The Type 2 Cannabinoid Receptor Regulates Bone Mass and Ovariectomy-Induced Bone Loss by Affecting Osteoblast Differentiation and Bone Formation

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The type 2 cannabinoid receptor (CB2) has been reported to regulate bone mass and bone turnover but the mechanisms responsible are incompletely understood. In this study we investigated the role that the CB2 pathway plays in bone metabolism using a combination of genetic and pharmacological approaches. Bone mass and turnover were normal in young mice with targeted inactivation of CB2 receptor \( (CB2^{-/-}) \), but by 12 months of age, they had developed high-turnover osteoporosis with relative uncoupling of bone resorption from bone formation. Primary osteoblasts from \( CB2^{-/-} \) mice had a reduced capacity to form bone nodules \textit{in vitro} when compared with cells from wild-type littermates and also had impaired PTH-induced alkaline phosphatase (ALP) activity. The CB2-selective agonist HU308 stimulated bone nodule formation in wild-type osteoblasts but had no effect in \( CB2^{-/-} \) osteoblasts. Further studies in MC3T3-E1 osteoblast like cells showed that HU308 promoted cell migration and activated ERK phosphorylation, and these effects were blocked by the CB2 selective inverse agonist AM630. Finally, HU308 partially protected against ovariectomy induced bone loss in wild-type mice \textit{in vivo}, primarily by stimulating bone formation, whereas no protective effects were observed in ovariectomized \( CB2^{-/-} \) mice. These studies indicate that the CB2 regulates osteoblast differentiation \textit{in vitro} and bone formation \textit{in vivo}. \textit{(Endocrinology 152: 2141–2149, 2011)}

The endocannabinoid system is now recognized to play an important role in the regulation of bone mass and bone remodeling \( (1) \). Bone cells have been reported to express the type 1 cannabinoid receptor (CB1), the type 2 cannabinoid receptor (CB2), and the orphan G protein-coupled receptor 55 (GPR55), which has recently been found to be activated by some cannabinoid receptor ligands \( (2) \). Young adult mice with targeted inactivation of the CB1 receptor \( (CB1^{-/-}) \) mice have raised peak bone mass due to a defect in osteoclast differentiation and reduced bone resorption \( (3) \). With increasing age, however, \( CB1^{-/-} \) mice develop osteoporosis in association with accumulation of bone marrow fat due to an osteoblast defect with increased differentiation of bone marrow stromal cells to form adipocytes \( (4) \). Mice with targeted inactivation of GPR55 also have increased bone mass due to a defect in osteoclastic bone resorption, but GPR55 does not appear to play a role in regulating bone formation \( (5) \). Analysis of the skeletal phenotype in mice lacking the CB2 receptor \( (CB2^{-/-}) \) mice has yielded more conflicting results. In one study, Ofek \textit{et al.} \( (6) \) reported that \( CB2^{-/-} \) mice had reduced peak bone mass and high bone turnover and went on to develop marked trabecular osteoporosis and cortical expansion by 12 months of age. However, in another study we reported that \( CB2^{-/-} \) mice on the same genetic background had normal peak bone mass and were

Abbreviations: ALP, Alkaline phosphatase; BrdU, bromodeoxyuridine; BV/TV, trabecular bone volume/tissue volume; Ct.Th, cortical thickness; Ct.BV, cortical bone volume; CB1, type 1 cannabinoid receptor; CB2, type 2 cannabinoid receptor; CTX, carboxy terminal cross-linked, telopeptide of type I collagen; DMSO, dimethylsulfoxide; GPR55, G protein-coupled receptor 55; JNK, c-Jun N-terminal kinase; MAR, mineral apposition rate; MicroCT, microcomputed tomography; Ob.MV, osteoblast number/bone surface; Oc.MV, osteoclast number/bone surface; Oc.SB, osteoclast surface/bone surface; pERK, phosphorylated ERK; P1NP, amino terminal peptide of type I procollagen; PTX, pertussis toxin; Tb.N, trabecular number; Tb.Th, trabecular thickness.
partly protected from ovariec-tomy-induced bone loss (7). In the present study, we attempted to try and clarify the role that the CB2 receptor plays in bone metabolism by genetic and pharmacological approaches both in vivo and in vitro. Our studies confirm that CB2 receptor signaling protects against age-related and ovariec-tomy induced bone loss, but we show that this is mainly medi-ated by an effect on bone formation rather than on bone resorption as was previously reported.

**Materials and Methods**

**Materials**

Reagents were obtained from Sigma Aldrich (Dorset, UK) unless otherwise indicated. The CB2-selective receptor ligands HU308 and AM630 used for the in vitro experiments were purchased from Tocris Biosciences (Bristol, UK). HU308 used for the in vivo studies was a kind gift from Dr. Roel J. Arends (Organon, Oss, The Netherlands). Tissue culture medium (αMEM) was obtained from Invitrogen (Paisley, UK), fetal calf serum was obtained from Fisher Scientific (Leicestershire, UK), and 1-glutamine and penicillin/streptomycin were obtained from Invitrogen. Vitamin C (ascorbic acid) was obtained from BDH Laboratory Supplies (Dorset, UK). Primary antibody for phosphorylated ERK (pERK) 1/2 was obtained from New England Biolabs. Immunoassay kits for measuring the biochemical markers of bone turn-over, amino-terminal propeptide of type I procolla-gen (P1NP), and carboxy terminal cross-linked telopeptide of type I collagen (CTX) were obtained from Immunodiagnostic Systems (Boldon, UK).

**Animals**

Mice with targeted inactivation of the CB2 receptor (CB2-/- mice) were generated as previously described (8) and were obtained from Dr. Susana Winfield (National Institutes of Health, Bethesda, MD). These are essentially the same strain of mice that were analyzed by Ofek et al. (6). The mice used in the experiments described here had been crossed with wild-type C57BL/6 mice for at least 10 generations and were generated by mating heterozygote breeding pairs. All animal experiments were con-ducted in accordance with U.K. Home Office regulations.

**TABLE 1.** Histomorphometry of trabecular bone at the proximal tibial metaphysis of 3- and 12-month-old CB2-/- and wild type mice

<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Oc.N/BS (cells/mm)</th>
<th>Oc.S/BS (%)</th>
<th>Ob.N/BS (cells/mm)</th>
<th>MAR (µm/d)</th>
<th>BFR (µm²/µm·d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 months</td>
<td>Wild type</td>
<td>0.47 ± 0.08</td>
<td>1.24 ± 0.20</td>
<td>18.6 ± 1.4</td>
<td>3.57 ± 0.17</td>
<td>1.74 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>CB2-/-</td>
<td>0.39 ± 0.06</td>
<td>1.12 ± 0.16</td>
<td>15.6 ± 0.9</td>
<td>3.68 ± 0.14</td>
<td>1.70 ± 0.12</td>
</tr>
<tr>
<td>12 months</td>
<td>Wild type</td>
<td>1.58 ± 0.31</td>
<td>4.18 ± 0.78</td>
<td>17.6 ± 2.0</td>
<td>1.90 ± 0.10</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>CB2-/-</td>
<td>2.49 ± 0.29a</td>
<td>7.19 ± 0.75b</td>
<td>22.6 ± 1.3a</td>
<td>2.17 ± 0.11</td>
<td>0.86 ± 0.07a</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM from seven to eight animals per group. BFR, Bone formation rate.

* P < 0.05 from wild type mice of same age.

† P < 0.02 from wild type mice of same age.
Ovariectomy-induced bone loss

Bilateral ovariectomy or sham surgery was carried out in 8-wk-old female mice according to standard methods, and the completeness of ovariectomy was verified postmortem by measuring uterine weight. Treatment with test agents was commenced 2 d after ovariectomy or sham operation by ip administration of the test agent in a solution of 5% (vol/vol) mannitol and 0.5% (wt/vol) gelatin prepared in distilled H2O. Controls received vehicle [dimethylsulfoxide (DMSO)] in mannitol/gelatin suspension. Treatment was continued for 19 d and the experiment was terminated on d 21. The mice also received two ip injections of 0.2% (wt/vol) calcein 4 d apart, 6 and 2 d before termination of the experiment for assessment of dynamic histomorphometry.

Analysis of bone density and structure by microcomputed tomography (MicroCT)

MicroCT analysis was performed at the left tibial metaphysis 1 mm distal to the primary spongiosa and at the left distal femoral metaphysis 2.25 mm proximal to the primary spongiosa using a SkyScan 1172 instrument set (Kontich, Belgium) at 60 kV, and 150 µA at a resolution of 5 µm. The images were reconstructed using the SkyScan NRecon program and analyzed using SkyScan CTAn software.

Bone histomorphometry

Bones were fixed for 24 h in 4% formaldehyde and stored in 75% ethanol prior to MicroCT scanning and embedding. They were embedded in methyl methacrylate, and processed for static and dynamic histomorphometry according to standard techniques. The bones were sectioned using a Leica microtome (Heidelberg, Germany), stained with Von Kossa, and counterstained with Paragon. Bone histomorphometry was assessed using a semiautomated image analysis system.

Osteoblast cultures and bone nodule cultures

Osteoblasts were isolated from the calvarial bones of 2-d-old mice by sequential collagenase/EDTA digestion and cultured in 75-cm² tissue culture flasks in MEM supplemented with 50 µg/ml vitamin C and 3 mM β-glycerophosphate in the presence of test agents or vehicle (DMSO 0.1%). The medium was replaced three times per week and cultures in standard αMEM supplemented with 50 µg/ml vitamin C and 3 mM β-glycerophosphate in the presence of test agents or vehicle (DMSO 0.1%). The medium was replaced three times per week and cultures were continued for up to 21 d. For the studies on alkaline phosphatase (ALP) activity, osteoblasts were isolated from bone marrow as previously described (4) and cultured in complete αMEM supplemented with 50 µg/ml vitamin C and 3 mM β-glycerophosphate in petri dishes for 5–7 d when they were detached using trypsin. The cells were then seeded into 96-well plates at a density of 1 × 10⁵ cells/well and exposed to test agents or vehicle (DMSO 0.1%) for 48 h. Cell lysates were then prepared and ALP activity measured as previously described (4). Mineralized nodules were detected using Alizarin Red staining, and bone nodule formation was quantified by destaining the cultures in 10% (vol/vol) cetylpyridinium chloride and dissolving the stain in 10 mM sodium phosphate (pH 7.0). The absorbance of the extracted stain was then measured by a spectrophotometer at 562 nm as previously described (9). Nodule formation was corrected for cell number as determined by the Alamar Blue assay.

Cell migration assay

The cell migration assay was performed using MC3T3-E1 cells, which were seeded into 48-well plates at a density of 5 × 10⁴ cells/well and allowed to grow to confluence over a period of 48 h. The gap was then made by drawing a 10-µl pipette tip through the confluent cell layer. Test compounds or vehicle were then added and the cultures terminated after 18 h. Migration of cells across the gap was visualized by phase-contrast light microscopy and the degree of cell migration quantitated using the ImageJ analysis program (http://rsb.info.nih.gov/ij/). Osteoblast number was assessed by the Alamar Blue assay, and osteoblast proliferation was assessed by bromodeoxyuridine (BrdU) incorporation using a kit purchased from Calbiochem (Nottingham, UK) according to the manufacturer’s instructions. These experiments were also performed in the presence of Mitomycin C, which was added at a concentration of 10 µg/ml, 2 h before test compounds were added.

FIG. 2. HU308 partially protects from ovariectomy-induced bone loss. A, BV/TV in wild-type (WT) and CB2−/− mice after ovariectomy (OVX) or a sham procedure, treated with HU308 1 mg/kg for 3 wk or vehicle. B, Tb.N. C, Tb.Th. For all variables, data are expressed as percent change relative to sham-operated vehicle-treated mice of the same genotype. Values are mean ± SEM from six to seven mice per group. #, P < 0.05 from sham-operated, vehicle-treated mice; *, P < 0.05 and **, P < 0.01, CB2−/− vs. wild-type mice with the same treatment; +++, P < 0.01 HU308 vs. vehicle.
Intracellular signaling

The intracellular signaling experiments were conducted using MC3T3-E1 osteoblast-like cells cultured between passages 4 and 18. The cells were seeded into 12-well plates at a density of $2 \times 10^5$ cells/well in standard αMEM and cultured for 2 d. The cells were transferred to serum-free medium and pretreated with U0126, LY294002, pertussis toxin (PTX) or AM630 for 60 min before stimulation with HU308 or vehicle. The cells were lysed after a 10-min exposure to the test agents and lysates prepared by adding a buffer containing 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 0.1% (wt/vol) sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2% (vol/vol) protease inhibitor cocktail, and 0.4% (vol/vol) phosphatase inhibitor cocktail to the cell layer. The protein content was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). Levels of pERK1/2 were detected using a rabbit polyclonal anti-pERK1/2 antibody and normalized to total ERK using a rabbit polyclonal anti-actin antibody and to total ERK using a polyclonal antiactin antibody (all at 1:1000 dilution). Membranes were then incubated with the appropriate secondary antibody coupled to horseradish peroxidase (1:5000 dilution), and the bands were visualized using a chemiluminescent detection system (Fisher Scientific) on a Syngene Genegnome imaging system (Fisher Scientific).

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL). Differences between groups were assessed using one-way ANOVA followed by Dunnett’s post hoc test. Differences between wild-type and CB2−/− mice or cultures derived from wild-type and CB2−/− mice were analyzed by Student’s t test. The significance level was set at $P = 0.05$.

Results

CB2−/− mice have normal peak bone mass but develop age-related osteoporosis

To investigate the role of CB2 in age-related bone loss, MicroCT analysis was performed at the proximal tibial metaphysis of wild-type and CB2−/− female mice at age 3, 6, and 12 months. There was a trend for lower trabecular bone volume/tissue volume (BV/TV) values in the CB2−/− mice at 3 months of age when compared with wild type, but this was not significant ($P = 0.057$) (Fig. 1A). Trabecular number (Tb.N) was significantly lower at in CB2−/− mice when compared with wild type at 3 months ($P = 0.008$) (Fig. 1C), but trabecular thickness (Tb.Th), cortical thickness (Ct.Th), and cortical bone volume (Ct.BV) were not significantly different between genotypes. At 6 months of age, there was no significant difference between BV/TV and threshold between BV/TV and Tb.N between genotypes, but Tb.Th and Ct.Th values were slightly but significantly greater in CB2−/− mice compared with wild type (Fig. 1, D and F). By 12 months of age, BV/TV values were about 30% lower in CB2−/− mice compared with wild type (CB2−/− vs. wild type, $P = 0.003$), and Tb.N values were about 40% lower ($P = 0.007$) (Fig. 1, A and C). We also performed MicroCT analysis at the distal femoral metaphysis in 3-month-old mice to determine whether we could detect a difference in BV/TV at this time point as reported by Ofek et al. (6). There was a trend for reduced BV/TV values in CB2−/− mice at 3 months of age, similar to that observed in the tibia, but the difference between genotypes was not statistically significant ($P = 0.054$).

Analysis of bone histomorphometry showed no significant difference in osteoblast numbers/bone surface (Ob.N/BS), osteoclast numbers/bone surface (Oc.N/BS), or osteoclast surfaces/bone surface (Oc.S/BS) between wild-type and CB2−/− female mice at 3 months of age (Table 1). By 12 months of age, however, Oc.N/BS and Oc.S/BS values were between 60 and 80% higher in CB2−/− mice than in wild-type littermates ($P = 0.042$ and $P = 0.01$, respectively). Although Ob.N/BS and bone formation rate were also higher in CB2−/− mice at 12 months of age when compared with wild-type ($P = 0.03$ and $P = 0.04$, respectively), the difference between genotypes (25–35%) was less than the indices of bone resorption (Table 1). In keeping with these observations, the biochemical markers of bone metabolism were analyzed by Student’s t test. The significance level was set at $P = 0.05$.

**FIG. 3.** HU308 partially protects from ovariectomy (OVX)-induced bone loss by increasing bone formation. A, Ob.N/BS (cells per millimeter) in wild-type (WT) and CB2−/− mice subjected to ovariectomy or sham operation and treated with vehicle or HU308 at 1 mg/kg for 3 wk, assessed by histomorphometry. B, MAR (millimeters per day). C, Oc.N/BS (cells per millimeter). D, Oc.S/BS (percentage). Values are means ± SEM from five to six mice per group. *, $P < 0.05$ and **, $P < 0.01$ from sham-operated mice of same genotype; †, $P < 0.05$ and ‡, $P < 0.01$ from ovariectomized, vehicle-treated mice of same genotype; $S$, $P < 0.01$ from wild-type mice with the same treatment.

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turnover CTX and P1NP were higher in CB2−/− mice when compared with wild-type at 12 months. The mean (± SEM) serum CTX values were 17.6 ± 1.3 ng/ml in CB2−/− mice compared with 12.6 ± 0.8 ng/ml in wild type (P = 0.007), and corresponding values for P1NP were 47.8 ± 2.1 ng/ml in CB2−/− mice compared with 41.2 ± 1.1 ng/ml in wild type (P = 0.02). These data indicate that CB2−/− mice develop trabecular osteoporosis with increasing age in association with increased bone turnover but with a greater increase in bone resorption than bone formation.

HU308 protects against ovariectomy induced bone loss mainly by affecting bone formation

We next investigated the role that CB2 signaling plays on bone loss that results from estrogen deficiency by studying the effect of HU308 in ovariectomized wild-type and CB2−/− mice. The changes in bone mass and bone structure that occurred in response to ovariectomy in CB2−/− and wild-type mice are shown in Fig. 2. After ovariectomy, BV/TV fell significantly in CB2−/− mice, but the degree of bone loss was significantly less than in wild type (P = 0.01). Administration of HU308 at 1 mg/kgd partially prevented the reduction in BV/TV and Tb.N after ovariectomy in wild-type mice (Fig. 2, A and B). The reduction in BV/TV was 47.7 ± 8.4% less in HU308-treated, ovariectomized wild-type mice when compared with vehicle (P = 0.005), and the reduction in Tb.N was 55.4 ± 6.5% less (P = 0.002). Although HU308 protected against trabecular bone loss after ovariectomy in wild-type mice, there was no significant effect on Ct.BV, Ct.Th, or medullary cavity diameter (Supplemental Fig. 1, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). There was no significant effect of HU308 on BV/TV, Tb.N, or Tb.Th or on cortical bone parameters in ovariectomized CB2−/− mice (Fig. 2 and Supplemental Fig. 1).

The changes in bone histomorphometry after ovariectomy and HU308 treatment in wild-type and CB2−/− mice are shown in Fig. 3. As expected, indices of bone resorption and bone formation increased in wild-type and CB2−/− mice after ovariectomy, but the increase in osteoclast number did not reach statistical significance in CB2−/− mice, consistent with the fact that these mice have previously been reported to have a mild defect in osteoclastic bone resorption (7) (Fig. 3). There was no significant difference in Ob.N/BS or mineral apposition rate (MAR) between wild-type and CB2−/− mice in response to sham operation or after ovariectomy. Administration of HU308 to ovariectomized wild-type mice caused a significant increase in Ob.N/BS and MAR when compared with vehicle treated ovariectomized mice, whereas no such effect was observed in CB2−/− mice (Fig. 3, A and B). Administration of HU308 slightly decreased Oc.N/BS and Oc.S/BS after ovariectomy in wild-type mice, but the changes did not reach statistical significance (P = 0.193 for osteoclast numbers and P = 0.135 for resorption surfaces) (Fig. 3, C and D). Surprisingly, HU308 slightly increased osteoclast numbers and active resorption surfaces in CB2−/− mice, but the differences from vehicle treated ovariectomized mice of the same genotype were not significant. Taken together, these data indicate that the protective effect of HU308 on ovariectomy-induced bone

FIG. 4. Primary osteoblasts from CB2−/− mice have defective bone nodule formation and impaired PTH-induced ALP activity. A, Bone nodule formation as assessed by alizarin red staining in neonatal calvarial osteoblasts from wild-type (WT) and CB2−/− mice. Values are expressed as percent of the values observed in WT cultures at wk 1. B, Representative photomicrographs from one of the cultures described in A. C, Effect of HU308 on bone nodule formation in calvarial osteoblast cultures. Data are expressed as percent of the values observed in wild-type, vehicle-treated cultures harvested after 10–12 d in culture. D, Cell numbers assessed by Alamar Blue assay in the cultures from C. E, ALP activity in bone marrow osteoblasts from WT and CB2−/− mice exposed to PTH (25–100 nM) for 24 h. F, Effect of HU308 on growth of calvarial osteoblasts from wild-type mice assessed by BrdU incorporation or Alamar Blue assay. The alizarin red staining in A and C and the ALP values in E are corrected for cell number. The values in the graphs are means ± SEM from three to four independent experiments. *, P < 0.05; **, P < 0.01 CB2−/− vs. WT; ***, P < 0.05; ****, P < 0.01 from vehicle-treated cultures.
loss that we observed was most probably mediated by a stimulatory effect on bone formation rather than an inhibitory effect on bone resorption, although we cannot completely exclude the possibility that HU308 exerts subtle effects on bone resorption in this model.

**Activation of the CB2 receptor stimulates osteoblast differentiation in vitro**

We went on to investigate the effects of CB2 signaling on osteoblast proliferation and differentiation using primary osteoblast cultures from wild-type and CB2−/− mice. Cultures from CB2−/− mice formed significantly fewer nodules than wild-type cultures in osteogenic medium, and the difference between the genotypes increased with time in culture (Fig. 4, A and B). Treatment of wild-type cultures with HU308 for 10–12 d over the concentration range of 10–30 nM stimulated bone nodule formation by about 10%, but these effects were blunted in CB2−/− cultures (Fig. 4C). In contrast, these concentrations of HU308 had no significant effect on cell number (Fig. 4D). We observed no significant difference in ALP activity in bone marrow stromal cell cultures from CB2−/− and wild-type mice under basal conditions but found that ALP levels failed to increase in CB2−/− cultures in response to PTH, contrasting with the concentration-dependent increase in PTH-induced ALP activity that was observed in wild-type cultures (Fig. 4E). We observed no effect of HU308 on growth of calvarial osteoblasts over 48 h in normal culture conditions at concentrations of up to 1000 nM using the Alamar Blue assay and the BrdU assay (Fig. 4F).

**The CB2-selective agonist, HU308, stimulates osteoblast migration**

Given that bone formation occurs only once osteoblast precursors migrate to the bone remodeling site, we studied the effects of the CB2-selective agonist HU308 on osteoblast migration in vitro using MC3T3-E1 cells. This showed that HU308 stimulated cell migration (Fig. 5, A and C) when compared with vehicle-treated cultures (P = 0.01), without affecting cell number (Fig. 5B). The stimulatory effect of HU308 on cell migration was blocked by the CB2-selective antagonist/inverse agonist AM630 (Fig. 5A), consistent with a CB2-mediated effect. These assays were repeated in the presence of Mitomycin C to determine whether the effects observed might have been due to an effect on osteoblast proliferation, but the results were similar to those observed in the absence of Mitomycin C (Supplemental Fig. 2). This indicates that HU308 promotes wound healing by an effect on cell migration rather than by an effect on osteoblast proliferation.

**The CB2-selective agonist HU308 activates the MAPK-ERK signaling pathway in osteoblast-like cells**

To explore the mechanisms by which CB2 signaling regulates osteoblast function, we studied the effects of HU308 on phosphorylation of p38 MAPK, c-Jun N-terminal kinase (JNK), and ERK1/2 in MC3T3-E1 osteoblast-like cells in view of the role that these pathways play in cell proliferation and function (10). We found that HU308 stimulated ERK1/2 phosphorylation at concentrations of 0.1 µM and greater (Fig. 6, A and B), but no effect was observed on levels of phospho-p38 or phospho-JNK (data not shown). The stimulatory effect of HU308 on ERK phosphorylation was significantly inhibited by the Gi/o inhibitor PTX; the CB2-selective antagonist/inverse agonist AM630 and by the PI3 kinase inhibitor LY294002 (Fig. 6, C and D and Supplemental Fig. 3). We also observed a nonsignificant trend for inhibition of ERK phosphorylation by PTX and AM630 in vehicle-treated cultures. Although LY294002 significantly inhibited ERK phosphorylation in the absence of HU308, the magnitude of inhibition was less that in HU308-treated cultures. Our interpretation of these experiments is that HU308 up-regulates ERK phosphorylation by a CB2-dependent pathway (as evidenced by the inhibition with PTX and AM630) and by a pathway that involves PI3 kinase (as evidenced by the inhibition with LY294002). It is possible that the modest inhibitory effects on ERK phosphorylation that were observed in
vehicle-treated cultures could also have been mediated through CB2 by blocking the effects of endocannabinoids, which are produced constitutively by MC3T3 cells (11). However, we acknowledge that we cannot exclude the possibility that PTX might have inhibited ERK phosphorylation by affecting other $G_{i/o}$-coupled receptors (such as CB1) or that LY294002 might have inhibited ERK phosphorylation by affecting other signaling pathways.

**Discussion**

The present study has confirmed that the CB2 receptor plays an important role in regulating bone mass and bone turnover. Although some of the results reported here are consistent with those described by Ofek et al. (6), who conducted similar experiments in CB2$^{-/-}$ mice of an identical strain (6), other findings differ and provide new insights into the role that CB2 plays in bone metabolism. In agreement with Ofek et al., we found that CB2$^{-/-}$ mice developed age-related osteoporosis in association with increased bone turnover, but we failed to detect any significant abnormalities in peak bone mass or bone turnover in young CB2$^{-/-}$ mice. These differences may be partly methodological in nature because we performed the MicroCT scanning at higher resolution than in the study by Ofek et al.

The lack of an effect on peak bone mass could also be gender related because the study by Ofek et al. (6) focused on male mice, whereas we studied female mice. We confirmed that HU308 partially protects against ovariectomy-induced bone loss but found that this was mainly due to a stimulatory effect on bone formation rather than an inhibitory effect on bone resorption as was previously reported (6). Another difference between this study and that of Ofek et al. (6) is that we failed to detect a protective effect of HU308 on cortical bone after ovariectomy. These differences might be explained by various factors including the use by Ofek et al. of a different vehicle for HU308; the use of C3H mice for the ovariectomy experiments, as opposed to the C57BL/6 mice used here; and the fact that HU308 was administered immediately after ovariectomy at a 10-fold higher dose than was used here.

Although the high doses of HU308 used by Ofek et al. could potentially have resulted in off-target effects, it is also possible that the higher dose may have exerted site specific effects in cortical bone by a CB2-dependent mechanism. Further dose-ranging studies in CB2$^{-/-}$ mice would be required to fully address this point.

The importance of the osteoblast as a target for the positive effects of CB2 on bone mass were confirmed by the observation that osteoblasts from CB2$^{-/-}$ mice had a reduced capacity to form bone nodules in vitro compared with cells from wild-type littermates, which is in agreement with the findings described by Ofek et al. (6) and also by the fact that the effects of PTH on ALP activity were blunted in osteoblasts cultured from CB2$^{-/-}$ mice. We were unable to detect any effect of HU308 on osteoblast

![Image](https://example.com/image.png)
proliferation either during the osteogenic cultures or when primary calvarial osteoblasts were cultured under normal conditions, which differs from the results reported by Ofek et al. (6, 12). Our data therefore suggest that the CB2 pathway primarily affects bone formation by an effect on osteoblast differentiation or function, rather than by stimulating proliferation of osteoblasts and their precursors. The ovariectomy studies also supported the hypothesis that the protective effect of HU308 on ovariectomy-induced bone loss was mediated by a stimulatory effect on bone formation rather than an inhibitory effect on bone resorption because histomorphometric analysis showed no effect of HU308 on resorption parameters but instead showed an increase in osteoblast numbers and MAR. Although osteoblast numbers and MAR were not significantly different between wild-type and CB2−/− mice under basal conditions, we found that HU308 when combined with ovariectomy significantly increased osteoblast numbers and MAR in wild-type but not mutant mice. These observations suggest that CB2 deficiency results in a subtle defect in bone formation that becomes apparent only under conditions of increased bone turnover and when CB2 receptors are stimulated with an agonist such as HU308. We also found that HU308 promoted the migration of MC3T3-E1 cells, raising the possibility that the CB2 pathway might play a role in regulating recruitment of osteoblast precursors to sites of bone formation.

To explore the downstream signaling pathways used by CB2 to regulate bone formation, we studied the effects of HU308 on p38 MAPK, ERK1/2, and JNK phosphorylation in MC3T3-E1 osteoblast-like cells in view of the important role that these pathways play in regulating osteoblast function (13). Our results showed that HU308 stimulated ERK1/2 phosphorylation but had no effect on JNK or p38 MAPK phosphorylation. The stimulatory effects of HU308 on ERK1/2 phosphorylation were abrogated by PTX, consistent with a G<sub>ia</sub>-mediated effect; by the CB2 selective antagonist/inverse agonist, AM630; and by the PI3 kinase inhibitor LY294002. Previous studies have shown that CB2 can couple to PI3 kinase/Akt in oligodendrocytes and to ERK in Chinese hamster ovary cells (14), but so far as we are aware, this is the first demonstration that CB2 might couple to these pathways in osteoblasts. Further work will be required, however, to determine whether the stimulatory effects of CB2 on osteoblast differentiation were mediated by activation of the PI3 kinase/ERK pathways as opposed to the adenylate cyclase pathway, which is responsible for mediating many of the effects of cannabinoids in other cell types. The stimulatory effect that we observed on ERK phosphorylation agrees with the results recently reported by Ofek et al. (12), although we failed to show a stimulatory effect of HU308 on proliferation of MC3T3-E1 cells in this study, which contrasts with the results reported in the above-mentioned paper.

The stimulatory effects of the CB2 pathway on bone formation reported here and by other workers (6) are of interest in light of our previous studies, which showed that the CB2−/− mice have a mild osteoclast defect (7) and that the CB2 selective inverse agonist AM630 protects against ovariectomy induced bone loss by inhibiting bone resorption (7). This suggests that the CB2 pathway plays a unique role in regulating bone turnover in that CB2 receptor inverse agonists inhibit bone resorption (7), whereas CB2 agonists stimulate bone formation (6). Taken together, these findings raise the possibility that pharmacological manipulation of the CB2 pathway might be of benefit in the prevention and treatment of bone diseases such as postmenopausal osteoporosis and age-related bone loss, in which these processes are uncoupled, and indicate the need for further clinical studies to address this issue.

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Disclosure Summary: S.H.R., R.J.v.H., and A.I.I. are inventors on patent applications to protect the use of cannabinoid receptor ligands as treatments for bone disease. A.S. and E.L.-B. have no interests to declare.

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