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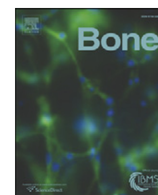

The endovanilloid/endocannabinoid system: A new potential target for osteoporosis therapy

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ABSTRACT

Human osteoclasts express functional TRPV1 channels, CB1/CB2 cannabinoid receptors and endocannabinoid/endovanilloid synthetic/catabolic enzymes. Pharmacologic manipulation of this system can modulate osteoclast activity. Here, through multidisciplinary approaches, we demonstrate that enzymes and receptors of the endocannabinoid/endovanilloid system are differently expressed in osteoclasts from menopausal women without or with osteoporosis. We report that in osteoclasts from osteoporotic patients, TRPV1 channels are upregulated and, if persistently stimulated with resiniferatoxin, become clustered to the plasma membrane while inducing a massive over-expression of CB2 receptors. By providing new evidence for a critical functional cross-talk between CB2 and TRPV1 receptors in osteoporosis, we speculate that TRPV1 desensitization, or its enhanced trafficking, together with TRPV1 agonist-induced CB2 receptor over-expression, might be critical to minimize calcium entry in osteoclasts, which could be in turn responsible of cell over-activation and higher bone resorption. Our data pave the way to the use of TRPV1 agonist together with CB2 agonists or CB1 antagonists in osteoporosis.

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Introduction

Osteoporosis (OP) is a skeletal disorder characterized by low bone mass and micro-architectural bone deterioration. The associated fractures and the subsequent morbidity and mortality make of OP as an enormous public health concern [1–3]. The etiology of OP is heterogeneous, the main predictors are age, gender and a positive family history. Indeed, while OP is thought to be a polygenic disorder and affects both sexes, postmenopausal women are at the highest risk [4,5]. Postmenopausal OP represents a classical type of primary OP, which is characterized by a progressive bone mass loss, with an onset overlapping with the beginning of menopause, and fractures that occur within 15–20 years from gonad function interruption [6]. Moreover, it represents the bone disease with the highest epidemiological impact, which makes it one of the most important public and economic health problems. Although a low bone mass index, lifestyle, dietary habits and age-related bone mass loss contribute to the establishment of OP, the main etiopathological mechanisms seem to be hormone-related [7,8]. Treatment options include general measures on lifestyle, calcium and vitamin D supplements, hormone therapy, raloxifene, and bisphosphonates [9–11].

Recently, we provided evidence that human osteoclasts (OCs), derived by peripheral blood monocyte (PBMC) from healthy volunteers, express functional transient receptor potential vanilloid subtype 1 (TRPV1) channels together with types 1 and 2 cannabinoid receptors (CB1/CB2) and anandamide (AEA) synthetic and catabolic enzymes, NAPE-PLD (*N*-acyl-phosphatidylethanolamine phospholipase) and FAAH (fatty acid amide hydrolase), respectively [12]. It was shown that cannabinoid/vanilloid agonists, alone or in combination with selective antagonists, are able to modulate osteoclast formation and activity. AEA has been recognized to have endovanilloid activity [13,14] and pharmacological blockade of FAAH, by enhancing AEA levels, causes TRPV1-mediated stimulatory effects on osteoclast formation and bone

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turnover [12]. Consistent with the data from rodents pointing to the endovanilloid/endocannabinoid (EV/EC) system as a novel therapeutic target for treating some bone diseases, i.e. OP, [15–19], in this study we have considered it interesting to investigate the co-expression of CB1/CB2 receptors and TRPV1 channels, as well as the specific synthetic or catabolic EV/EC enzymatic machinery, in OCs derived by PBMC from menopausal women screened by Dual Energy X-ray Absorptiometry (DEXA) to measure Bone Mineral Density (BMD), and then categorized using WHO criteria. Accordingly, subjects from three different population were selected and three corresponding groups of OCs were obtained: i) from control women (BMD<1.0 SD below the mean), ii) from women with osteopenia (1.0>BMD<2.5 SD), and iii) from women with OP (BMD>2.5 SD). In these three different human OCs cultures we have performed: 1) biomolecular and immunohistochemical analysis to reveal a possible alteration in the coexpression of CB1, CB2 and TRPV1; or in the expression of the enzymes NAPE-PLD and FAAH, but also of DAGL-α (diacylglycerol lipase alpha), and MAGL (monoacylglycerol lipase), involved in the biosynthesis and catabolism, respectively, of the endocannabinoid 2-arachydonoylglycerol (2-AG), which has little activity at TRPV1; 2) TRPV1 functional activity assays as measured by capsaicin-induced intracellular calcium entry in vitro; 3) measurements of the levels of the EV/EC AEA, the EV *N*-oleoylethanolamine (OEA), the EC 2-AG, and the AEA congener, *N*-palmitoylethanolamine (PEA), which, among others, can enhance AEA effects at cannabinoid and TRPV1 receptors; these measures were made with and without application of URB597, a FAAH blocker; 4) enzymatic and biomolecular assay for evaluating the activity of Tartrate-Resistant Acid Phosphatase (TRAP), a specific biomarker of osteoclast activation, before and after pharmacological manipulation of TRPV1, CB1 or CB2 receptors, or of the enzyme FAAH. Finally, immunohistochemical analyses to reveal co-expression of native CB1, CB2 and TRPV1 receptors in OCs from osteoporotic human bone were also performed.

Materials and methods

Patients

Post-menopausal women ($n = 142$) admitted for the first time to Menopause Unit of Department of Gynaecology of the Second University of Naples, were enrolled after formal approval of the Ethics Committee. A signed study informed consent was obtained from all enrolled women. Patients were screened by DEXA to evaluate bone loss and to measure BMD. DEXA results were categorized on the basis of *T*-scores and *Z*-scores using WHO criteria. According to these criteria, all enrolled menopausal women were divided in 3 subgroups: OP ($n = 53$) (BMD>2.5 SD below the mean for adult women); osteopenia ($n = 45$) (1.0>BMD<2.5 SD); normal ($n = 44$) (BMD<1.0 SD). Past history of fractures and bone disease treatments were excluded. For each experimental procedure the following number of patients were used: cell-signalling, $n = 6$ for each group; molecular biology, $n = 15$ for each group; endocannabinoid level measurements, $n = 6$–8 for each group; immunocytochemistry, $n = 4$ for each group; TRAP assays, $n = 6$ for each group). Seventy-four patients were genotyped for the rs35761398 variant of the *CNR2* gene encoding for CB2 receptor. Moreover, pre-menopausal women ($n = 50$) were also enrolled, in order to confirm previously reported data ($n = 18$) [12] and to check genotype distributions of the investigated SNP. All experiments were conducted according to the Declaration of Helsinki.

Human cell cultures

OCs were obtained from PBMC of menopausal women and differentiated for 21 days as described [12]. The mature fully differentiated OCs, strongly adherent to plastic, were characterized by reverse transcriptase-polymerase chain reaction (RT-PCR) for the expression of the specific biomarker TRAP. Seeding density was: 4×10^7 cells for biomolecular experiments; 3×10^6 cells for immunocytochemical and calcium assay experiments; 5×10^5 cells for TRAP assay experiments.

RNA isolation

Before RNA extraction, cell cultures were trypsinized to remove early or late OCs precursors. mRNA extraction was performed by using an RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH), according to the manufacturer's instructions. RNA concentrations were determined by UV spectrophotometer (NanoDrop ND 1000, NanoDrop Technologies, LLC, Wilmington, USA).

DNA isolation

According to manufacturer's protocol, genomic DNA was extracted from whole blood by using the Wizard Genomic kit (Promega, Milan, Italy) or from the residual phase of RNA extraction by using the Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH).

RT–PCR, semiquantitative amplification, real-time quantitative PCR

Reverse transcriptase from Avian Myeloblastosis Virus (Promega, Madison, WI, USA) was used [12]. Specific amplification reactions for TRAP (alias ACPS, GeneID54), TRPV1 (transcript variants 1 and 3) (GeneID7442), CB1 (isoforms a and b) (alias CNR1, GeneID1268), CB2 (alias CNR2 GeneID1269), FAAH (GeneID2166), NAPE-PLD (GeneID222236), DAGL-α (GeneID221955), MAGL (alias MGLL, GeneID11343), CRIP1A (alias CNRIP1 GeneID25927) and NF-κB (GeneID4720) and for the housekeeping β-actin cDNAs were carried out. In addition, mRNA levels were normalized also with respect to osteoclast number.

Amplimers were resolved into 2.0% agarose gel, detected by the "Gel Doc 2000 UV System" (Bio-Rad, Hercules, CA, USA) and verified by sequencing using the Big-Dye Terminators reaction kit and an ABI PRISM 310 (Applied Biosystem, Forster City, USA).

Three serial 2× cDNA dilutions obtained from 300 ng and 750 ng total mRNA were amplified by real-time PCR, using Sybr green as fluorophore, in order to quantify the expression levels of CB1, CB2 and TRPV1 respect to β-actin. Assays were performed in triplicate. A twenty-five-microliter reaction contained: 2 μl cDNA, 12.5 μl Sybr green Master Mix (Biorad), 10 μl primers mix (10 mM). The thermal cycling program was: 95 °C–10 min, followed by 40 cycles of 95 °C–15 s and 60 °C–1 min. Gene expression profiling was achieved using the comparative cycle threshold method of relative quantization to the housekeeping gene. Real-time PCR products were analyzed by Icycler software (Biorad).

TRAP assay

TRAP was valuated as specific OCs biochemical activity marker and quantified using the ACP method (Takara Bio, Japan) as previously described [12]. Experiments included positive and negative controls.

Cell counting

We counted TRAP(+) multinucleated ($n \geq 3$) OCs in three different wells *per* each group of treatment using an optical microscope (Nikon Eclipse TS100, Nikon Instruments, Badhoevedorp, The Netherlands).

Western blot

TRPV1 channels and CB2 receptors in total lysates from osteoclast cultures were analyzed by Western blot experiment. Membrane strips

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