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Bisphosphonates activate nucleotide receptors signaling and induce the expression of Hsp90 in osteoblast-like cell lines

Milena Romanello [a,1], Nicoletta Bivi [a,1], Alex Pines [a], Marta Deganuto [a], Franco Quadrifoglio [a], Luigi Moro [b,c], Gianluca Tell [a,*]

[a] *Department of Biomedical Sciences and Technologies and the Center for Regenerative Medicine (C.I.M.E.), University of Udine, p.le Kolbe 4, 33100 Udine, Italy*
[b] *Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Trieste, Italy*
[c] *Center for the Study of Metabolic Bone Diseases, Gorizia, Italy*

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Abstract

Bisphosphonates are the most important drugs used in the treatment of osteoporosis as they inhibit osteoclast resorption and stimulate proliferation of osteoblasts. However, the molecular mechanisms responsible for these effects are still poorly elucidated. It is known that nucleotide receptors-mediated signaling plays a central role in modulating osteoblasts growth in response to mechanical stress. By using osteoblast-like cell lines (i.e., HOBIT, MG-63, ROS P2Y), which express P2Y receptors, we found that the treatment with risedronate promotes non-lytic ATP release leading to activation of ERKs through the involvement of P2Y receptors triggering. A major role in this signal transduction pathway seems to be the involvement of $P2Y_1$ and $P2Y_2$ receptors, since the stimulatory effect of risedronate on ERKs is not appreciable in ROS 17/2.8 cells, which do not express these two receptors. Differential proteomics analysis identified Hsp90 upregulation as a result of risedronate effect on HOBIT and MG-63 cells. The stimulatory effect is dependent on ERKs activation involving nucleotide receptors triggering and leads to increased proliferation of osteoblast-like cells. In fact, functional inactivation of Hsp90 by the specific inhibitor 17-AAG prevents the bisphosphonate-induced mitogenic effects in osteoblasts. These findings show that bisphosphonates, by inducing ATP release, may also act through nucleotide receptors signaling leading to ERKs activation and may exert their mitogenic role on osteoblasts through the involvement of Hsp90.

Keywords: Osteoblasts; P2 receptors; Bisphosphonates; ATP release; Hsp90

Introduction

Bisphosphonates (BPs), like risedronate and alendronate, are small charged molecules highly used in osteoporosis treatment as they prevent bone loss by inhibiting osteoclasts resorption [1–3]. It is known that BPs are able to trigger osteoclasts apoptosis by inhibiting prenylation of survival proteins such as Ras [4,5]. However, a growing body of evidences suggests that BPs effects on bone are not exclusively restricted to their toxic action on osteoclasts [6–8]. In fact, in the concentration range 10^{-9} to 10^{-6} M, BPs prevent etoposide and cortisone-induced osteoblasts and osteocytes apoptosis by a mechanism involving active ERKs phosphorylation [6–8] and induce cellular proliferation and expression of specific osteoblastic genes [9,10]. Moreover, it has been demonstrated that biological effects of bisphosphonates on osteoblasts proliferation is mediated by L-type voltage-sensitive Ca^{2+} channels (VSCC) through ERKs activation [9]. In all cases, the molecular mechanisms of such effects are poorly understood. As far as the ERKs activation is concerned, a recent work by Plotkin et al., pointed to an involvement of Cx-43 hemichannels transient opening as the first event triggered by BPs treatment. The authors suggested that BPs, due to their charged nature, exert part of their effects by using a sort of 'membrane-receptor triggering' by means of Cx-43 direct binding [7].

Abbreviations: 17-AAG, 17-demethoxygeldanamycin; 18-GA, 18α-glycyrrhetinic acid; BPs, bisphosphonates; CBX, carbenoxolone; Cx-43, connexin-43; ERKs, extracellular signal regulated kinases; Hsp90, heat shock protein-90; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; MTT, 3-dimethylthiazol-2,5-diphenyltetrazolium bromide; PGE_2, prostaglandin E_2; PKC, protein kinase C; VSCC, L-type voltage-sensitive Ca^{2+} channels.

* Corresponding author. Fax: +39 0432 494301.
E-mail address: gtell@mail.dstb.uniud.it (G. Tell).

[1] First two authors equally contributed to the present work.

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Dynamic mechanical loading increases bone density and strength and promotes osteoblast proliferation, differentiation and matrix production, by acting at the gene expression level. Several evidences point to extracellular nucleotides (i.e., ATP and UTP) as soluble factors released, by non-lytic mechanisms, in response to mechanical stimulation in different cell systems, including osteoblasts [11–19]. These soluble mediators play a major role in modulating cellular response to mechanical stress through activation of signaling pathways upon binding to their own receptors (i.e., nucleotide receptors). We have previously shown that mechanical stress induces both the expression and the activity of Egr-1 and Runx2 transcription factors via ATP release and stimulation of P2Y receptors in the human osteoblastic HOBIT cell line [19,20]. Extracellular nucleotides exert stimulatory effects on eukaryotic cells via the P2 family of membrane-bound receptors, which comprises metabotropic P2Y (G-protein-coupled) and ionotropic P2X (ligand-gated ion channel) receptors. Extracellular ATP improves the mitogenic action of several growth factors in a variety of cell types [21,22] through the control of transcriptional activators, such as AP-1 [23] and Egr-1 [19], via stimulation of P2Y receptors. These effects are mainly played through the simultaneous increase of two important second messengers, Ca^{2+} ion and 1,2 diacylglycerol, a physiological activator of protein kinase C (PKC) [24]. Molecular mechanisms responsible for the non-lytic release of ATP are poorly known. Very recently, Genetos et al. provided evidences that activation of VSCC is centrally involved in the fluid-shear stress-induced non-lytic extrusion of ATP that may act, in turn, as an autocrine factor in the PGE_2 release by osteoblasts [11].

In light of these evidences and considering the hypothesis that BPs action is mediated by an involvement of nucleotide receptors, we investigated the possible existence of cross-talks between BPs effects and the activation of P2 receptors. By using the human osteoblast-like HOBIT and the MG-63 cell lines, together with other different osteoblast-like cell lines (i.e., ROS P2Y and ROS17/2.8), stimulated with risedronate or alendronate, we demonstrated that, indeed, BPs activation of ERKs phosphorylation is mediated by P2 receptors triggering. Interestingly, this effect is due, at least in part, to the ability of BPs to significantly promote non-lytic ATP release by stimulated cells. Moreover, the activatory role exerted on ERKs phosphorylation is mainly due to $P2Y_1$ and $P2Y_2$ receptors activation, as demonstrated by the inability of ROS17/2.8 cell line, which do not express these receptors, to actively respond to BPs treatment in contrast to ROS P2Y cells. In addition, by using a differential proteomic approach to find early targets of BPs action in HOBIT cells, we identified an unsuspected molecular target of BPs stimulatory effects, i.e., Hsp90, that could explain the proliferative effect exerted by these drugs on osteoblasts. Therefore, these data open completely new views in the comprehension, at the molecular level, of the BPs effects on osteoblasts physiology.

Materials and methods

Cell culture and chemicals

HOBIT cells were kindly provided by Prof. B. Lawrence Riggs, Mayo Foundation, Rochester, MN, USA and were grown in Dulbecco's Minimal Essential Medium F-12 (DMEM/F-12) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. ROS 17/2.8 and ROS P2Y were kindly provided by Prof. R. Civitelli, Washington University School of Medicine, St. Louis, MO, USA. MG-63 cells were kindly provided by Dr. G. Girasole, Dept. of Internal Medicine and Biomedical Sciences, University of Parma, Italy. MG-63 and ROS17/2.8 cells were grown in DMEM culture medium containing 10% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were seeded in 100 mm dishes and grown at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Both ATP and bisphosphonates treatments were performed on cells after 48 h of serum starvation and in medium w/o serum.

2′,7′-bis(carboxyethyl)-5-(6′)-carboxyfluorescein (BCECF-AM), Fura-2-AM and Pluronics were obtained from Molecular Probes (Eugene, OR, USA).

All the other chemicals described below were from Sigma Aldrich Co. (Milan, Italy) unless otherwise specified. The bisphosphonates used in this study were provided by Procter and Gamble Pharmaceuticals (Cincinnati, OH, USA).

Cell viability assay

An MTT assay was used for growth experiments in a 96-well plate in hexaplicate. The MTT assay assesses cell viability by measuring the mitochondrial function [25]. Cells at 80% confluence were harvested with 1× trypsin/EDTA solution and seeded into a 96-well plate at $3–5 \times 10^3$ cells/well and maintained o/n in medium w/o serum plus 0.1% w/v BSA. After incubation with risedronate ±17-(allylamino)-17-demethoxygeldanamycin (17-AAG) for 24 h, 48 h, or 72 h, a 1/10 volume of MTT solution (4 mg/ml in PBS) was added and incubated for 3 h under 5% CO_2/95% air at 37°C. Then, the supernatant was aspirated and an equal volume of DMSO was added to the cells, and the MTT formazan was dissolved by pipetting. The absorbance was measured on an enzyme-linked immunosorbant assay (ELISA) plate reader (EL808 Ultra Microplate Reader Bio-Tek Instruments, Inc., Winooski, VT, USA) with a test and reference wavelength of 570 and 690 nm, respectively. Confirmatory data were obtained by assessment of cell proliferation through direct cell counting [10].

Evaluation of apoptosis by annexin V staining

Osteoblast-like cells were seeded in 35 mm dishes and grown up to 70% confluence. Cells were then maintained in medium w/o serum plus 0.1% BSA for 24 h and further treated with vehicle, risedronate (10^{-7} M), 17-AAG (10 μM) or risedronate (10^{-7} M) plus 17-AAG (10 μM) for 24 h, 48 h or 72 h. Cells were then harvested with trypsin to evaluate apoptosis. Apoptosis was assessed by staining of phosphatidylserine exposed on cell membranes with FITC labeled Annexin V [26], according to manufacturer's instruction (Roche Diagnostic Italia, Monza, Italy). Samples were analyzed by flow cytometry [27] by using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA).

Luciferin–luciferase bioluminescence assay

Cells were plated at a density of 2×10^4 cells/cm^2 onto 6-well plates. 24 h after plating, the cells were rinsed with phosphate buffered saline (PBS) and the medium was replaced with 1 ml of fresh, serum-free DMEM/F12 medium in which the cells were grown for 48 h. At the end of starvation, the cells were treated with 10^{-7} M alendronate or risedronate or with vehicle for 2, 5 and 10 min at 37°C. Particular care was taken to minimize mechanical perturbation of cells during these procedures. Luciferin bioluminescence was measured in the cells supernatants. The extracellular ATP concentration was determined using the ATP-Determination kit CLS II from Roche (Roche Diagnostics, Mannheim, Germany). 50 μl of the luciferin–luciferase assay medium, dissolved in DMEM/F12 medium, were added to 50 μl of sample into the cuvette. The resulting light signal was immediately measured by a Turner TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). A calibration curve was generated for each luciferase assay using serial dilution of an ATP standard. All reagents used to stimulate cells were tested in control experiments. Each experiment was repeated at least 4 times in triplicate. Statistical differences among groups were evaluated by unpaired *t* test.

Membrane leakage after BPs treatments was evaluated by measuring lactate dehydrogenase (LDH) activity in the medium, indicating possible mechanically or drug induced cell damage, and was measured according to the recommendations reported in [28]. In addition, trypan blue exclusion and loss of intracellular