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Alendronate affects calcium dynamics in cardiomyocytes *in vitro*

Naomi Kemeny-Suss [a,1], Amanda Kasneci [b,c,1], Daniel Rivas [c], Jonathan Afilalo [d], Svetlana V. Komarova [a], Lorraine E. Chalifour [b,c], Gustavo Duque [c,e,*]

[a] *Faculty of Dentistry, McGill University, Montreal, Quebec, Canada H3A 2B2*
[b] *Bank of Montreal Center for the Study of Heart Disease in Women, Montreal, Quebec, Canada H3T 1E2*
[c] *Lady Davis Institute for Medical Research, McGill University, Montreal, Quebec, Canada H3T 1E2*
[d] *Division of Cardiology, Jewish General Hospital, McGill University, Montreal, Quebec, Canada H3T 1E2*
[e] *Aging Bone Research Program, Nepean Clinical School, University of Sydney, Penrith, NSW, Australia, 2750*

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ABSTRACT

Therapy with bisphosphonates, including alendronate (ALN), is considered a safe and effective treatment for osteoporosis. However, recent studies have reported an unexpected increase in serious atrial fibrillation (AF) in patients treated with bisphosphonates. The mechanism that explains this side effect remains unknown. Since AF is associated with an altered sarcoendoplasmic reticulum calcium load, we studied how ALN affects cardiomyocyte calcium homeostasis and protein isoprenylation *in vitro*. Acute and long-term (48 h) treatment of atrial and ventricular cardiomyocytes with ALN (10^{-8}–10^{-6}M) was performed. Changes in calcium dynamics were determined by both fluorescence measurement of cytosolic free Ca^{2+} concentration and western blot analysis of calcium-regulating proteins. Finally, effect of ALN on protein farnesylation was also identified. In both atrial and ventricular cardiomyocytes, ALN treatment delayed and diminished calcium responses to caffeine. Only in atrial cells, long-term exposure to ALN-induced transitory calcium oscillations and led to the development of oscillatory component in calcium responses to caffeine. Changes in calcium dynamics were accompanied by changes in expression of proteins controlling sarcoendoplasmic reticulum calcium. In contrast, ALN minimally affected protein isoprenylation in these cells. In summary, treatment of atrial cardiomyocytes with ALN-induced abnormalities in calcium dynamics consistent with induction of a self-stimulatory, pacemaker-like behavior, which may contribute to the development of cardiac side effects associated with these drugs.

1. Introduction

Osteoporosis is characterized by reductions in bone quantity and quality, leading to fractures and increased mortality in older adults (Lane et al., 2006). Currently, bisphosphonates are the most effective treatment for osteoporosis (Zizic, 2004), and considered to be relatively safe (Diel et al., 2007). However, recent studies have reported an unexpected increase in the incidence of serious atrial fibrillation (AF) in patients treated with zoledronate (Black et al., 2007) and alendronate (ALN) (Cummings et al., 2007). Some of the subsequent population studies confirmed this association (Heckbert et al., 2008), whereas some did not (Sorensen et al., 2008). In the studies reporting AF as a side effect of bisphosphonate treatment, two features are important: first, treatment with either zoledronate or ALN was associated with an increase in the number of serious AF events (characterized as life threatening, resulting in hospitalization or death), but not in total number of AF events (Black et al., 2007; Cummings et al., 2007), potentially explaining why some studies did not find the association. Second, most cases of AF did not occur immediately, but were delayed by 30 or more days (Black et al., 2007; Heckbert et al., 2008). Although the absolute risk of AF remains very small, the wide use of bisphosphonates necessitates a better understanding of the potential arrythmogenic action of these drugs to better identify the population at risk and develop strategies of prevention of this unexpected side effect.

When given orally or intravenously, bisphosphonates are rapidly removed from the circulation, are targeted to bone surfaces by their attraction to elemental calcium and are taken up by active osteoclasts (Kimmel, 2007; van Beek et al., 1999a). Plasma ALN concentration can reach micromolar concentrations for several hours, and then stabilize at 100–1000 fold lower levels due to a slow release from the skeletal sites (Porras et al., 1999; Yun et al., 2006). After this significant redistribution, small amounts could remain in soft tissues being gradually released over a period of several days to weeks (Porras et al., 1999). In their traditional target, osteoclasts, nitrogen-containing bisphosphonates inhibit farnesyl pyrophosphate synthase, reducing protein isoprenylation (farnesylation and geranylgeranylation) of

* Corresponding author. Nepean Clinical School, Level 5-South Block, Nepean Hospital, Penrith, NSW, 2750, Australia. Tel.: +61 2 47344279; fax: +61 2 47341817.
E-mail address: gduque@med.usyd.edu.au (G. Duque).
[1] Contributed equally to this work.

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small GTPases leading to cytoskeletal disruption, inhibition of osteoclast differentiation and activity, and increased apoptosis (Kimmel, 2007; Luckman et al., 1998; Reszka and Rodan, 2004). After administration, bisphosphonates (including ALN) can cause transient hypocalcemia and increased inflammatory cytokine release, which although resolve quickly (Hewitt et al., 2005), have been proposed as the potential mechanisms of AF in patients treated with bisphosphonates (Black et al., 2007; Cummings et al., 2007). However, considering that these are transient effects and that in all studies the risk for AF was found to increase with time, the possibility of a long-term effect of bisphosphonates on cardiomyocytes is plausible.

Cytosolic free calcium ($[Ca^{2+}]_i$) is critical for cardiomyocyte contraction and cardiac rhythm (Bers, 2008; Knollmann and Roden, 2008). Generation of the action potential at the cardiomyocyte membrane leads to a rise in $[Ca^{2+}]_i$ that initiates cell contraction. Several calcium-handling proteins regulate calcium dynamics in cardiomyocytes (Bers, 2008; Knollmann and Roden, 2008). Calcium removal from the cytosol to the sarcoendoplasmic reticulum (SER) by sarcoendoplasmic reticulum ATPase (SERCA2a) is necessary for relaxation. Calcium is stored in the SER by binding with the low-affinity, high-capacity calsequestrin (CSQ), whereas calreticulin (CRT) is the main calcium storage proteins of the endoplasmic reticulum. Dysfunction in these cardiomyocyte calcium-handling mechanisms may lead to spontaneous calcium activity, which has been associated with arrythmogenesis (Knollmann and Roden, 2008; Lakireddy et al., 2005; Salama, 2006). For instance, isoproterenol stimulation of failing hearts led to delayed after-depolarizations and after-contractions because of an increase in SER calcium load (Desantiago et al., 2008). Spontaneous calcium release in the absence of membrane depolarization can occur during SER calcium overload. Finally, mutations or reductions in CSQ2 are associated with catecholaminergic polymorphic ventricular tachycardia that can deteriorate into fibrillation and sudden death (di Barletta et al., 2006; Knollmann et al., 2006). These data indicate that changes in the amount of proteins involved in controlling SER calcium load may be involved in inducing fibrillation.

In order to elucidate the potential mechanisms that explain the observed arrythmogenic effect of ALN, in this study, we have used *in vitro* models to assess the effect of acute and chronic (48 h) ALN treatment on protein isoprenylation, expression of SER calcium-handling proteins and calcium dynamics in cardiomyocytes of both atrial and ventricular origin. Our findings report a novel mechanism of action of bisphosphonates, which may contribute to the development of cardiac side effects associated with these drugs.

2. Materials and methods

2.1. Reagents

ALN was purchased from Sigma-Aldrich (St. Louis, MI, USA). Farnesyl transferase inhibitor (FTI-277), geranylgeranyltransferase inhibitor (GGTI-298) and anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were from Sigma-Aldrich (St. Louis, Missouri, USA). Calsequestrin (CSQ), Calreticulin (CRT) and sarcoendoplasmic reticulum ATPase 2a (SERCA2a) polyclonal antibodies were from Affinity BioReagents (Golden, CO, USA). HDJ-2 antibody was from Lab Vision (Fremont, CA, USA) and RAP-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies and enhanced chemiluminescent detection kits were from Pierce (Rockford, IL, USA). Fura-2 was from Invitrogen, (Carlsbad, CA, USA). Other reagents were from Sigma-Aldrich (St. Louis, MI, USA) unless otherwise noted.

2.2. Cell culture

Mouse atrial cardiomyocyte HL-1 cells were cultured on gelatin–fibronectin coated dishes as previously described (Claycomb et al., 1998). Cells were maintained in Claycomb Media (SAFC Biosciences, Lenexa, KA, USA) supplemented with 10% fetal bovine serum, 4 mM L-glutamine and 10 mM noradrenaline as described by the media manufacturer. Rat ventricular cardiomyocyte H9c2 cells (Menard et al., 1999) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For cardiac differentiation of H9c2 cells, the media was removed when the cells were 70% confluent, replaced with differentiation media (DMEM, 1% fetal bovine serum) and retinoic acid (10^{-8} M) was added daily for 4 days (Hescheler et al., 1991).

2.3. Drug treatments

In acute experiments, vehicle (PBS), ALN (10^{-8}–10^{-6} M), or caffeine (0.5 mM) was added to normal Tyrodes solution and calcium dynamics were analyzed immediately. In chronic HL-1 experiments, vehicle (PBS), ALN (10^{-8}–10^{-6} M), FTI-277 (5–10 μM) or GGTI-298 (5–10 μM) was diluted in water and added to the culture media and the cells were analyzed 48 h later. In chronic H9c2 experiments, drugs were added 2 days after differentiation induction and the cells were analyzed 48 h later.

2.4. Measurement of cell proliferation

HL-1 and H9c2 cells were seeded at a density of 4×10^2 cells/well in 96-well cluster plates (Falcon, Becton-Dickinson, NJ, USA). Cells were treated with either vehicle (PBS), ALN (10^{-8}—10^{-6} M), FTI-277 (5–10 μM) or GGTI-298 (5–10 μM) as previously described. Cell proliferation was assessed at timed intervals (24, 48 and 72 h) using MTS-Formazan (Promega Biosciences, St. Louis Obispo, CA). This method assesses mitochondrial function by the ability of viable cells to convert soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) into an insoluble dark blue Formazan reaction product measured photometrically as previously described (Duque et al., 2002). A stock solution of MTS was dissolved in PBS at a concentration of 5 mg/ml and was added in a 1:10 ratio (MTS/DMEM) to each well incubated at 37 °C for 4 h and the optical density was determined at a wavelength of 570–630 nm on a microplate reader model 3550 (Bio-Rad, Hercules, CA). In preliminary experiments the absorbance was found to be directly proportional to the number of cells over a wide range (2×10^2 to 50×10^3 cells/well). The percent survival was defined as $[(\text{experimental}_{\text{absorbance}} - \text{blank}_{\text{absorbance}})/(\text{control}_{\text{absorbance}} - \text{blank}_{\text{absorbance}})]\times100$, where the $\text{control}_{\text{absorbance}}$ is the optical density obtained for 10×10^3 cells/well (number of cells plated at the start of the experiment), and $\text{blank}_{\text{absorbance}}$ is the optical density determined in wells containing medium and MTS alone.

2.5. Protein isolation and immunoblot analysis

For DnaJ 2 (HDJ-2), and Rab-RP1 (RP-1), cells were lysed in 20 mM tris–HCl, pH 7.5, 200 mM DTT, 200 mM KCl, 0.5 ml glycerol and protease inhibitor tablets (Roche Diagnostics Canada, Laval, QC, Canada), freeze-thawed 3 times in a dry ice–ethanol bath and centrifuged at 11,500 rpm for 15 min to remove insoluble material. Cell lysates were dissolved in SDS electrophoresis buffer (Bio-Rad, Hercules, CA, USA) and proteins separated on SDS-polyacrylamide gels and subsequently electrotransferred to polyvinylidene difluoride membranes. Membranes were blocked for 2 h with PBST + milk (50 mM Na phosphate pH 7.4, 150 mM NaCl, 0.1% Tween 20, 10% non-fat dry milk). DnaJ 2 (HDJ-2), unprenylated Rab-RP1 (RAP-1), GAPDH or a-tubulin antibodies were diluted 1:1000 in PBST + milk and incubated overnight at 4 °C. For calcium homeostasis proteins, cells were homogenized in SDS lysis buffer (62.5 mM Tris pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue). Equal amounts of protein were electrophoresed through SDS-PAGE then transferred to membranes. Membrane staining with Ponceau S