Journal of Molecular and Cellular Cardiology 100 (2016) 83–92

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Vascular action of bisphosphonates: In vitro effect of alendronate on the regulation of cellular events involved in vessel pathogenesis

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ARTICLE INFO

Article history:
Received 27 June 2016
Received in revised form 19 August 2016
Accepted 24 August 2016
Available online 2 October 2016

Keywords:
Nitric oxide
Alendronate
Endothelial monocyte adhesion
Platelet adhesion and aggregation
Vascular calcification
Osteogenic markers

ABSTRACT

In this work we investigate whether, despite the procalcific action of alendronate on bone, the drug would be able to regulate in vitro the main cellular events that take part in atherosclerotic lesion generation. Using endothelial cell cultures we showed that Alendronate (1–50 μM) acutely enhances nitric oxide production (10–30 min). This stimulatory action of the bisphosphonate involves the participation of MAPK signaling transduction pathway. Under inflammatory stress, the drug reduces monocytes and platelets interactions with endothelial cells induced by lipopolysaccharide. Indeed the bisphophonate exhibits a significant inhibition of endothelial dependent platelet aggregation. The molecular mechanism of alendronate (ALN) on leukocyte adhesion depends on the regulation of the expression of cell adhesion related genes (VCAM-1; ICAM-1); meanwhile the antiplatelet activity is associated with the effect of the drug on nitric oxide production. On vascular smooth muscle cells, the drug exhibits ability to decrease osteogenic transdifferentiation and extracellular matrix mineralization. When vascular smooth muscle cells were cultured in osteogenic medium for 21 days, they exhibited an upregulation of calcification markers (RUNX2 and TNAP), high alkaline phosphatase activity and a great amount of mineralization nodules. ALN treatment significantly down-regulates mRNA levels of osteoblasts markers; diminishes alkaline phosphatase activity and reduces the extracellular calcium deposition. The effect of ALN on vascular cells differs from its own bone action. On calvarial osteoblasts ALN induces cell proliferation, enhances alkaline phosphatase activity, and increases mineralization, but does not affect nitric oxide synthesis. Our results support the hypothesis that ALN is an active drug at vascular level that regulates key processes involved in vascular pathogenesis through a direct action on vessel cells.

1. Introduction

Osteoporosis and cardiovascular disease (CVD) are multifactorial clinical entities that often coexist in postmenopausal women. Osteoporotic fractures, coronary and peripheral artery disease and stroke resulting from atherosclerosis, are common conditions particularly in the elderly. Epidemiological and clinical studies have shown an interesting relationship between osteoporosis and CVD mortality [1,2]. Disorders in bone metabolism and reduced bone mineral density reversely correlate with vascular calcification and this link is thought to contribute to high CVD mortality [3]. However, although these diseases have been traditionally considered as independent processes associated with age and ovarian status, increasing evidence confirms that both entities share pathophysiological mechanisms [2].

Bisphosphonates (BPs) are the most widely used category of therapeutic agents in osteoporosis [4]. These drugs are synthetic analogues of pyrophosphate in which the two phosphates are connected by an atom of carbon instead of oxygen. The presence of a nitrogen atom in the side chain [alendronate (ALN), pamidronate, ibandronate, risedronate, zoledronate] enhances their affinity for bone tissue. Traditionally, the bone protective effects of BPs have been attributed to induce osteoclasts apoptosis leading to decrease bone resorption. However, it has been recently reported that they also act directly on osteoblasts and osteocytes, to preserve their viability and to prolong their life span [5]. BPs have also been approved for the treatment of cancer-related skeletal complications, bone metastases, pain, nerve compression and fractures. Long bones are the most common site of spread to tumours such as breast, prostate, kidney and haematological cancers. The antineoplastic action induced by BPs involves disruption of intracellular vesicle transport, which reduces the ability of the cells to migrate and invade surrounding tissue [6]. Meanwhile, the proposed mechanisms for the beneficial effect of the BPs against hypercalcemia bone

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http://dx.doi.org/10.1016/j.yjmcc.2016.08.017
0022-2828/

pain include inhibition of bone resorption, mechanical stabilisation, and enhancement of pH of the bone turnover microenvironment which results in decreased acid-sensing ion channel stimulation [7].

In concern with vascular action of BPs, existing data mostly belongs from clinical trials carried out in patients with chronic kidney disease and diabetes, where it is proposed that BPs could reduce the thickening of arterial vessels, and inhibit vascular calcification [8,9]. Although BPs seem to have an inhibitory effect on atherosclerosis process, the results are conflicting and it is not comprehensible whether the BPs act directly on vascular cells or indirectly through their action on bone system [10].

Atherosclerosis is a chronic inflammatory disease characterized by loss of vascular architecture, vascular injury (atheromatous generation), and finally, occlusion of the affected blood vessels [11]. The process starts with endothelial dysfunction, an event characterized by imbalance in the production of vasodilator and vasoconstrictor factors, followed by a marked decrease in the bioavailability of nitric oxide (NO) which leads to a pro-oxidant, proinflammatory and prothrombotic features [12]. The initial response to vascular injury is mediated by an increase of endothelial permeability and inflammatory cytokine secretion that consequently causes platelet adhesion to the activated endothelium. In turn, activated platelets promote the recruitment of monocytes, enhancing their transendothelial migration, and macrophage activation. The later stage of atherosclerotic lesion involves vascular tissue replacement by osteogenic like cells, extracellular matrix mineralization and intima calcification. This occurs as result of osteogenic transdifferentiation of vascular cells induced by the inflammatory microenvironment. Approximately 15% of human atherosclerotic plaques exhibit full calcification, with histological structure almost indistinguishable from bone trabecular architecture.

Bone mineralization and vascular calcification are cell-mediated processes sharing common mechanisms. The idea that pharmacological agents that inhibit bone loss could also provide benefits in terms of slowing the progression of atherosclerosis have grown up in the last years. The aim of the present work was to evaluate whether, despite the procalcific action of ALN on bone, the drug would be able to regulate in vitro the main cellular events that take part in atherosclerotic lesion generation. The direct effect of ALN either on endothelial cells (EC) and vascular smooth muscle cells (VSMC) was evaluated.

2. Materials and methods

2.1. Materials

Griess reagents were purchased from Britania Laboratories (Buenos Aires, Argentina). Trypsin/EDTA (10×), L-glutamine (100×), amphotericin B (0.25 mg/mL), penicillin/streptomycin (100×), and fetal calf serum (FCS) were obtained from PAA Laboratories (Pasching, Austria). Dulbecco's modified Eagle's medium (DMEM), ALN, and all other reagents were purchased from Sigma Chemical Company (St Louis, MO, USA).

2.2. Cell cultures and in vitro calcification

EC and VSMC cultures were obtained from aortic rings explants isolated from young Wistar rats (3–5 weeks old) as previously described [13]. Briefly, animals were killed by cervical dislocation and the full length thoracic aorta was aseptically removed. Immediately after, the aorta was cleaned of adherent connective tissue, and cut into small ring-shaped segments. Ring explants were seeded on 60-mm matrix-coated petri dishes containing phenol red-free DMEM supplemented with 20% (*v*/v) FCS, 60 μg/mL penicillin, 10 μg/mL streptomycin, 2.5 μg/mL amphotericin-B, 2 mM L-glutamine, and 1.7 g/L sodium bicarbonate. Explants were incubated at 37 °C in 5% CO_2 atmosphere. In order to establish a pure EC culture, after 5 days of culture, ring explants were removed and transferred into new culture dishes with fresh DMEM supplemented with 10% (*v*/v) FCS. Additional transfer of the ring explants resulted in pure cultures of VSMC. At last, the rings were discarded and EC and VSMC cultures were allowed to reach confluence. EC and VSMC identity was performed as previously reported (Supplementary Data) [13–15]. Cells from passages 2 to 5 were used for all experiments. Fresh medium containing 10% (*v*/v) FCS was replaced every 72 h. To perform cellular treatments ALN were dissolved in phosphate buffered saline (PBS) as vehicle.

In order to induce VSMC osteogenic differentiation, VSMC were seeded into 24-well plates and cultured for 21 days in DMEM containing 4 mM $CaCl_2$ and 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid (osteogenic medium), as described [16]. The osteogenic medium was replaced by fresh medium every 3 days. The cells were exposed to the bisphosphonate (BP) or vehicle (control) in the last 24 h of culture. All the procedures were performed in accordance with the guidelines published in the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. All procedures involving animals and their care were performed at the Unit of Animal Care belonging to the Biology, Biochemistry and Pharmacy Department of the University. The Animal Care Use Committee of this Unit approved the protocol employed.

2.3. Osteoblast isolation and culture

Calvaria osteoblasts were obtained from 5-day-old neonatal rats as described by Santillán et al. [17]. Briefly, calvarias were incubated at 37 °C in PBS containing 4 mM EDTA for 10 min (two periods). The cultures medium were discarded. Subsequently, calvarias were rinsed in PBS and submitted to digestion in PBS containing 200 IU/mL collagenase for 15 min (four periods). Cells released during the first digestion were discarded, and those released during the subsequent digestions were spun down and collected after centrifugation (10 min at 1500 rpm). Then, cells were cultured at 37 °C in 5% CO_2 atmosphere in DMEM supplemented with 15% FCS, 2.15 g/L β-glycerophosphate, and 0.05 g/L ascorbic acid, 60 μg/mL penicillin, and 10 μg/mL streptomycin. After 24 h, the medium was replaced by fresh DMEM containing 10% FCS, and cells were cultured until 80% of confluence.

2.4. Measurement of NO production

Cells were seeded on 24-multiwell culture plates at a density of 3.5×10^4 cells/well in DMEM containing 10% (*v*/v) FCS. Prior treatment, culture medium was changed by fresh DMEM containing 1% (v/v) FCS. In vitro treatments were performed by of ALN or vehicle (PBS) addition. When specific antagonist/inhibitors were used, they were added to culture medium 1 h before ALN treatment. Nitrites were measured in the incubation media as a stable and non-volatile breakdown product of the NO released, employing the spectrometric Griess reaction [14]. Briefly, once finished treatment, aliquots of culture medium supernatant were mixed with Griess reagent (1% sulphanilamide and 0.1% naphthylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated 10 min at room temperature. Absorbance was measured at 548 nm in a microplate reader (Biotek Synergy-HT). Nitrite concentration in the samples was determined with reference to a sodium nitrite ($NaNO_2$) standard curve performed in the same matrix. Cells were dissolved in 1 M NaOH, and protein content was measured by Lowry method [18]. The results were expressed as nmol of NO per mg of protein. The quantification of NO production through the indirect method of Griess, was validated against direct measurements of nitric oxide synthase (NOS) activity using the ^{3}H–arginine to ^{3}H–citrulline conversion method [19]. Similar results were obtained with both assays.

2.5. Monocyte adhesion assay

2.5.1. Monocyte isolation

Peripheral blood mononuclear cells were obtained using density gradient (Ficoll-Paque Plus) and monocytes were isolated by adherence to plastic dishes as previously described [20]. Briefly, heparinized whole blood diluted with PBS (1:1) was carefully layered onto Ficoll-Paque