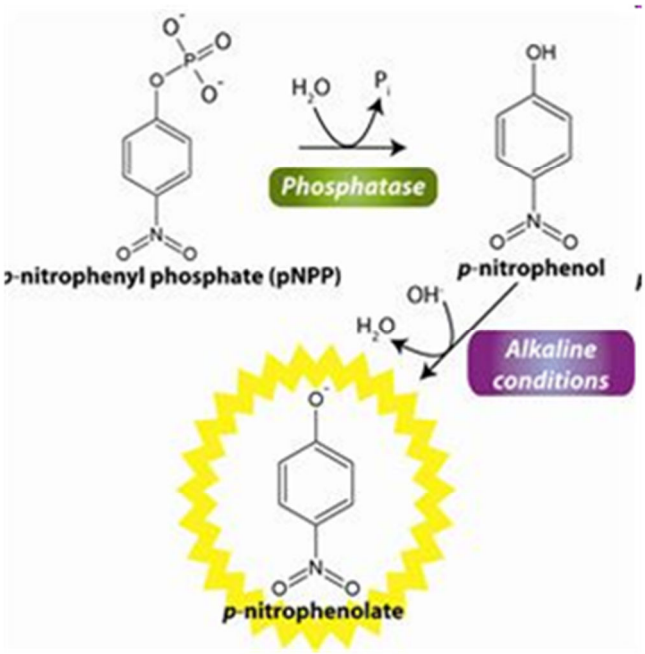


**Figure 1. Reaction Mechanism for TRAP Assay** The TRAP produced by osteoclasts and osteoclast-like cells catalyzes a reaction where p-nitrophenyl phosphate is converted to p-nitrophenol. In basic conditions, p-nitrophenol loses a hydroxyl group and becomes a yellow compound, p-nitrophenolate, can be measured by spectrophotometry. TRAP staining uses a principle similar to the TRAP assay, but pNPP is soluble and will not stain cells. Instead, napthol AS-B1 phosphoric acid is used. When hydrolyzed by TRAP, naphthol AS-B1 phosphoric acid couples with a dye (Fast garnet GBC) and produces a reddish precipitate at the site of enzyme activity.

TRAP activity was used to measure osteoclastogenesis by the method depicted in Figure 1. Briefly, the medium was aspirated and the cells were washed with 100 μL saline (0.9% NaCl). The cells were then lysed with 100 μL 0.2% Triton X-100 in water (v/v) and incubated at room temperature for ten minutes. Fifty microliters of the lysate from each well was transferred to a 96-well spectrophotometric microplate. Substrate solution (50 μL) made up of 0.1M HEPES (pH 5.2) containing 50 mM Na-tartrate (an inhibitor of non-tartrate resistant acid phosphatase) and 4.8 mg/mL p-nitrophenyl phosphate (pNPP) was added to each well. Blank wells were made by combining 50 μL of lysis solution with 50 μL of substrate solution. The plate was incubated at 37°C with gentle shaking

for 30 to 60 minutes. Fifty microliters of stop solution (0.5 M NaOH) was added to each well to stop the reaction and produce the yellow p-nitrophenolate. The intensity of the yellow color, and therefore TRAP activity, was measured using a SpectraMax microplate reader set at 405 nm (Molecular Devices). The protein concentration of the lysates was determined by performing a micro-BCA assay according to the manufacturer's instructions (Pierce, Rockford, IL) for the purposes of normalizing TRAP activity. The TRAP activity was calculated as the $A_{405}$/mg protein for each sample and presented as the mean ±SD of quadruplicate treatments or as the fold induction of TRAP activity compared to the mean of untreated controls.

*Method B:* TRAP Staining

Osteoclast activity in cultured cells was visualized by TRAP staining using a Leukocyte Acid Phosphatase TRAP Kit from Sigma-Aldrich (St. Louis, MO, USA). The staining was performed according to manufacturer's instructions and osteoclasts were visualized by light microscopy using an Olympus BX41 equipped with a CCD digital camera. Osteoclasts were defined as TRAP-positive cells with three or more nuclei. The numbers of osteoclasts in four random microscopic fields (20X magnification) were quantified and the data presented as the mean ±SD for each condition.

Generation of Osteoblasts from MOVAS-1 Cells

MOVAS-1 cells were seeded at 10,000 cells per well in12-well plates in standard growth media. After one day of incubation, the standard media was replaced with osteogenic media (standard media supplemented with 50 μg/mL L-ascorbic acid and 2.5 mM β-glycerophosphate) or osteogenic media supplemented with the desired concentration of CB2 agonists and

antagonists. Cells were incubated for 21-28 days with the media changed every second or third day before they were screened for osteoblastogenesis. Osteoblastogenesis was determined through two methods.

*Method A*: Alkaline Phosphatase (ALP) Activity Assay

ALP activity of osteoblasts and committed precursors was measured using a method similar to that used to measure TRAP activity in osteoclastogenesis. Cells were rinsed three times with saline and then lysed using 0.2% TritonX-100. The mixture was clarified of cellular debris by centrifugation at 12,000 x g for 15 min at 4°C. Ten microliters of supernatant from each sample was placed in a well of a 96-well spectrophotometric plate reader plate containing 40 μL of lysis buffer. Fifty μL of substrate solution (0.2 M Tris-HCl, pH 9.5, 1 mM $MgCl_2$ and 4.8 mg/mL pNPP) was added to each well followed by incubation for 30 min at 37°C. Blanks were made using 50 μL of lysis buffer and 50 μL of substrate solution. The reaction was stopped by the addition of 50 μL of stop solution (0.5M NaOH) and the plates were read using a spectrophotometer at 405 nm. The protein concentration of the lysate was determined using a microBCA protein assay as described above. ALP activity was calculated as the $A_{405}$/mg protein for each sample and presented as the mean ±SD of triplicate treatments or as fold-induction compared to the mean of untreated controls.

*Method B*: Alizarin red staining for calcium deposition.

Calcification of MOVAS-1 cells was determined by Alizarin red staining as described by Byon et. al [21]. Briefly, the cell monolayers were rinsed with PBS and fixed with 4% paraformaldehyde for 5 min at 4°C. The cells were then stained with 2% Alizarin red

(pH 4.2) for 5 min at room temperature. After rinsing 5 times with distilled water and digitally photographed the alizarin red was extracted with 10% cetylpyridinium chloride for 10 minutes and the absorbance at 562 nm was determined using a microplate reading spectrophotometer. The protein concentration was measured by micro-BCA assay as described above and the data represented as $A_{562}$/mg protein.

# Results

Role of CB2 in Osteoclastogenesis

It was found that treatment of RAW264.7 cells with CB2 agonists reduced TRAP activity and the number of multi-nucleate TRAP-positive cells in culture. Co-treatment of cells with a CB2 antagonist countered the inhibitory effects of agonists, indicating that CB2 signaling has an inhibitory effect on osteoclastogenesis of RAW264.7 cells.

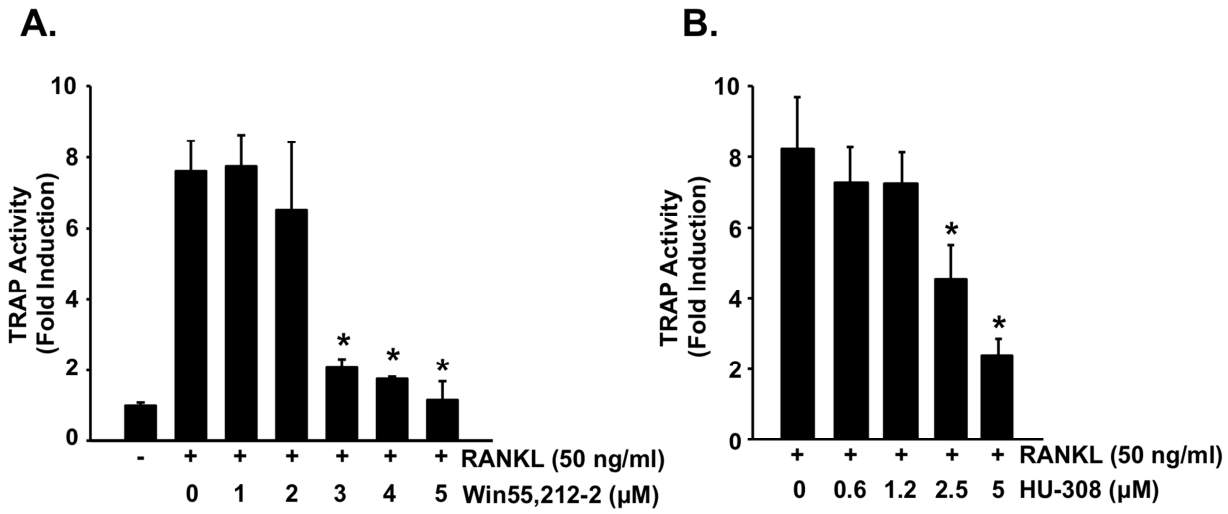


**Figure 2. CB2 agonists inhibit RANKL-stimulated tartrate-resistant acid phosphatase activity (TRAP) in RAW264.7 cells.** RAW264.7 cells were cultured in media containing RANKL and increasing amounts of a nonselective CB1/CB2 agonist, Win55,212-2 (**Panel A**), or a CB2-selective agonist, HU-308 (**Panel B**) as indicated. After 4 days, osteoclastogenesis was evaluated by assaying TRAP activity, a marker osteoclast enzyme. Data are means ± SD from quadruplicate determinations. ***** indicates $p<0.05$ compared to cells treated with RANKL alone.

RAW264.7 cells undergo osteoclastogenesis when stimulated with RANKL [26]. To evaluate the effect of CB2 signaling on osteoclastogenesis, RAW264.7 cells were incubated in the presence of RANKL and various CB1/CB2 agonists. TRAP activity in RAW264.7 cells increased dramatically (~7.5 fold compared to untreated controls) when stimulated with RANKL (Figure 2). In comparison, cells co-treated with Win55,212-2, a CB1/CB2 mixed agonist showed a dose-

dependent and significant reduction in RANKL-stimulated TRAP activity (Figure 2a). RANKL-induced TRAP activity was also dose-dependently and significantly decreased in RAW264.7 cells by co-treatment with HU-308, a CB2-selective agonist (Figure 2b). These results suggest that CB2 signaling decreases osteoclastogenesis in RAW264.7 cells.

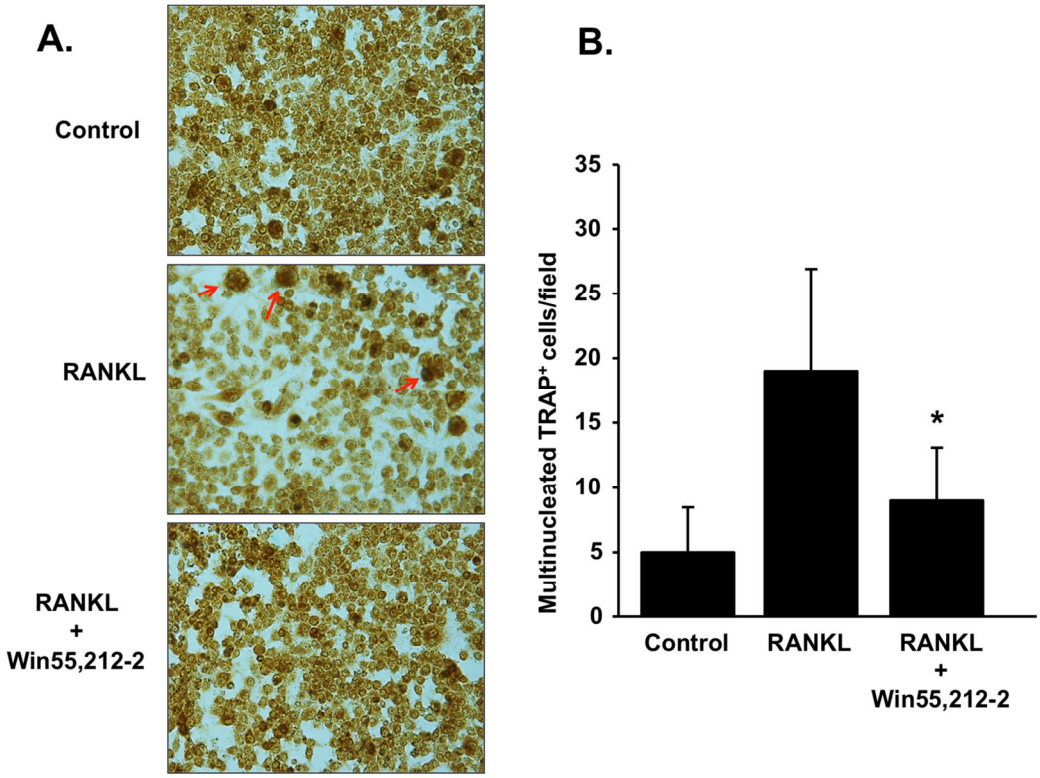


**Figure 3. RANKL-induced formation of TRAP positive osteoclast-like cells is reduced by Win55,212-2.** RAW264.7 cells were cultured on multi-chambered slides in media alone (control) or media containing RANKL (50 ng/ml) in the absence or presence of Win55,212-2 (5 μM), as indicated. After 5 days, the cells were fixed and stained for TRAP. **Panel A** shows representative photomicrographs for each treatment. Arrows indicate TRAP-positive multinucleated cells. **Panel B** depicts quantitation of the number of TRAP-positive multinucleated cells in four random microscopic fields (40X) for each treatment. Data are means ± SD. * indicates $p<0.05$ compared to cells treated with RANKL alone.

We next sought to confirm the TRAP assay results by visual quantitation of osteoclast-like cells (TRAP-positive, multi-nucleate) within cultures of RANKL-stimulated RAW264.7 cells treated with and without Win55,212-2. Cells were incubated for five days in media alone,

media containing RANKL, or media with RANKL plus 5 μM Win5,212-2, stained for TRAP, and examined by light microscopy (Figure 3). After TRAP staining, microscopic observation revealed the presence of large, multi-nucleated TRAP+ cells in RAW264.7 cultures treated with RANKL (Figure 3a). Cultures co-treated with RANKL and Win55,212-2 showed a significant decrease in the number of osteoclasts per microscopic field compared to cells treated with RANKL alone (Figure 3a). Visual quantitation of osteoclasts in four random microscopic fields for each treatment revealed that RANKL treatment resulted in an approximate four-fold increase ($5.0 \pm 3.5$ versus $19.0 \pm 7.9$, $p \leq 0.05$) in the mean number of osteoclast-like cells compared to untreated controls. Co-treatment with RANKL and Win55,212-2 significantly reduced the number of osteoclast-like cells compared to treatment with RANKL alone ($9.0 \pm 4.1$ versus $19.0 \pm 7.9$, $p \leq 0.05$) (Figure 3b).

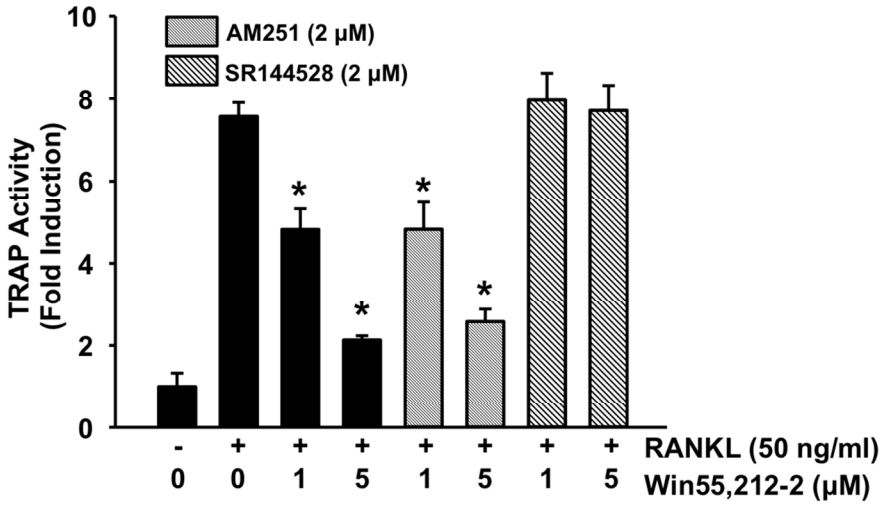


**Figure 4. Inhibition of RANKL-induced TRAP activity by Win55,212-2 is sensitive to CB2-selective antagonism.** RAW264.7 cells were cultured in media containing RANKL and Win55,212-2 alone or in the presence of SR144524, a CB2-selective antagonist, or AM251, a CB1-selective antagonist, as indicated. After 4 days, osteoclastogenesis was evaluated by assaying TRAP activity. Data are means ± SD from quadruplicate determinations. * indicates $P<0.05$ compared to cells treated with RANKL alone.

Since Win55,212-2 is a mixed CB1/CB2 agonist, we next sought to determine if the observed repression of RANKL-induced osteoclastogenesis in RAW264.7 by Win55,212-2 was mediated by CB1 or CB2. RAW264.7 cells were treated with RANKL for four days in the presence of Win55,212-2 alone or in combination with AM251, a CB1-selective antagonist, or with SR144528, a CB2-selective antagonist [30, 29]. Co-treatment with RANKL and Win55,212-2 (1 μM and 5 μM) significantly reduced TRAP activity compared to cells treated with RANKL alone (Figure 4). Supplementation with SR144528 completely blocked the effect of Win55,212-2 on RANKL-induced TRAP activity at either dose. In contrast, co-treatment with AM251 had no effect on the ability of Win55,212-2 to repress RANKL-induced TRAP activity. This result indicates that the pathway by which Win55,212-2 inhibits osteoclastogenesis is sensitive to CB2-selective antagonism, but not CB1-selective antagonism, and therefore is likely mediated by CB2 activation.

Role of CB2 in Osteoblastogenesis

Treatment of MOVAS-1 cells with CB2 agonists was found to increase ALP activity and calcium deposition *in vitro*. Co-treatment of cells with CB2 antagonists reduced both ALP and calcium deposition relative to agonists alone, indicating that CB2 signaling encourages osteoblastogenesis of MOVAS-1 cells.

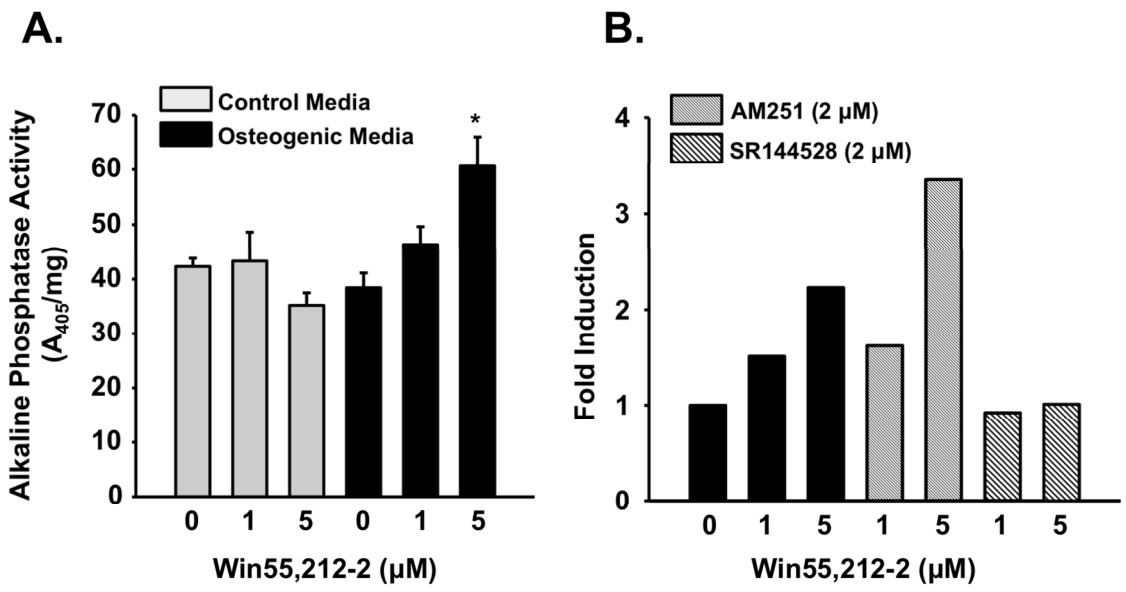


**Figure 5. Win55,212-2 dose-dependently increases the activity of ALP, an osteoblast marker enzyme, in MOVAS-1 cells by a mechanism sensitive to CB2 antagonism.** MOVAS-1 cells were cultured in control media and osteogenic media (2.5 mM β-glycerophosphate and 50 μg/ml ascorbic acid) containing increasing concentrations of Win55,212-2 (**Panel A**), and in osteogenic media containing Win55,212-2 alone or in the presence of a CB1 antagonist (AM251) or a CB2 antagonist (SR144528) as indicated (**Panel B**). After 21 days, osteoblastogenesis was evaluated by assaying ALP activity. Data are means ± SD of triplicate determinations for Panel A and means of duplicate samples for Panel B. * indicates $p<0.05$ compared to control.

Previous studies indicate that CB2 signaling encourages osteoblastogenesis by promoting expression of osteogenic genes in precursor cells [31]. MOVAS-1 cells are a murine vascular smooth muscle cell line capable of spontaneous differentiation into osteoblast-like cells when cultured in media containing β-glycerophosphate and ascorbic acid [32]. Osteoblastogenesis was evaluated by measuring the induction of an osteoblast marker enzyme, alkaline phosphatase (ALP). As shown in figure 5, culturing MOVAS-1 cells for 21 days in osteogenic media supplemented with increasing concentrations of Win55,212-2 resulted in a dose dependent increase in ALP activity compared to MOVAS-1 cells cultured in standard media. To determine if CB2 played a role in Win55,212-2 enhancement of ALP activity in MOVAS-1 cells we also cultured cells in osteogenic media containing Win55,212-2 and either AM251 or SR144528

(Figure 5b). Co-treatment with the CB2-selective antagonist SR144528 reduced ALP activity in Win55,212-2 treated cultures to that of untreated controls. In contrast, ALP activity in MOVAS-1 cells co-treated with the CB1 antagonist AM251 was similar to or greater than MOVAS-1 cells treated with Win55,212-2 alone (Figure 5b). Similar results were obtained when MOVAS-1 cells were cultured in osteogenic media containing another synthetic CB2-selective agonist, HU-308, or an endocannabinoid, 2-arachidonyl glycerol (data not shown). These results indicate that CB2 signaling up-regulates osteoblastogenesis in MOVAS-1 cells by a CB2-dependent mechanism.

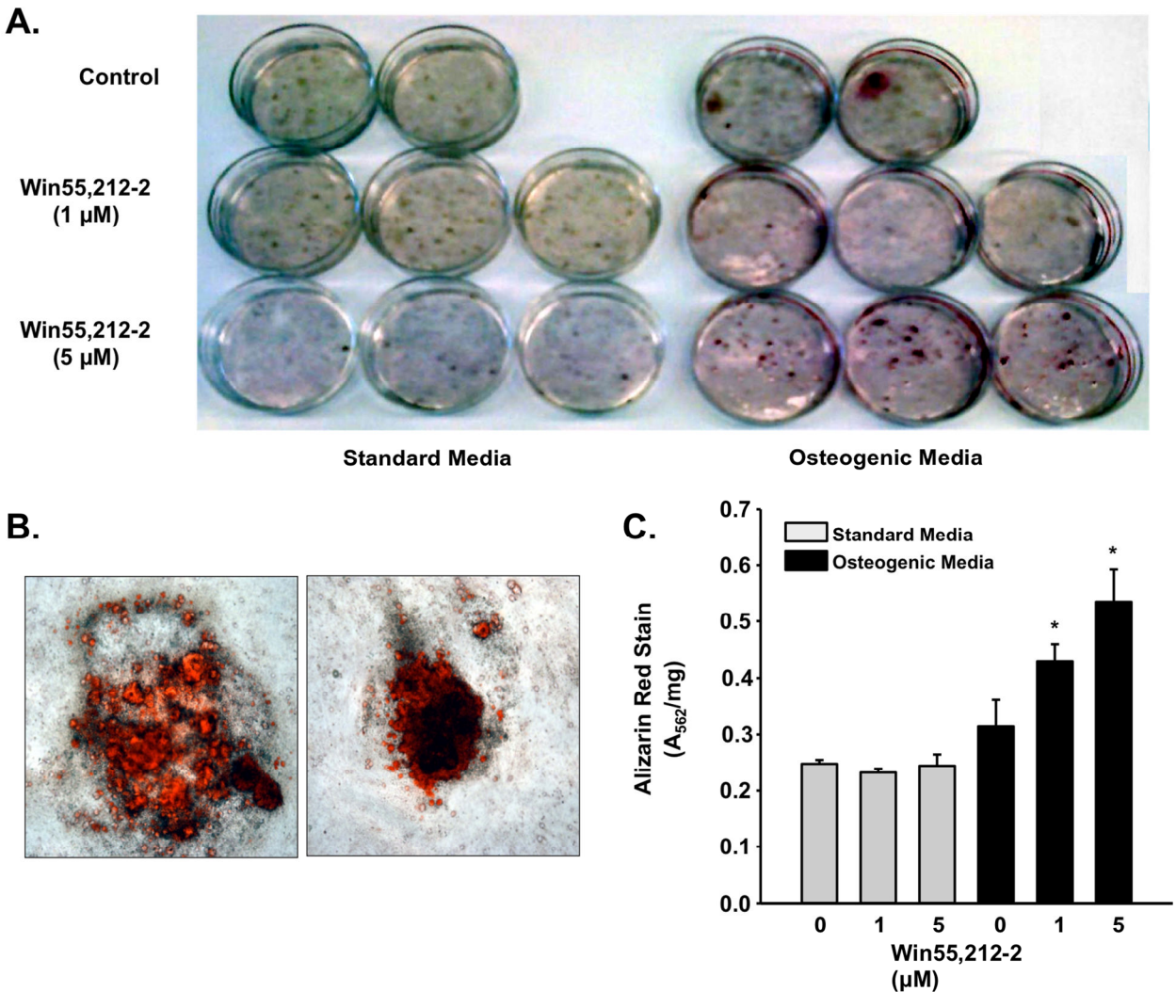

**Figure 6. Win55,212-2 induces osteoblast-like cell activity in MOVAS-1 cells.** MOVAS-1 cells were cultured on 35 mm plates in standard and osteogenic media supplemented with a mixed CB1/CB2 agonist, Win55,212-2, as indicated. After 21 days, the osteoblast-like cell activity was evaluated by staining for calcium deposition with Alizarin red-S (**Panel A**). Photomicrographs (40X) of representative nodules of mineralization following Alizarin red staining are shown in **Panel B**. After photography, bound Alizarin red-S was eluted with 10% cetylpyridinium chloride and quantified by measuring absorbance at 562 nm (**Panel C**). Data are means ± SD. ***** indicates $P<0.05$ compared to cells untreated controls.

In order to confirm osteoblast-like activity in Win55,212-2-treated MOVAS-1 cultures we stained for calcium deposition with Alizarin red. Alizarin red staining in MOVAS-1 cells cultured under standard (non-calcifying) conditions was negligible in the absence or presence of Win55,212-2 (Figure 6). In contrast, Alizarin red staining visibly increased in a dose-dependent manner with Win55,212-2 treatment in MOVAS-1 cells grown in osteogenic media (Figure 6a). Spectrophotometric quantitation of Alizarin red stain extracted with 10% cetylpyridinium confirmed that Alizarin red staining increased significantly in MOVAS-1 cells cultured in osteogenic media supplemented with Win55,212-2 compared to cells cultured in osteogenic media alone (Figure 6c). These results show that the addition of Win55,212-2 increased calcium deposition above that observed in untreated cells confirming the induction of an osteoblast-like cell activity.

To determine if Win55,212-2 enhanced osteoblast-like activity was due to CB2 activation, the effect of CB2-selective antagonism on Win55,212-2 induced Alizarin red staining was also examined. MOVAS-1 cells co-cultured for 21 days in osteogenic media containing Win55,212-2 and SR144528 (a CB2-selective antagonist), showed a notable decrease in Alizarin red staining compared to cells cultured with Win55,212-2 alone (Figure 7). Furthermore, treatment of MOVAS-1 cells in osteogenic media with HU-308, a CB2-selective agonist, also enhanced Alizarin red staining (data not shown).

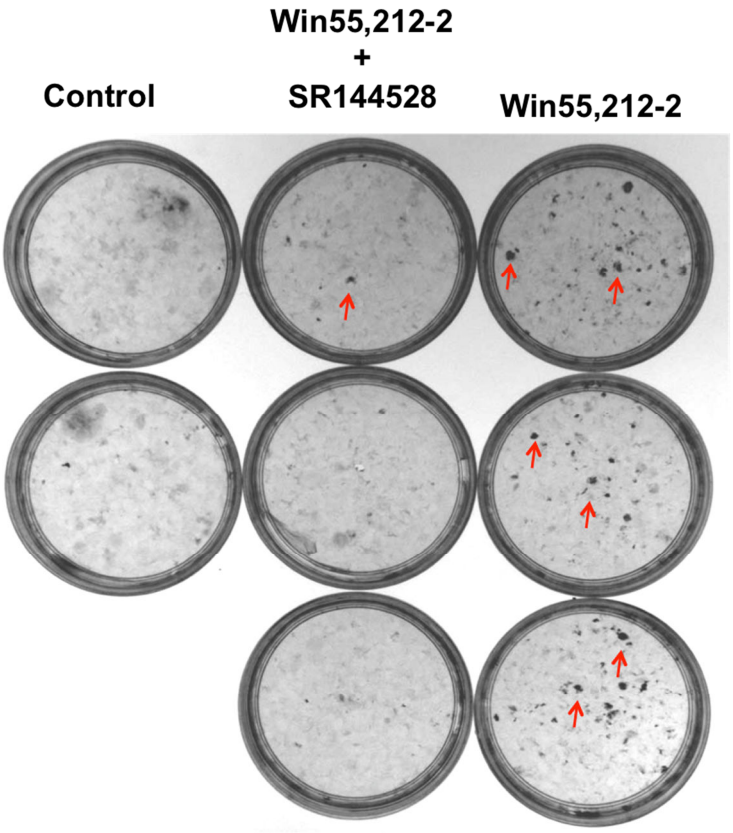


**Figure 7. Win55,212-2 stimulated osteoblast-like cell activity in MOVAS-1 cells is inhibited by CB2 antagonism.** MOVAS-1 cells were cultured on 35 mm dishes in osteogenic media supplemented with Win55,212-2 (5 μM) in the presence and absence of SR144528 as indicated. After 21 days, osteoblast-like activity was evaluated by Alizarin red staining for calcium deposition. Arrows indicate representative examples of Alizarin-stained nodules of mineralization.

## Discussion

Atherosclerosis is a vascular disease that presents serious health risks to many people in Western countries. Roughly 35.3% of Americans (36.7 % of men and 34% of women) suffer from some form of vascular disease. Atherosclerosis is the underlying cause of the majority of clinical cardiovascular events, and has no known cure [33]. It is a slow progressive disease that may start as early as childhood and can cause severe health issues in adults beginning in their thirties and becoming increasingly likely in their fifties and sixties [34]. Current treatments are only able to slow lesion growth by controlling clinically relevant risk factors such as blood LDL levels, obesity and diabetes. However, there are currently no treatment options for the calcification that takes place in advanced lesions partly because the mechanisms for this lesion calcification as well as reasons for its occurrence are not well understood. The goal of this project was to better understand the causes of lesion calcification, especially the effects of CB2 activation on this process. The data from this investigation supports our hypothesis that CB2 signaling influences lesion calcification by affecting osteogenic processes in lesions to reduce osteoclastogenesis and activate osteoblastogenesis, thus promoting lesion calcification.

We observed that osteoclastogenesis was inhibited by CB2 signaling in RAW264.7 cells *in vitro*. When cells were treated with both Win55,212-2 (a synthetic CB1/CB2 non-selective agonist) and HU-308 (a synthetic CB2-selective agonist), there was a significant dose-dependent decrease in RANKL-induced osteoclastogenesis (Figures 2 and 3). This indicates that CB2 signaling performs an inhibitory role in osteoclast formation. This conclusion is supported by the fact that Win55,212-2 inhibition of osteoclastogenesis is sensitive to CB2 antagonism (Figure 4). TRAP staining verified these results. Treatment of RAW264.7 cells with Win55,212-2

caused a significant decrease in osteoclastogenesis when compared to cells treated with RANKL alone (Figure 3).

In contrast, CB2 signaling was found to promote osteoblastogenesis of MOVAS-1 cells *in vitro*. When MOVAS-1 cells were treated with Win55,212-2 a significant and dose-dependent increase in osteoblastogenesis, as determined by ALP induction and calcium deposition, resulted (Figures 5 and 6). The pathway by which promotion of osteoblastogenesis occurred was completely inhibited by CB2 antagonism, indicating that it was the CB2 and not the CB1 receptor involved in promoting osteoblastogenesis (Figures 5 and 7). There is a possibility that CB1 signaling may play a role in increasing osteoblastogenesis, as ALP activity of MOVAS-1 cells increased when cells were co-treated with Win55,212-2 and CB1-selective antagonist AM251 (Figure 5b). However, further investigation is necessary before any conclusive results regarding the involvement of CB1 in osteoblastogenesis of MOVAS-1 cells.

Together, our results show that CB2 signaling promotes osteoblastogenesis and inhibits osteoclastogenesis in murine vascular precursor cells in vitro. These results indicate that activation of CB2 signaling within lesions could alter osteogenic processes and promote calcification of atherosclerotic lesions. These results also underscore the need for future *in vivo* analysis of CB2 signaling in murine models of atherosclerosis. Confirmation of a role for CB2 in lesion calcification would be a potentially major discovery in atherosclerotic research and help clarify the little-understood phenomenon of advanced lesion calcification. Drugs that target CB2 receptors may have a potential for use in treatment of advanced calcified lesions, however, further research is necessary to understand the local and systemic effects of pharmacologically targeting CB2 signaling. Both bone density and immunosuppression have been shown to be greatly affected by CB2 signaling [32, 14, 17], so treatments designed to target CB2 receptors in

lesions could potentially adversely affect patients in other areas. Despite the potential problems, a better understanding of the mechanisms of lesion calcification could improve current treatments and potentially provide new targets for treatments.

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