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Research Article

Osteoblastic protein tyrosine phosphatases inhibition and connexin 43 phosphorylation by alendronate

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ABSTRACT

Bisphosphonates (BPs), potent inhibitors of bone resorption which inhibit osteoclasts, have also been shown to act on osteocytes and osteoblasts preventing apoptosis via connexin (Cx) 43 hemichannels and activating the extracellular signal regulated kinases ERKs. We previously demonstrated the presence of a saturable, specific and high affinity binding site for alendronate (ALN) in osteoblastic cells which express Cx43. However, cells lacking Cx43 also bound BPs. Herein we show that bound [^{3}H]-alendronate is displaced by phosphatase substrates. Moreover, similar to Na_3VO_4, ALN inhibited the activity of transmembrane and cytoplasmic PTPs, pointing out the catalytic domain of phosphatases as a putative BP target. In addition, anti-phosphotyrosine immunoblot analysis revealed that ALN stimulates tyrosine phosphorylation of several proteins of whole cell lysates, among which the major targets of the BP could be immunochemically identified as Cx43. Additionally, the transmembrane receptor-like PTPs, RPTPμ and RPTPα, as well as the cytoplasmic PTP1B, are highly expressed in ROS 17/2.8 cells. Furthermore, we evidenced that Cx43 interacts with RPTPμ in ROS 17/2.8 and ALN decreases their association. These results support the hypothesis that BPs bind and inhibit PTPs associated to Cx43 or not, which would lead to the activation of signaling pathways in osteoblasts.

Introduction

The bisphosphonates (BPs) are small molecular size (<300 Da) organic pyrophosphate analogues in which two phosphates are connected by a carbon atom (P–C–P) with various side chains. This chemical structure gives them resistance to enzymatic degradation.

BPs can be very powerful inhibitors of bone resorption and their potency vary according to their structure. Alendronate (ALN) is a potent amino-BP commonly used in the treatment of osteoporosis.

Abbreviations: BPs, bisphosphonates; ALN, alendronate; OPD, olpadronate; ETI, etidronate; PPi, inorganic pyrophosphate; PTP, protein tyrosine phosphatase; RPTP, receptor-like PTP; *p*NPP, *p*-nitrophenylphosphate; AF-ALN, fluorescently labeled (Alexa Fluor-488) analog of alendronate; [^{3}H]-ALN, tritiated alendronate; Cx43, connexin 43; phosphatase inhibitor: NaF, sodium fluoride; phosphatase inhibitor: Na_3VO_4, orthovanadate

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It is established that BPs inhibit resorption by acting on osteoclasts to reduce their activity or to increase the rate of apoptosis [1]. Several pieces of evidence support the notion that BPs also influence directly the function of cells of the osteoblastic lineage, and that this effect may in turn contribute to the reduction in osteoclast formation and activity. However, the molecular target of ALN, as well as of other BPs, in osteoblasts is still unknown.

It has been previously reported that BPs inhibit osteocyte and osteoblast apoptosis [2]. Moreover, we evidenced that both BPs and the protein-tyrosine phosphatase (PTP) inhibitor Na_3VO_4, increased proliferation of osteoblasts [3]. In addition, we demonstrated in osteoblasts the existence of a saturable, specific and high affinity binding site for the bisphosphonates Olpadronate (OPD) [3] and ALN [4]. In view of these observations, in the present paper we have addressed the possibility that PTPs contain a binding site for BPs.

Protein-tyrosine phosphorylation plays an important role in the signal transduction pathways that control cellular growth, differentiation, and metabolism. Tyrosine phosphorylation levels are controlled by the dynamic equilibrium between the activity of protein-tyrosine kinases (PTK) and PTPs. The latter comprise a diverse family of tyrosine phosphatases which is divided into two main groups: the receptor-like forms, such as CD45 (leukocyte common antigen), RPTPα and RPTPμ; and the cytoplasmic forms, such as PTP1B. Each of the PTPs contains at least one conserved segment of 240 amino acid residues corresponding to the catalytic domain [5]. This study provides information on the expression of these PTP subclasses in ROS 17/2.8 osteoblast-like cells.

Short-term studies in ewes showed that low doses of the phosphatase inhibitor NaF stimulate bone formation by increasing the recruitment and lifespan of osteoblasts [6]. Furthermore, the specific PTP inhibitor orthovanadate, as well as NaF, has been used as anti-osteoporotic drugs in rats and humans, respectively [7,8]. Therefore, we also evaluated herein the effect of the anti-osteoporotic ALN on PTP activity.

The prosurvival effect of BPs in osteoblasts and osteocytes is strictly dependent on the expression of the C-terminal cytoplasmic domain of connexin (Cx) 43, by which the protein interacts with Src kinase, an upstream activator of ERK [9]. However, it is dispensable for ALN cellular binding [4]. The tyrosine phosphatase inhibitor pervanadate inhibits Cx43-based gap junction communication, accompanied by enhanced tyrosine phosphorylation of Cx43 [10,11], but the identity of the PTP(s) that acts (act) on Cx43 remains unknown.

Within the above context, in the present work we propose to elucidate the molecular mechanism involved upstream the signal transduction pathway activated by BPs in osteoblastic cells.

Materials and methods

Reagents

Alendronate was provided by Gador S.A. (Buenos Aires, Argentina); *p*-nitrophenylphosphate, phenol red-free αMEM and Na_3VO_4, inhibitor of protein tyrosine phosphatase were from Sigma–Aldrich Co. (St. Louis, MO, USA). Bovine calf serum and fetal bovine serum were from Hyclone (Logan, UT, USA). Alendronate sodium salt [2, 3-^{3}H] was from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). AF-488 was provided by Invitrogen Life Technologies (Grand Island, NY, USA). Rabbit polyclonal antibody recognizing Cx43 and phosphoCx43 (Tyr265) or anti-actin mouse polyclonal antibody were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Anti-RPTPα rabbit and anti-PTP1B goat polyclonal antibodies, anti-rabbit and anti-mouse peroxidase-conjugated secondary antibodies and protein-G PLUS agarose were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-RPTPμ mouse monoclonal antibody was from Cell Signaling (Danvers, MA, USA). Anti-phospho-tyrosine mouse monoclonal antibody was from UBI (NY, USA). Protein size markers, Immobilon P (polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, England). All other reagents used were of analytical grade.

Cell culture

ROS 17/2.8 osteoblasts (rat osteosarcoma-derived) and HeLa cells were cultured at 37 °C in phenol red-free α-MEM supplemented with 10% FBS, 1% each of penicillin, streptomycin and glutamine, under humidified air (5.5% CO_2) and grown at 70–80% of confluence; 1% minimum essential amino acids were added to the medium for HeLa cells.

Binding assay

Binding of [^{3}H]-alendronate was performed in intact cell monolayers. ROS 17/2.8 cells were incubated with 30 nM [^{3}H]-alendronate (specific activity 30 Ci/mmol) for 120 min at 30 °C in the absence (total binding) or presence (non-specific binding) of 200 μM unlabeled BPs (Alendronate, ALN; Etidronate, ETI and Olpadronate, OPD), 8 mM *p*-nitro-phenylphosphate or 8 mM α-naftylphosphate, two common protein phosphatase substrates. Results are expressed as a percent of total binding. Each value is the mean ± SD of results from three separate experiments performed in triplicate.

Synthesis of fluorescently labeled alendronate

Alendronate labeled with Alexa Fluor-488 (AF-488) was synthesized in our laboratory following the method described by Thompson et al. [12].

Confocal microscopy

To analyze the uptake of AF-ALN by ROS 17/2.8 cells and its co-localization with RPTPμ, cell cultures grown onto glass coverslips were incubated with AF-ALN for 30 min at the concentration indicated. Subsequently, cells were washed in PBS and fixed for 10 min in 4% (v/v) *p*-formaldehyde and permeabilized with 0.05% Triton X-100. Non-specific sites were blocked with PBS, 5% BSA. Then, cells were incubated with the specific anti-RPTPμ antibody for 1 h, washed twice with PBS and incubated for additional 1 h in the presence of secondary FITC-conjugated antibody (1:200, room temperature). To determine co-localization of Cx43 and RPTPμ, osteoblastic cells treated with vehicle (control) or 0.1 μM ALN were fixed and incubated with a solution containing anti-Cx43 and anti-RPTPμ antibodies. Then washed and labeled with specific