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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.

Keywords

bisphosphonates; Cx43 hemichannels; AF-ALN uptake; osteoblast survival

INTRODUCTION

Bisphosphonates (BPs) are small molecular size (<300 Da) stable analogues of natural inorganic pyrophosphate, with a carbon atom replacing the oxygen atom that connects the

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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. 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, 18 α-glycyrrhetinic acid, glycyrrhizic acid, oleamide, carbenoxolone and inhibitors of protein phosphatase (NaF, Na_3VO_4, 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 4mM 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 1,500 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 pNPP 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. 48 h 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 48h, the medium was replaced by medium without FBS and the cells were starved for 18–21h. 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 were incubated 30 min at 4°C with 12% trichloroacetic acid (TCA) per well and then washed three times with cold 12% TCA. The samples were dissolved with 1 N NaOH and the radioactivity was counted in a liquid scintillation counter. Proteins were quantified by the Bradford method [13]. [^{3}H]-thymidine incorporation was quantified as cpm per milligram of protein and the results expressed as % stimulation with respect to the control.

Binding assay

Binding of [^{3}H]-alendronate was performed in intact cell monolayers. ROS 17/2.8 cells pretreated with vehicle (control) or agents that disassemble Cx hemi-channels (100 μM 18 α-glycyrrhetinic acid (AGA), 100 μM glycyrrhizic acid (GA), 1 μM oleamide or 100 μM carbenoxolone), MLO-Y4 osteocytic cells and HeLa 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 ALN. Results are expressed in fmol/mg protein.

For the saturation analysis rat calvaria-derived osteoblasts or ROS17/2.8 cells were incubated for 120 min at 30°C with increasing concentrations of [^{3}H]-alendronate (4–4000nM) in the presence (nonspecific) or absence of 200 μM unlabeled alendronate. After extensive washes with ice-cold PBS, cells were lysed with 1 ml 0.8 N NaOH, and cell-bound [^{3}H]-alendronate was determined by measuring the radioactivity in a liquid scintillation counter and normalized per mg of protein. Scatchard plots of specific [^{3}H]-alendronate binding were obtained with the Prism GraphPad program. Each value is the mean ± SEM of results from three separate experiments performed in triplicate.

Synthesis of fluorescently labeled alendronate

Alendronate labeled with Alexa Fluor-488 (AF-488, Invitrogen) was synthesized in our laboratory following the method described by Thompson et al. [6].

Confocal microscopy

Confocal microscopy was used to analyze AF-ALN uptake by ROS 17/2.8 and HeLa cells. Cell cultures were incubated with AF-ALN for 30 min at the concentrations indicated. Subsequently, cells were washed in PBS and fixed for 10 min in 4% (v/v) para-formaldehyde. To determine the specificity of BP cellular internalization, HeLa cells were treated with AF-ALN together with unlabeled ALN in excess. Cells were examined on a Zeiss LSM 5 Pascal confocal microscope.

Statistical analysis

Statistical significance of data was evaluated using Student's t-test [14] and probability values below 0.05 ($P < 0.05$) or 0.01 ($P < 0.01$) were considered significant or highly significant, respectively. Quantitative data are expressed as mean ± standard deviation (SD) from the indicated set of experiments. Data for apoptosis of cultured cells (Fig. 5) were analyzed by one-way analysis of variance (ANOVA), and the Student-Newman-Keuls method was used to estimate the level of significance of differences between means.

RESULTS

Specific and saturable binding site for [^{3}H]-ALN in osteoblastic cells

We examined the existence of BP binding sites in ROS 17/2.8 cells and primary cultures of rat osteoblasts by competitive binding assays with tritiated alendronate ([^{3}H]-ALN). As a first step we optimized the experimental conditions performing time-course and cellular density assays in the presence of 30 nM [^{3}H]-ALN. The results suggested that [^{3}H]-ALN-specific binding equilibrium was reached seeding 1×10^5 cells/well and performing the binding assay at 30°C for 90 to 180 min, with a nonspecific binding lower than 1% of the total binding (supplemental data). Saturation analysis was carried out in whole rat calvaria-derived osteoblasts or ROS17/2.8 cells. After incubation for 120 min at 30°C with increasing concentrations of [^{3}H]-ALN (4–4000 nM) in the presence (nonspecific) or absence of 200 μM of unlabeled ALN, radioactivity of the cell lysates was determined and specific binding was computed by subtracting background counts from total cpm. The results show a saturable and specific site, with high affinity for ALN binding (Fig. 1A). Scatchard plot of specific [^{3}H]-ALN binding was obtained with the Prism GraphPad program which yielded a Kd= 0.65 M ± 0.2 M and Bmax.= 3986±432 fmol/mg protein (Fig. 1B). The Scatchard plot obtained was linear, indicating that [^{3}H]-ALN binds to a single site in ROS 17/2.8 cells.

Cx43 is not required for bisphosphonate binding

To determine whether Cx43 is necessary for BP binding to osteoblastic cells we performed a competitive binding assay in HeLa cells that do not express Cx43 and ROS 17/2.8 cells pretreated with 18 α-glycyrrhetinic acid (AGA), glycyrrhizic acid (GA), oleamide or carbenoxolone; agents that disassemble Cx hemichannels, at the concentrations indicated (Fig. 2A). We found that the specific binding of [^{3}H]-ALN to osteoblastic cells was not modified by pretreatment with any of the disassembling agents studied. In addition, we demonstrated that [^{3}H]-ALN also bound specifically to cells that do not express Cx43. We next studied the requirement of Cx43 expression for bisphosphonate binding in cultured osteocytic cell line by silencing the expression of Cx43 using shRNA. Cx43 expression was decreased in MLO-Y4 cells treated with Cx43 shRNA to 58% of cells treated with

scrambled shRNA (control) (Fig. 2C). We found no significative differences of [^{3}H]-ALN specific binding to control cells as well as in MLO-Y4 in which Cx43 was silenced (Fig. 2B). These results are consistent with those observed in HeLa cells and osteoblasts pretreated with agents that disassembled hemichannels and indicate that BPs bind to an entity different from Cx43.

ALN induces osteoblasts proliferation independently of Cx43 expression

ALN stimulated the synthesis of DNA in ROS 17/2.8 cells, as revealed by [^{3}H]-thymidine incorporation assays (Fig. 3A). Cell exposure to 10 and 100 μM ALN for 48 h increased the synthesis of DNA by ~40 and ~60% over the control, respectively. We did not observe responses employing either a lower concentration of ALN (1 μM) or a shorter incubation time of cells with the BP (24 h). According to previous studies of our laboratory [10], we employed phosphatase inhibitors as positive controls of cellular proliferation. Both, NaF and Na_3VO_4, stimulated [^{3}H]-thymidine incorporation at 24 and 48 h of treatment.

To evaluate if Cx43 mediates the stimulation of cell proliferation by bisphosphonates, we used HeLa cells which lack Cx43 expression [5] and determined [^{3}H]-thymidine incorporation into DNA (Fig. 3B). Treatment of HeLa cells with ALN resulted in a significant increase in cell proliferation that was dose-dependent. Maximal stimulation of DNA synthesis was achieved with 100 μM ALN after 24 h treatment (35.7% over control), whereas at 48 h the increment was lower (19.1% over control). Similarly to ALN, the tyrosine phosphatase inhibitor Na_3VO_4 significantly increased proliferation of HeLa cells in a dose-dependent manner after 24 h of treatment. NaF, a Ser/Thr phosphatase inhibitor, also induced a significant increase in DNA synthesis, that was independent of the dose employed, as well as Okadaic acid, a potent inhibitor of the serine threonine protein phosphatases PP2A and PP1. On the other hand, no significant proliferative effects were observed with any of these inhibitors at 48 h. These results altogether indicate that Cx43 is not involved in the proliferative effects exerted by ALN and suggest the participation of a PTP.

Alendronate is internalized by cells and Cx43 is not the route of access

It is well known that BPs are internalized by osteoclasts during the resorption process of bone, however the ability of non-resorptive bone cells to incorporate these drugs remains unknown. We have previously reported that [^{3}H]-olpadronate uptake is a fast event, as 56% of total binding was taken up within 5 min. In addition, we observed specific [^{3}H]-olpadronate binding to different subcellular fractions obtained from osteoblastic cells [10] indicating non selective intracellular localization of the drug. To further study the osteoblastic BP uptake we synthesized ALN labeled fluorescently with Alexa Fluor 488 (AF-ALN) following the method described by Thompson et al. [6]. ROS 17/2.8 cells were incubated in the presence of 0.1 μM AF-ALN for 30 min. Phase contrast image showed that this protocol did not affect the characteristic morphology of osteoblasts (Fig. 4A, I). Green fluorescence could be observed throughout the cytosol although mostly distributed in the peri-nuclear region (Fig. 4A, II), indicating that ALN is rapidly incorporated by the cells. The latter was confirmed by the merge of images (Fig. 4A, III). When ROS 17/2.8 cells were treated with a higher concentration of AF-ALN (10 μM), we observed a similar pattern of fluorescence distribution within the cells (*data not shown*). Comparable results were found in primary culture of osteoblasts obtained from rat calvaria [15] (Fig. 4B).

To determine if Cx43 hemichannels are the route by which ALN is internalized by osteoblastic cells from bone microenvironment, we used HeLa cells to evaluate AF-ALN cellular uptake. HeLa cells were incubated with 0.1 μM AF-ALN for 30 min in the absence (*left images*) or presence of 500 μM unlabeled ALN (*right images*) and then analyzed by

microscopy (Fig. 4C). Cells that lack Cx43 expression also showed intracellular AF-ALN, distributed throughout the cytoplasm and completely excluded from nuclei. This cellular BP incorporation was significantly decreased in the presence of high concentrations of unlabeled ALN, indicating that there is a specific AF-ALN internalization by HeLa cells.

Anti-apoptotic effect of ALN requires Cx43 and is reversed by the presence of a phosphatase substrate

It has been previously shown that BP prevention of apoptosis in osteoblasts and osteocytes requires Cx43 hemichannel opening [5]. We evaluated the role of Cx43 in prevention of apoptosis in HeLa cells that express or not Cx43 and the effect of the presence of a phosphatase substrate. As demonstrated before, ALN was not capable to prevent apoptosis induced by etoposide in vector-transfected HeLa cells. However transfection of HeLa cells with Cx43 conferred responsiveness to the bisphosphonate. In addition, the phosphatase substrate p-nitrophenyl phosphate (p-NPP) reverses the anti-apoptotic effect of ALN, suggesting that a phospatase must be involved (Fig. 5A). A similar response was observed in MLO-Y4 osteocytic cells (Fig. 5B).

DISCUSSION

Bisphosphonates are well established as successful antiresorptive agents for prevention and treatment of osteoporosis. Differences among BPs exist and reside in their biochemical targets within the osteoclast inducing their apoptosis [16]. Some BPs, including ALN, trigger osteoclast apoptosis by inhibiting the mevalonate pathway which prenylate survival proteins, such as Ras, whereas others form cytotoxic analogs of ATP [17].

Over the years, different biochemical mechanisms for how bone resorption is inhibited have been proposed; including effects on phosphatases, kinases, proton pumps, and lysosomal enzymes [18].

It has been demonstrated that osteocytes are important target cells for BPs in bone and reported that many BPs protect osteocytes and osteoblasts from apoptosis induced by glucocorticoids, etoposide and TNFα *in vitro* [4]. The ability of BPs to inhibit apoptosis in osteocytes contrasts with their ability to induce apoptosis in osteoclasts and is mediated through connexin 43 (Cx43) hemichannels opening and subsequent activation of ERKs [19].

We have recently reported the existence of a recognition target molecule for olpadronate (OPD) in osteoblasts [10]. Taking into account the differences in BPs actions according to their chemical structure and that the specific activity for [^{3}H]-ALN is much lower than that for [^{3}H]-OPD employed, in this work it was necessary to set again the experimental conditions for ALN. Subsequently, through saturation studies we confirmed the existence of a single population of high affinity and specific binding sites for ALN in osteoblasts.

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. However, herein we demonstrate that, although Cx43 is necessary for ALN-induced survival of osteoblastic cells, this protein is dispensable for BP cell binding. Thus, [^{3}H]-ALN bound specifically to HeLa cells that do not express Cx43 and to ROS 17/2.8 osteoblastic cells pretreated with agents that disassemble Cx hemichannels, suggesting that BPs bind to another moiety that, in turn, interacts with Cx43. In addition, we found no significative differences on BP specific binding when performing binding assay in MLO-Y4 cells that express endogenous Cx43 compared to those in which Cx43 expression was efficiently silenced using shRNA. These results indicate that ALN specifically bound not only to osteoblastic cells but also to osteocytic cell lines. Moreover,

we confirmed that ALN binding to both cells type is independent of the presence of Cx43 hemichannel. Because bisphosphonate binding to osteoblastic cells was displaced by protein phosphatase substrates [10] the putative target molecule which links BPs with Cx43 could be a phosphatase. Interestingly, recent studies have shown that PTPμ interacts with the regulatory C-terminal tail of Cx43 [20].

Earlier work [21] as well as more recent evidence [10] have shown the proliferative effect of other BPs, such as OPD, in osteoblastic cells. Similarly, in this report we show that the treatment of ROS 17/2.8 cells with ALN for 48 h resulted in a significant increase in the rate of [^{3}H]-thymidine incorporation into DNA with a response profile comparable to that of PTP inhibitors. These results suggest that the stimulatory action of ALN on osteoblast proliferation can also be mediated by PTP inhibition. To perform these studies we used a wide range of ALN concentrations (10^{-7}- 10^{-4} M) since in bone tissue the osteoblastics cells are exposed to variable pharmacological doses of BP which depend on the amount of drug resorbed from the bone surface by active osteoclasts. In addition, it was reported that higher doses of BP are toxic for osteoblasts [22] and cells undergo apoptosis. As a positive control of cellular proliferation we employed different phosphatase inhibitors according to a previous report in which the same experimental approach was applied [10].

The observation that ALN promoted proliferation of HeLa cells lacking Cx43 expression implies that, even though the importance of the pore formation region and the C-terminal domain of this protein for BP prevention of osteoblast/osteocyte apoptosis, Cx43 is excluded from the signaling pathway that leads to BP proliferative effect of bone forming cells.

It is thought that BPs buried in the bone matrix reach the osteoclast interior following osteoclastic bone resorption [7]. Thus, using a fluorescently labeled analog of ALN (AF-ALN) it was demonstrated that osteoclastic uptake of BPs requires fluid-phase endocytosis [6]. However, the mechanism involved in BP incorporation by non resorbing cells is not completely clear. The absolute requirement of Cx43 for the anti-apoptotic effect of BPs on osteoblasts and osteocytes [5] together with the evidence that shows OPD binding sites in different subcellular fractions obtained from osteoblastic cells [10], raised the possibility that BPs might be internalized through Cx43 hemichannels. This hypothesis is supported by the fact that Cx hemichannels permit the passage of hydrophylic molecules with size lower than 1000 Da. To prove this, we synthesized AF-ALN and then incubated cells that express Cx43 or not with low concentrations of this drug. Interestingly, we found that an osteoblastic cell line as well as in calvarial osteoblasts are capable to incorporate AF-ALN from the medium. Likewise, cells lacking Cx43 expression showed intracellular AF-ALN, indicating that Cx43 hemichannels are not the route of access of AF-ALN into the cells. We observed ALN uptake within 30 min of incubation but this could be a more rapid process since [^{3}H]-OPD radioactivity has been detected intracellularly at 5 min of incubation [10]. These results suggest that although BPs are likely to be membrane impermeable due to their high negative charge, they can be internalized by cells. In addition, Felix et al. demonstrated that these compounds are taken up by calvarial cells in vitro [23]. Further studies should be performed employing specific markers to determine the precise intracellular distribution of this compound and the organelles involved.

Because BPs resemble to the naturally occurring pyrophosphate (PPi), their access to osteoblastic cytoplasm might occur via a PPi transporter. Although this topic should be studied in depth, a molecular candidate for a pyrophosphate transporter, ANKH, has been identified [24]. There is growing evidence that this transmembrane protein may play roles in regulating calcification within many tissues, including joints, bones and kidney [25].

Lucifer Yellow uptake by both adherent cells or cells maintained in suspension increased upon addition of ALN or removal of extracellular Ca^{2+} by the addition of EGTA [5], an established maneuver that opens Cx hemichannels [26]. The precise mechanism by which exposure to ALN leads to opening of Cx43 hemichannels in osteocytes/osteoblasts is a matter of conjecture at the present time. BPs are effective calcium chelators and in vivo are rapidly cleared from circulation [27] because their affinity for Ca^{2+} containing-bone mineral surfaces at sites of active bone remodeling [28]. In this regard, Thompson et al. found that the presence of bivalent ions such as Ca^{2+} increased osteoclast uptake of AF-ALN and enhanced the inhibitory effect of ALN on protein prenylation. Furthermore, addition of EGTA or a molar excess of the bisphosphonate clodronate, which does not inhibit protein prenylation [17, 29], reversed ALN effects due to Ca^{2+} chelation [6]. The possibility that hemichannel opening in the presence of ALN is the result of calcium local decrease in the proximity of the hemichannel should be investigated.

Earlier work by our group demonstrated that BPs inhibit apoptosis induced by several pro-apoptotic stimuli, including the inhibitor of topoisomerase etoposide, the activator of death receptors TNF alpha, and glucocorticoids [4]. The anti-apoptotic effect has been extensively studied and confirmed that involves blockade of the increases in membrane permeability, nuclear fragmentation and chromatin condensation, and activation of caspases [30, 4]. Consistent with these reports, we showed that ALN prevented etoposide-induced apoptosis in HeLa Cx43-transfected cells and osteocytic MLO-Y4 cells. Because ALN does not prevent apoptosis of HeLa cells lacking Cx43 or Cx43-expressing cells pre-treated with Cx channel disassembling agents [5] we conclude although this protein is necessary for the anti-apoptotic effect of ALN, BPs bind to an entity different from Cx43. Moreover, the fact that the phosphatase substrate p-nitro-phenylphosphate inhibited BP-induced anti-apoptosis of HeLa cells transfected with Cx43 supports the hypothesis that a phosphatase must be involved in ALN mechanism of action [10]. On the other hand, BP binding is not sufficient to induce survival, based on the specific [^{3}H]-ALN binding found in HeLa cells lacking Cx43 expression which are not responsive to ALN. Thus, BPs might bind to a protein that, in turn, induces Cx43 hemichannel transduction of survival signals.

Based on our results and other lines of supporting evidence discussed above, we propose that although the same receptor entity might mediate the proliferative and anti-apoptotic effects of ALN, they become dissociated at one point of the bisphosphonate signaling pathway. Indeed, we demonstrated that despite the fact that Cx43 is required for triggering intracellular signaling by BPs, it is dispensable for ALN-induced proliferation. Moreover, we suggest that after opening of Cx43 hemichannels by ALN, the BP enters to osteoblastic cells by a mechanism independent of Cx43. As we mentioned above, we believe that the unknown molecule which links BPs with Cx43 could be a phosphatase and its identification will be the subject of future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

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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HIGHLIGHTS

- Alendronate cellular specific binding does not depend on Cx43 expression.
- Non-resorbing bone cells are able to incorporate bisphosphonate from the medium.
- Alendronate cellular uptake is not mediated via Cx43 hemichannels.
- Cx43 is required for prevention of apoptosis by ALN in osteoblasts and osteocytes.

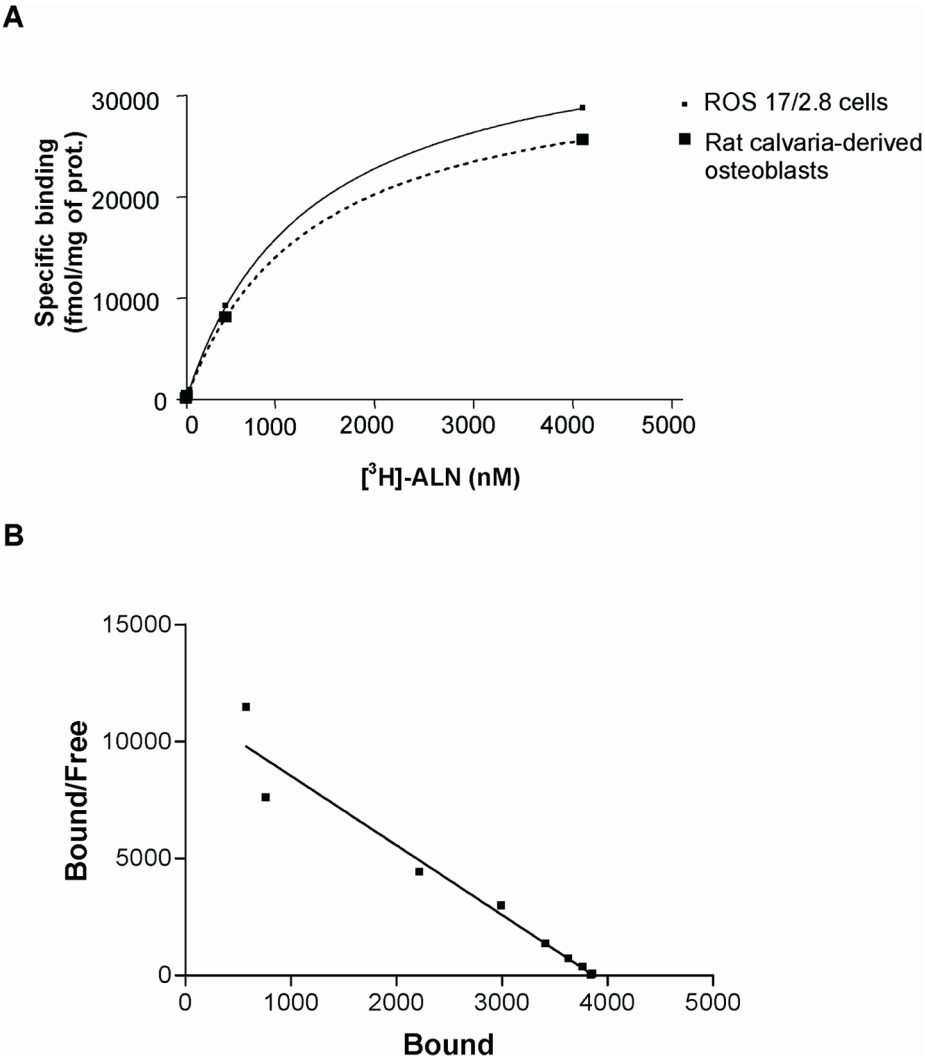

Fig.1. Specific and saturable binding site for [^{3}H]-ALN in osteoblastic cells

A) Saturation analysis. Whole rat calvaria-derived osteoblasts or ROS17/2.8 cells were incubated for 120 min at 30°C with increasing concentrations of [^{3}H]-ALN (4–4000 nM; specific activity 30 Ci/mmol) in the presence (nonspecific binding) or absence (total binding) of 200 μM of unlabeled ALN. Each value is the mean ± SEM of results from three separate experiments performed in triplicate. **B)** Scatchard plot of specific [^{3}H]-ALN binding obtained with the Prism GraphPad program. K_d= 0.65 ± 0.2 μM and B_{max}= 3986 ± 432 fmol/mg of protein.

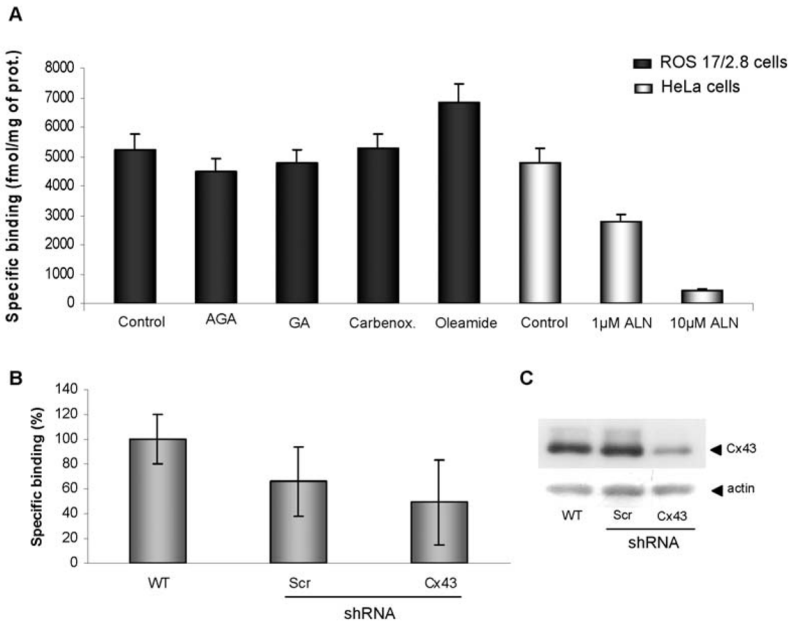

Fig.2. Cx43 is not required for bisphosphonate binding

A) ROS 17/2.8 cells pretreated with AGA, 18 α-glycyrrhetinic acid (100 M); GA, glycyrrhizic acid (100 M); carbenoxolone (100 M) or oleamide (1 M), agents that disassemble connexin hemichannels, and HeLa cells that do not express Cx43, were incubated for 120 min at 30°C with 30 nM [^{3}H]-ALN in the presence (nonspecific binding) or absence (total binding) of 200 μM of unlabeled ALN. Results are expressed as specific binding (fmol/mg of protein) = total bound- nonspecific bound. **B)** Cells were incubated for 120 min at 30°C with 30 nM [^{3}H]-ALN in the presence (nonspecific binding) or absence (total binding) of 200 μM of unlabeled ALN. Results of specific binding are expressed as percentage of control (WT; wild type cells). Each value is the mean ± SEM of results from three separate experiments performed in triplicate. **C)** The expression of Cx43 was silenced in MLO-Y4 osteocytic cells using short hairpin RNA (shRNA)-containing virus. As controls, cells were infected with scrambled (scr) shRNA. Cx43 protein levels were determined by Western blotting.

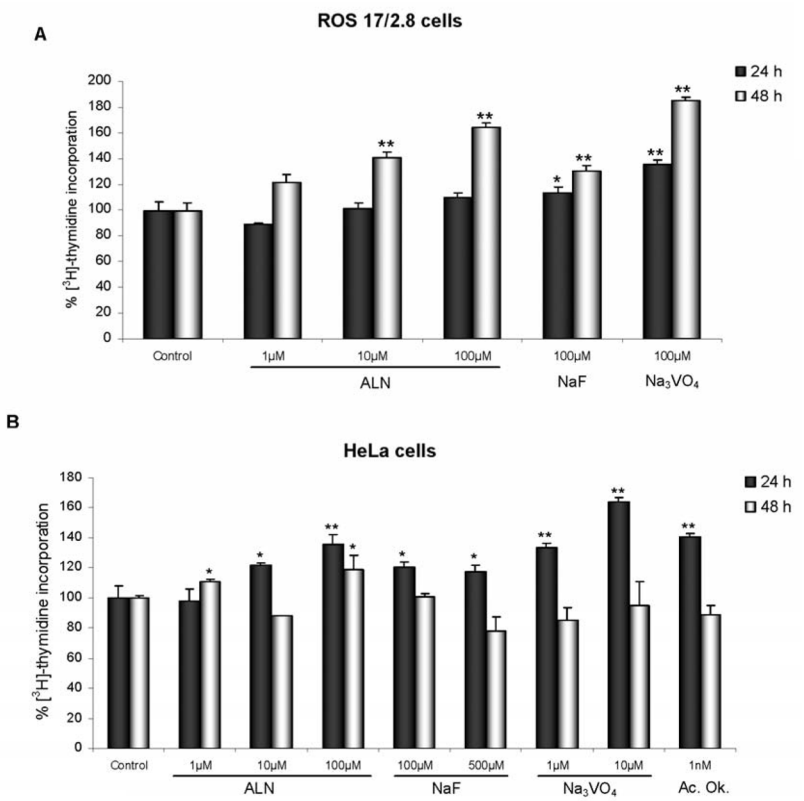

Fig.3. ALN induces proliferation of osteoblasts independently of Cx43 expression

ROS 17/2.8 (**A**) and HeLa (**B**) cells were grown in α-MEM medium containing 10% FBS during 72 h. Then, the medium was replaced with serum and phenol red-free α-MEM. After 24 h, cells were treated with vehicle (controls), ALN or phosphatase inhibitors, sodium fluoride (NaF), orthovanadate (Na_3VO_4) and okadaic acid (Ok. Ac.) at the indicated concentrations for 24 or 48 h, followed by the determination of [^{3}H]-thymidine cellular incorporation as indicated in Materials and Methods. The results are expressed as percentage of control (100%). Each value is the mean ± SEM of results from three separate experiments performed in quadruplicate. * $p < 0.05$, ** $p < 0.01$.

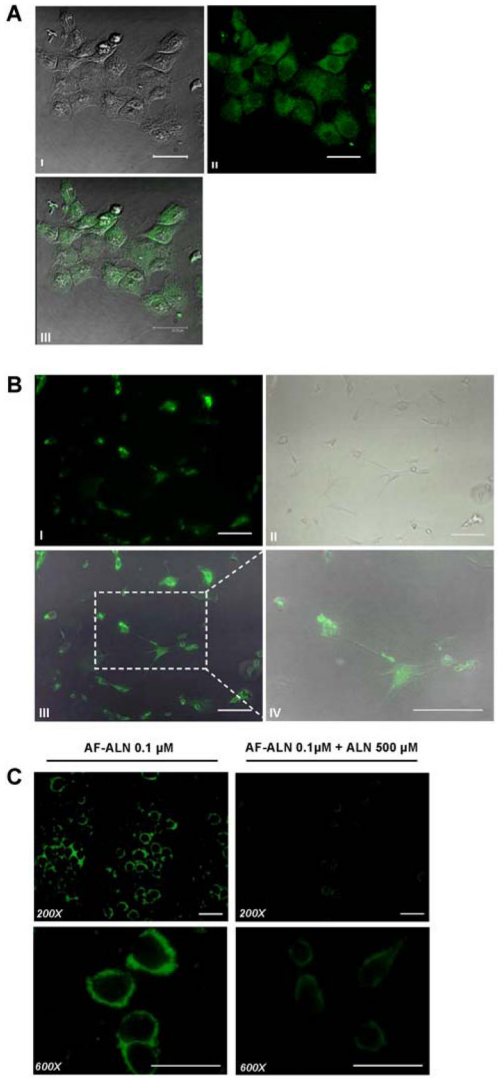

Fig.4. ALN is internalized by osteoblastic cells and Cx43 is not the route of access

A) ROS 17/2.8 cells were incubated for 30 min with 0.1 μM AF-ALN, then fixed and analyzed by confocal microscopy (scale bar, 31.75 μm). Phase contrast image (I); green fluorescence (II); merge (III). **B)** Primary osteoblastic cells were obtained from calvaria and treated as described in A. Green fluorescence image (I); phase contrast image (II); merge (III); higher magnification view of area indicated by square in III (IV). **C)** HeLa cells were treated as described in A. At the same time, cells were incubated with 0.1 μM AF-ALN together with a high concentration of unlabeled ALN (500 μM), fixed and then analyzed as described above.

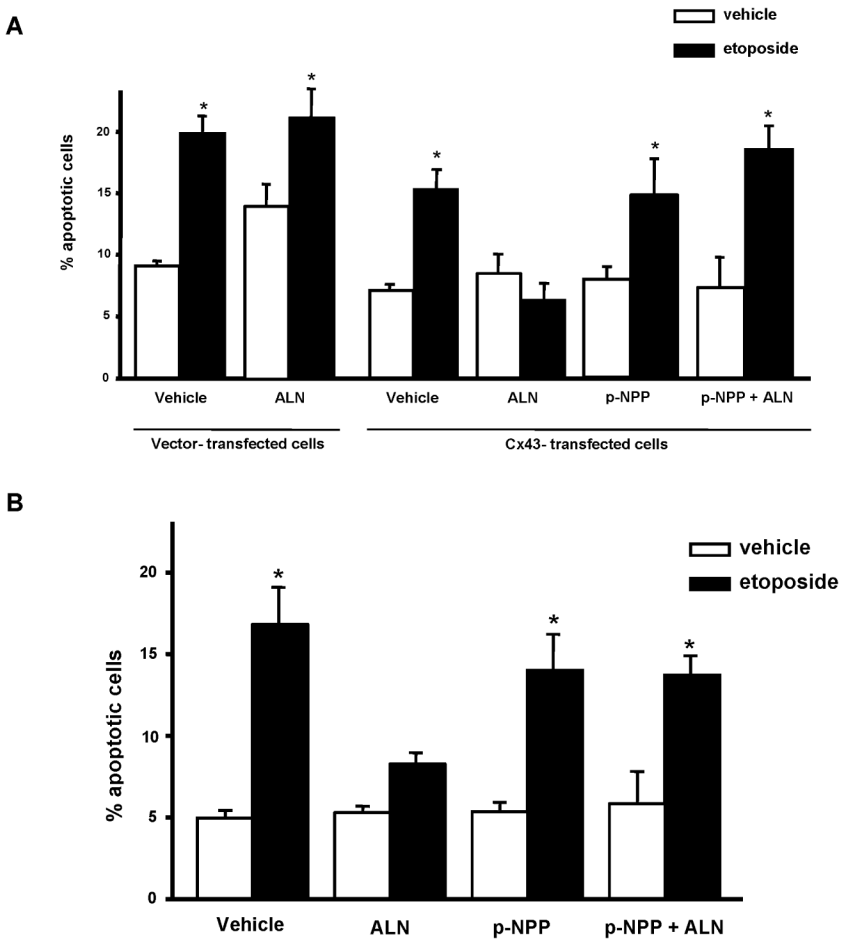

Fig.5. Anti-apoptotic effect of ALN requires Cx43 and is reversed by the presence of a phosphatase substrate

A) HeLa cells were transiently transfected with Cx43 or vector along with nuclear green fluorescent protein, in order to evaluate apoptosis only in transfected cells. Cells were treated with vehicle (PBS) or 8 mM p-NPP for 30 min. Subsequently 10^{-7} M ALN or vehicle was added and 30 min later, vehicle (DMSO) or 50 mM etoposide. Cells were cultured for additional 6 h and fixed with neutral buffer formalin. Apoptosis was evaluated by examining nuclear morphology of fluorescent cells. Cells exhibiting chromatin condensation and/or nuclear fragmentation were considered apoptotic. **B**) MLO-Y4 osteocytic cells were treated as described in (A) and apoptosis was evaluated by trypan blue uptake, as indicated in Methods. Data were analyzed by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls method was used to estimate the level of significance of differences between means, * indicate $p<0.05$ versus vehicle.