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Perspective

PKCβII-mediated cross-talk of TRPV1/CB2 modulates the glucocorticoid-induced osteoclast overactivity

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ABSTRACT

In this study, we investigated the role of the endovanilloid/endocannabinoid system in the glucocorticoid-induced osteoclast overactivity.

Receptorial and enzymatic component of the endovanilloid/endocannabinoid system are expressed in bone cells, and dysregulated when bone mass is reduced. Moreover, blockade or desensitization of vanilloid receptor 1 (TRPV1) and/or stimulation of cannabinoid receptor 2 (CB2) are beneficial for reducing number and activity of the bone cells modulating resorption, the osteoclasts.

We have treated in vitro healthy woman derived osteoclasts with methylprednisolone in presence or not of CB2 or TRPV1 agonists/antagonists, analysing the effect on osteoclast function and morphology through a multidisciplinary approach. Moreover, a treatment with a protein kinase C inhibitor to evaluate osteoclast activity and endovanilloid/endocannabinoid component expression levels was performed in osteoclasts derived from healthy subjects in presence of not of methylprednisolone.

Our results show, for the first time, that the endovanilloid/endocannabinoid system is dysregulated by the treatment with methylprednisolone, that the osteoclast activity is increased and that pharmacological compounds stimulating CB2 or inhibiting TRPV1 might reduce, possible inhibiting protein kinase C beta II, the methylprednisolone–induced osteoclast over-activation, suggesting their therapeutic use for protecting from the glucocorticoid-induced bone mass loss.

1. Introduction

Since their discovery, glucocorticoids have been used for inflammatory and autoimmune diseases treatment [1], despite the onset of many adverse effects arising by their use [2].

The administration of glucocorticoids for prolonged periods affects, both directly and indirectly, the bone cells activity leading to a bone mass reduction, and, in turn, to skeletal fragility and osteoporosis [3–13]. Indeed, the glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis that occurs, independently of sex or age, in 30–50% of treated patients [14]. Thus, whatever the disease for which the patient assumes glucocorticoids, a preventive pharmacological strategy counteracting the loss of bone mass, occurring from the start of steroid therapy, could represent an important achievement.

Recent studies have implicated the endovanilloid/endocannabinoid (EV/EC) system in the regulation of bone cell differentiation, survival and activity [15–18].

The EV/EC system comprises the cannabinoid receptors type 1 and 2 (CB1, CB2), the transient receptor protein cation channel vanilloid subtype 1 (TRPV1) and their endogenous ligands, the hybrid EV/EC anandamide (AEA) and the EC 2-arachidonoylglycerol (2-AG) [19–21].

CB1, CB2 and TRPV1 and the enzymes for EV/EC metabolism are expressed in bone cells, their expression is altered in osteoporo-

Abbreviations: MP, methylprednisolone; RTX, resiniferatoxin; I-RTX, 5′-iodo-resiniferatoxin; AM630, (6-iodo-2-methyl-1-(2-morpholinoethyl)-1H-indol-3-yl)(4-methoxyphenyl)methanone; JWH-133, (6AR,10AR)-3-(1,1-DIMETHYLBUTYL)-6A,7,10,10A-TETRAHYDRO-6,6,9-TRIMETHYL-6H-DIBENZO[B,D]PYRAN; DMSO, dimethyl sulfoxide; OCs, osteoclasts; OP, osteoporosis; CB1, cannabinoid receptors type 1; CB2, cannabinoid receptors type 2 the; TRPV1, Transient receptor protein cation channel vanilloid subtype 1; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; EV, endovanilloid; EC, endocannabinoid; PKCβII, protein kinase C beta II.

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sis and compounds acting on these receptors influence bone cell activity [17,18]. In particular, TRPV1 levels are enhanced, whereas those of CB2 are dramatic reduced, in osteoclast derived from osteoporotic subjects [18] and the genetic ablation, blockade or desensitization of TRPV1 and/or the CB2 stimulation are beneficial for reducing osteoclast over-activity [22].

Several studies indicate that glucocorticoids up-regulate protein kinase C beta II (PKCβII) expression [23,24]. PKCβ-activation increases osteoclastogenesis [25], and, accordingly, a protein kinase C inhibitor administration reduces bone resorption [26].

To highlight a possible role of the EV/EC system in the glucocorticoid-induced osteoporosis, we treated in vitro healthy woman derived osteoclasts (OCs) with Methylprednisolone (MP) in presence or not of CB2 or TRPV1 agonists/antagonists, analysing the effect on OCs function and morphology through a multidisciplinary approach. Moreover, a treatment with a PKCβII inhibitor to evaluate osteoclast activity and EV/EC component expression levels was performed in OCs derived from healthy subjects in presence of not of MP.

Our results show, for the first time, that the EV/EC system is dysregulated by the treatment with MP, that the osteoclast activity is increased by MP, and that pharmacological compounds stimulating CB2 or inhibiting TRPV1 might reduce, possible inhibiting PKCβII, the MP-induced OCs overactivation, suggesting their therapeutic use for protecting from the glucocorticoid-induced bone mass loss.

2. Material and methods

2.1. Human cell cultures

Osteoclasts were obtained from unfractionated peripheral blood mononuclear cells (PBMCs) of healthy subjects. A signed study informed consent was obtained from all healthy subjects with the approval of Ethics Committee of the Second University of Naples and in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki). PBMCs were isolated by centrifugation over Histopaque 1077 density gradient (Sigma Chemical, St Louis, MO), diluted at 1×10^6 cells/mL in α-Minimal Essential Medium (α-MEM) (Lonza, Verviers, Belgium) and supplemented with 10% fetal bovine serum (FBS) (Euroclone, Siziano,Italy),100 IU/mL penicillin, and 100 g/mL streptomycin and L-glutamine (Gibco Limited,Uxbridge, United Kingdom) and plated in 12-well Cell Culture Multiwell for molecular biology experiments, in 96-well Cell Culture Multiwell for TRAP assay and in chamber slides for immunocytofluorescence (Corning Inc., Corning, NY, USA).

In order to obtain fully differentiated human osteoclasts, the PBMCs were then cultured for about 21 days in the presence of 25 ng/mL recombinant human macrophage colony-stimulating factor (rh-MCSF) (Peprotech, London,UK) and 50 ng/mL RANK-L, (R&D Systems, Minneapolis, MN, USA). Mature OCs were identified as tartrate-resistant acid phosphatase positive (TRAP+) multinucleated cells containing 3 or more nuclei at the end of the culture period. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 21 days and culture medium was replaced every three days with fresh medium supplemented or less with the drugs described above.

2.2. mRNA analysis

Before RNA extraction, cell cultures were trypsinized to remove early or late osteoclasts precursors. The cells persisting in culture following trypsin treatment were fully differentiated OCs. Total mRNA was extracted by using RNA Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA), and retrotranscripted by using the RT High Capacity cDNA Reverse Transcription kit (Applied biosystems, Foster City CA, USA). Real-time RT-PCR analysis was carried out by using the Fast SYBR Green Master mix kit (Applied biosystems, Foster City CA, USA) on an 7900 HT Sequence Detection System (Applied Biosystems, Foster City CA, USA). Primers, picked with Primer3 software, and thermal conditions are available on request. Specific amplification reactions for *TRPV1* (GeneID7442), *CB1* (alias *CNR1*, GeneID1268), *CB2* (alias *CNR2* GeneID1269), *TRAP* (alias *ACPS*, GeneID54), PKCβII (GeneID5579) and for the housekeeping *β-actin* (GeneID60) cDNAs were carried out. 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 5x cDNA dilutions obtained from 250 ng total mRNA were amplified by Real-time PCR, using Sybr green as fluorophore, in order to quantify the expression levels of *CB1, CB2, TRPV1, TRAP and PKCβII* respect to *β-actin*. Assays were performed in triplicate. Twenty-five μl reaction contained: 2 μl cDNA, 12.5 μl SYBR green Master Mix (Bio-Rad, Hercules, CA, USA), 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 (Bio-Rad, Berkeley, USA).

2.3. TRAP assay

TRAP was evaluated as specific biochemical activity marker of osteoclasts. Cell cultures were performed in a 96-multiwell plate (Corning Inc., Corning, NY, USA) by adding 200 μl of the cell suspension to each well (650,000 cells per well). TRAP positivity was quantified using the ACP method (Takara Bio, Japan), adapted to a 96-multiwell plate. After cell fixation (citrate buffer pH 5.4, containing 60% acetone and 10% methanol for 5 min, at room temperature), 50 μl of chromogen substrate solution (naphtol-AS-BI-phosphate substrate/fast red violet LB), spiked with 0.1 vol of sodium tartrate, was added to each well. The TRAP enzyme cleaves the substrate, forming a red azoic dye with purplish red color that can be detected with an optical microscope (Nikon Eclipse TS100, Nikon Instruments, Badhoevedorp, The Netherlands). Each experiment included a positive and a negative control to ensure functionality of the assay.

2.4. Cell counting

We counted TRAP-positive multinucleated ($n \geq 3$) osteoclasts in three different wells per each group of treatment using an optical microscope (Nikon Eclipse TS100, Nikon Instruments, Badhoevedorp,The Netherlands). Multinucleated TRAP-positive cells were identified as osteoclasts.

2.5. Bone resorption assay

The osteoclast bone resorption assay was performed by using a commercially available bone resorption assay kit (CosMo Bio, Tokyo, Japan). OCs were differentiated in calcium phosphate coated 24-multiwell in presence or not of MP [50 nM] co-treated or not with JWH-133 [100 nM] and AM630 [10 μM]. The drugs were added from day 14. In order to measure the pit areas, 5% NaOCl was used to remove cells at day 21. The resorbed areas on the plate were visualized under light microscopy (Nikon Eclipse TS100, Nikon Instruments, Badhoevedorp, The Netherlands).