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Role of connexin 43 in the mechanism of action of alendronate: Dissociation of anti-apoptotic and proliferative signaling pathways

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ABSTRACT

Bisphosphonates (BPs) inhibit osteocyte and osteoblast apoptosis via opening of connexin (Cx) 43 hemichannels and activating the extracellular signal regulated kinases ERKs. Previously, we hypothesized that intracellular survival signaling is initiated by interaction of BPs with Cx43. However, using whole cell binding assays with [^{3}H]-alendronate, herein we demonstrated the presence of saturable, specific and high affinity binding sites in the Cx43-expressing ROS 17/2.8 osteoblastic cells, authentic osteoblasts and MLO-Y4 cells expressing Cx43 or not, as well as in HeLa cells lacking Cx43 expression and ROS 17/2.8 cells pretreated with agents that disassemble Cx channels. In addition, both BPs and the PTP inhibitor Na_3VO_4 increased proliferation of cells expressing Cx43 or not. Furthermore, although BPs are internalized and inhibit intracellular enzymes in osteoclasts, whether the drugs penetrate non-resorptive bone cells is not known. To clarify this, we evaluated the osteoblastic uptake of AF-ALN, a fluorescently labeled analog of alendronate. AF-ALN was rapidly internalized in cells expressing Cx43 or not indicating that this process is not mediated via Cx43 hemichannels. Altogether, these findings suggest that although required for triggering intracellular survival signaling by BPs, Cx43 is dispensable for cellular BP binding, its uptake, as well as the proliferative effects of these agents.

Introduction

Bisphosphonates (BPs)[1] are small molecular size (<300 Da) stable analogues of natural inorganic pyrophosphate, with a carbon atom replacing the oxygen atom that connects the two phosphates. The R1 and R2 side-chains attached to the carbon atom are responsible for the large range of activity observed among the BPs [1]. Although the potent amino-BP alendronate (ALN) is widely used for treating diseases associated with increased bone resorption, such as postmenopausal osteoporosis, Paget's disease, among others, its mechanism of action is not completely understood.

It is now known that BPs act directly on osteoclasts and interfere with specific intracellular biochemical processes such as isoprenoid biosynthesis and subsequent protein prenylation to inhibit cell activity [2]. Specifically, it was reported that nitrogen-containing BPs inhibit farnesyl pyrophosphate synthase in osteoclasts [3]. However, recent studies suggest that osteocytes may be important target cells for BPs in bone. Many BPs protect osteocytes and osteoblasts from apoptosis induced by glucocorticoids in vitro [4]. The inhibition of osteocyte apoptosis by BPs requires opening of connexin 43 (Cx43) hemichannels and subsequent activation of extracellular signal-regulated kinases (ERKs) [5]. However, the events elicited by BPs upstream of hemichannel opening remain unknown.

All bisphosphonate drugs, by virtue of their P–C–P backbone structure, target to calcified tissues, where they are released and internalized by bone-resorbing osteoclasts [6]. Cellular uptake of bisphosphonates by osteoclasts, like other negatively charged compounds, probably occurs initially by endocytosis [7]. However, additional steps must be involved in osteoclasts in order to translocate the compounds from intracellular, endocytic vacuoles to their site of action in the cytosol. Recent studies suggest the presence of a transport mechanism or recognition step on osteoclasts and macrophages, either on the plasma membrane or on vacuolar membranes [8].

Despite the recent major advances in understanding the molecular mechanisms of action of BPs, the route by which they are internalized by non-resorbing bone cells such as osteoblasts and osteocytes is still not understood. In the present study, we synthesized a fluorescently labeled analog of alendronate (AF-ALN) to visualize the cellular uptake of ALN by confocal microscopy.

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[1] *Abbreviations used:* BPs, bisphosphonates; OPD, olpadronate; ALN, alendronate; PPi, inorganic pyrophosphate; PTP, protein tyrosine phosphatases; pNPP, *p*-nitrophenylphosphate; AF-ALN, fluorescently labeled analog (AlexaFluor-488) of alendronate; Cx43, connexin 43; AGA, glycyrrhetinic acid; GA, glycyrrhizic acid.

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Bisphosphonates, due to their small molecular size, could enter cells upon inducing opening of the hemichannels. We then examined whether AF-ALN cellular internalization depends on Cx43 expression.

As a result of the low concentration of BP required to induce its effect, the existence of a receptor entity was initially proposed by Fleisch [9], nevertheless this molecular BP target in osteoblastic cells has not been elucidated. In this regard, we have recently reported that olpadronate specifically binds to osteoblastic cells [10]. The requirement of Cx43 for antiapoptosis by BPs has raised the possibility that interaction of BPs with Cx43 present in the cell membrane results in hemichannel opening, thereby initiating intracellular survival signaling. Herein we evaluate [^{3}H]-ALN specific binding to osteoblastic cells pre-treated with Cx disassembling agents and HeLa cells lacking Cx43 expression.

Some studies reveal that culture of osteoblastic cells in the presence of BPs increases proliferation, stimulates differentiation towards the osteoblastic lineage, and enhances mineralization [11]. Within this context, we examined the anabolic effect of ALN in cells that express Cx43 or not, by determining the rate of DNA synthesis in ROS 17/2.8 and HeLa cells. In this work we propose the dissociation, in terms of Cx43 participation, of both proliferative and anti-apoptotic BPs effects in bone forming cells and we support the involvement of a phosphatase in the mechanism of action of BPs.

Materials and methods

Reagents

Alendronate was provided by Gador S.A. (Buenos Aires, Argentina); *p*-nitrophenylphosphate, etoposide, calf skin collagen type I, 18α-glycyrrhetinic acid, glycyrrhizic acid, oleamide, carbenoxolone and inhibitors of protein phosphatase (NaF, Na_3VO_4, and okadaic acid) and rabbit polyclonal antibody recognizing Cx43 or anti-actin mouse polyclonal antibody were from Sigma–Aldrich Co. (St. Louis, MO, USA). Anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein size markers, Immobilon P (polyvinylidene difluoride) membranes and ECL chemiluminescence detection kit were from GE Healthcare (Little Chalfont, Buckinghamshire, England). Bovine calf serum and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA). Phenol red-free α-minimum essential medium (αMEM), trypsin–EDTA and Lipofectamine Plus were obtained from Gibco BRL (Carlsbad, CA, USA). [2,3-3H]-alendronate, sodium salt was purchased from Moravek Biochemicals and Radiochemicals (Brea, CA, USA). The constructs encoding Cx43 and the corresponding empty vector (provided by Dr. Civitelli, Washington University, St. Louis, MO, USA) and the nuclear green fluorescent protein (nGFP) were described previously [4,5]. All other reagents used were of analytical grade.

Cell culture

The murine long bone-derived osteocytic cell line MLO-Y4 was cultured as previously described [4]. HeLa cells and ROS 17/2.8 osteoblastic cells (rat osteosarcoma-derived) were cultured at 37 °C in phenol red-free α-MEM supplemented with 10% FBS, 1% each of penicillin, streptomycin, glutamine, under humidified air (5.5% CO_2) and grown at 70–80% of confluence. For HeLa cells, 1% minimum essential amino acids was added to the medium. Calvarial osteoblasts were obtained from 5-day-old neonatal rats. Briefly, calvaria were incubated in PBS containing 4 mM EDTA at 37 °C for two 10-min periods and the supernatants were discarded. Subsequently, calvaria were rinsed in PBS and subjected to digestion with 200 U/ml collagenase in PBS for four 15-min periods. Cells released during the first digestion were discarded and those released during the subsequent digestions were spun down, collected and combined after centrifugation for 10 min at 1500 rpm. Then, cells were cultured at 37 °C in α-MEM supplemented with 10% FBS, 1% penicillin and streptomycin under humidified air (5.5% CO_2).

Silencing of Cx43 expression

The expression of Cx43 in MLO-Y4 osteocytic cells was silenced using MISSION short hairpin (sh)RNA lentiviral particles (Sigma), following the manufacturer's instructions [12]. Briefly, cells were infected with lentiviral particles carrying either scrambled or Cx43-specific shRNA. Stable cell lines were established by selection with puromycin (Sigma). The efficiency of deletion was determined by measuring Cx43 protein by Western blotting.

Western blot

Protein lysates from MLO-Y4 cells were prepared as previously reported [4]. Proteins were separated on 10% SDS–polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. Immunoblottings were performed using a rabbit anti-Cx43 antibody or mouse anti-actin antibody. After incubation with primary antibodies, blots were exposed to anti-rabbit or anti-mouse antibody conjugated with horseradish peroxidase and developed using a chemiluminescence substrate.

Quantification of apoptotic cells

Trypan blue uptake

Cells were treated with 8 mM *p*-NPP for 30 min. Subsequently, vehicle (PBS) or 10^{-7} M ALN were added and 30 min later, vehicle (DMSO) or 50 mM etoposide. Cells were cultured for additional 6 h. Non-adherent cells were combined with adherent cells released from the culture dish using trypsin–EDTA, re-suspended in medium containing serum, and collected by centrifugation. Subsequently, 0.04% trypan blue was added and the percentage of cells exhibiting both nuclear and cytoplasmic staining was determined using a hemocytometer. At least 500 cells from fields selected by systematic random sampling were examined for each experimental condition.

Nuclear morphology

HeLa cells were transiently transfected with Cx43 or vector along with nuclear green fluorescent protein, in order to evaluate apoptosis only in transfected cells. Forty-eight hour after transfections cells were treated with vehicle (PBS) or 10^{-7} M ALN for 30 min. Subsequently, vehicle (DMSO) or 50 mM etoposide were added. Cells were cultured for additional 6 h and fixed with neutral buffer formalin. Apoptosis was evaluated by examining nuclear morphology of fluorescent cells and those exhibiting chromatin condensation and/or nuclear fragmentation were considered apoptotic.

[^{3}H]-thymidine incorporation assay

ROS 17/2.8 and HeLa cells were cultured in 24-multiwell plates at a density of 14,000 cells per well. After 48 h, the medium was replaced by medium without FBS and the cells were starved for 18–21 h. Cell treatment was performed in quadruplicate by adding the indicated agonists or vehicle. Before the treatment was finished, 0.5 μCi [^{3}H]-thymidine/well was added and the cells were further incubated for 1 h at 37 °C. The treatment was stopped by aspirating the medium and washing three times with PBS. Cells