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Short communication

THE EFFECTS OF DISODIUM PAMIDRONATE ON HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND PLATELETS: AN *in vitro* STUDY

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Abstract: Recent reports have indicated that, as well as having antiresorptive effects, bisphosphonates could have an application as anti-inflammatory drugs. Our aim was to investigate whether this anti-inflammatory action could be mediated by the nitric oxide (NO) released by the leukocytes migrating to the site of inflammation. In particular, we investigated *in vitro* the intracellular calcium concentration ($[Ca^{2+}]_i$), the level of NO released by PMN and platelets, and the PMN myeloperoxidase activity after incubation with disodium pamidronate, since there was a postulated modulatory effect of this amino-substituted bisphosphonate on leukocytes both *in vitro* and *in vivo*. Our data shows that the pamidronate treatment provoked a significant increase in the $[Ca^{2+}]_i$ parallel to the enhancement in NO release, suggesting a possible activation of constitutive nitric oxide synthase, while the myeloperoxidase activity was significantly reduced. In conclusion, we hypothesized that treatment with pamidronate could stimulate NO-production by cells present near the bone compartment, thus constituting a protective mechanism against bone resorption occurring during inflammation. In addition, PMN- and platelet-derived NO could act as a negative feed-back signal to restrict the inflammatory processes.

Key words: Pamidronate, Polymorphonuclear leukocytes, Platelets, Nitric oxide, Intracellular calcium, Myeloperoxidase activity

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Abbreviations used: APD – disodium pamidronate; BP – bisphosphonates; $[Ca^{2+}]_i$ – intracellular calcium concentration; MPO – myeloperoxidase; PMN – polymorphonuclear leukocytes; ROS – reactive oxygen species

INTRODUCTION

Bisphosphonates (BP) are synthetic analogues of the endogenous pyrophosphate. They inhibit bone resorption by interfering with osteoclast recruitment and activity, and thus can be used in the treatment of a variety of bone diseases (e.g. Paget's disease, metastatic bone disease, post-menopausal osteoporosis) [1-3]. Recent studies have suggested that BP do not affect osteoclasts only: in addition to their antiresorptive effects, some BP have been shown to affect inflammatory responses [4-7]. It was previously demonstrated that osteoclast differentiation and activation are modulated by reactive oxygen species (ROS) [8], and it is well known that the activation of polymorphonuclear leukocytes (PMN) during inflammation processes results in the release of complement components, ROS, and lysosomal enzymes, including myeloperoxidase (MPO). Consequently, it has been hypothesized that the elimination of superoxide anions through a reaction with nitric oxide (NO) could constitute a protective mechanism against bone resorption during pathological conditions, such as inflammation [9, 10].

The migration of inflammatory cells might be a potential source of NO at the site of inflammation. Polymorphonuclear leukocytes are of particular interest as they have the highest level of NO production [11, 12]. Since chronic inflammatory diseases, such as rheumatoid arthritis and periodontitis, are associated with the accumulation and sustained presence of inflammatory cells adjacent to the bone with subsequent bone resorption, a drug that inhibited or neutralized ROS production or MPO activity in these cells would be a useful therapeutic agent.

The aim of our study was to examine whether the anti-inflammatory actions of BP could be mediated by an increased nitric oxide production. An anti-inflammatory profile for NO was previously suggested [13, 14]. In particular, we investigated *in vitro* the intracellular calcium concentration ($[Ca^{2+}]_i$), the level of NO released by PMN and platelets, and the PMN myeloperoxidase activity after incubation with disodium pamidronate (APD), since there was a postulated modulatory effect of this amino-substituted bisphosphonate on the leukocytes both *in vitro* and *in vivo* [6, 15]. Our study focused on PMN and platelets because these are the predominant cell types found in the microcirculation after an acute inflammatory injury [16].

MATERIALS AND METHODS

PMN isolation

Peripheral blood was obtained from 33 healthy adult donors (mean age = 34.48 ± 11.19, 9 women, 24 men) who had not ingested any drug for at least one week, following informed consent. Heparin (15 U/ml) was used as an anticoagulant for the separation of PMN and citrate dextrose (ACD, pH 6.8) was used for platelet recovery.

PMN were isolated using a double density gradient purification method (Histopaque®-1077 and Histopaque®-1119) and subsequently purified by hypotonic lysis of the contaminating erythrocytes [17]. As per the manufacturer's instructions, a double gradient was formed by layering an equal volume of Histopaque®-1077 over Histopaque®-1119. Whole blood was carefully layered onto the upper Histopaque®-1077 medium. The tubes were centrifuged at 700 x g for 30 min, and then PMN were harvested at the 1077/1119 interphase. The thus-obtained final preparation contained more than 95% PMN, as estimated by Giemsa staining under a microscope, with a viability exceeding 97%, as determined by the trypan blue exclusion test.

Platelet isolation

Platelets were isolated from peripheral blood recovered with the anticoagulant citrate dextrose, pH 6.8 (ACD) by differential centrifugation in an anti-aggregation buffer (Tris-HCl 10 mM; NaCl 150 mM; EDTA 1 mM; glucose 5 mM; pH 7.4) according to the method of Rao [18]. The method involved a preliminary centrifugation step (200 x g for 10 min) to obtain platelet rich plasma (PRP). The platelets were then washed three times in the anti-aggregation buffer and centrifuged in order to remove any residual erythrocytes. A final centrifugation at 2000 x g for 20 min was performed to isolate the platelets as previously reported [19].

Experimental procedure

The PMN were incubated with 50 μM disodium pamidronate (3-amino-1-hydroxypropylidene-1,1-bisphosphonate acid, 2 Na; APD; Aredia®, Novartis Farma S.p.A., Origgio, Varese, Italy) in RPMI medium containing 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml streptomycin and 100 μg/ml penicillin for 3, 6 and 24 h at 37ºC in an humified atmosphere with 5% CO_2. The platelets were treated with the same drug concentration in the anti-aggregation buffer for 3 h. A concentration of 50 μM was chosen in this study because a dose-related inhibitory effect of 30-100 μM pamidronate on *in vitro* bone resorption in the absence of any significant inhibitory effect on cellular activity was previously shown [20]. The control samples were incubated for the same periods in RPMI medium or anti-aggregation buffer without APD.

Intracellular Ca^{2+} concentration

The intracellular Ca^{2+} concentration was measured in both intact PMN and platelets using the fluorescent probe FURA 2-AM as previously described [21]. The determinations were performed using a Perkin-Elmer LS 50 B spectro-fluorometer at 37ºC according to the method of Rao [18]. The fluorescence intensity was evaluated at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). The calibration was carried out as described by Grynkiewicz *et al.* [21] with the equation:

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \times \frac{S_{f2}}{S_{b2}}$$

where K_d is the dissociation constant of the Ca^{2+}-FURA 2 interaction in the cytosolic environment; R is the ratio of the fluorescence intensities at the excitation wavelengths 340 and 380 nm; R_{min} and R_{max} are the ratios of the fluorescence intensities without Ca^{2+} and with saturating levels of Ca^{2+}, respectively; and S_{f2} and S_{b2} are the fluorescence intensities at 380 nm without Ca^{2+} and with saturating levels of Ca^{2+}, respectively. R_{min} and S_{f2} were measured after cellular lysis with 1% Triton X-100 and the addition of 10 mM EGTA, pH 8.3. R_{max} and S_{b2} were determined after lysis and the addition of $CaCl_2$. The autofluorescence was subtracted prior to performing the Ca^{2+} calibration procedure. The $[Ca^{2+}]_i$ was normalized per cell number.

Nitric oxide (NO) production

The NO released by the cells was measured in the supernatants as nitrite production, via the Griess reaction [22]. Briefly, in this assay, equal amounts of 1% sulphanilic acid and 0.1% *N*-(1-naphthyl)ethylene diamine were added to the samples, and the resulting absorbance was measured at 543 nm with a Secoman Anthelie spectrophotometer. The blank (background) was determined in each experiment utilizing a medium incubated without cells. The amount of nitrate in each sample was determined using a standard curve of serial dilutions of $NaNO_2$; the protein concentration was determined with the Bradford BioRad protein assay using serum albumin as a standard [23]. The results were expressed as nmol nitrite/mg protein.

Myeloperoxidase (MPO) activity

The MPO activity was determined according to the method of Suzuki *et al.* [24]. Briefly, the reaction mixture (PMN supernatants, 1.6 mM tetramethylbenzidine, 0.3 mM H_2O_2, 80 mM sodium phosphate buffer pH 5.4, 8% N,N-dimethyl-formamide, and 40% PBS; total volume 500 µl) was incubated for 3 min at 37ºC, and then immersed into an ice bath. The reaction was stopped by the addition of 1.75 ml of 200 mM sodium acetate buffer (pH 7.3). The rate of oxidized product formation was measured by monitoring the absorbance increase at 655 nm in a Secoman Anthelie spectrophotometer. The MPO activity was expressed as U/mg prot./h.

Statistical analysis

All the quantitative data was analyzed using the Student t-test. The results were presented as means ± standard deviations. All the experiments were performed at least four times. Values of *P* less than 0.05 were considered statistically significant.

Source of materials

All the reagents were from Sigma-Aldrich S.r.l., Milano, Italy.

RESULTS

Our results reveal that the $[Ca^{2+}]_i$ was significantly higher in the PMN incubated with 50 μM disodium pamidronate than in the controls at 3, 6 and 24 hours (PMN: 3 h = 50.86 ± 3.25 nM/10^6 cells; 6 h = 51.73 ± 5.07 nM/10^6 cells; 24 h = 52.02 ± 6.34 nM/10^6 cells. PMN + APD: 3 h = 101.49 ± 4.51 nM/10^6 cells; 6 h = 122.37 ± 10.41 nM/10^6 cells; 24 h = 107.13 ± 12.02 nM/10^6 cells; $p < 0.001$; Fig. 1A). This increase in $[Ca^{2+}]_i$ was parallel to the enhancement of NO production; in fact, our data shows that the level of NO released by PMN was significantly higher in APD-treated cells at 3, 6 and 24 hours of incubation (PMN: 3 h = 2.17 ± 0.27 nmol nitrite/mg protein; 6 h = 3.62 ± 1.09 nmol nitrite/mg protein; 24 h = 0.85 ± 0.09 nmol nitrite/mg protein. PMN + APD: 3 h = 5.20 ± 0.75 nmol nitrite/mg protein; 6 h = 6.25 ± 1.51 nmol nitrite/mg protein; 24 h = 3.92 ± 1.11 nmol nitrite/mg protein; $p < 0.001$; Fig. 1B).

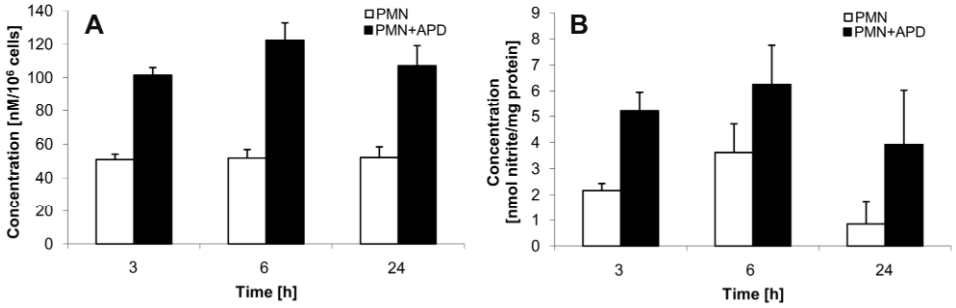

Fig. 1. The levels of $[Ca^{2+}]_i$ and NO production in PMN after incubation with disodium pamidronate. A: The $[Ca^{2+}]_i$ concentration in human PMN incubated with disodium pamidronate (PMN + APD) in comparison with the controls (PMN) at 3, 6 and 24 h. The means ± S.D. are shown. $p < 0.001$. B: The level of NO produced by human PMN after 3, 6 and 24 h of incubation with disodium pamidronate (PMN + APD) with respect to the controls (PMN). The means ± S.D. are shown. $p < 0.001$.

For the platelets, the levels of both $[Ca^{2+}]_i$ and NO production were significantly higher after 3 h of incubation with disodium pamidronate relative to the controls (Tab. 1).

Tab. 1. $[Ca^{2+}]_i$ and NO production in the platelets after 3 h of incubation with disodium pamidronate (PLT + APD) in comparison with the controls (PLT).

	PLT	PLT + APD
$[Ca^{2+}]_i$ (nM/10^6 cells)	57.98 ± 12.68	162.83 ± 38.65*
NO (nmol nitrite/mg protein)	4.72 ± 0.54	5.02 ± 0.59**

*$p < 0.001$; **$p < 0.05$

In this study, we also evaluated the PMN myeloperoxidase activity and found a significant decrease after 3, 6 and 24 hours of incubation with disodium pamidronate in comparison with the untreated cells (PMN: 3 h = 5.06 ± 0.57 U/mg prot./h; 6 h = 8.32 ± 2.36 U/mg prot./h; 24 h = 4.08 ± 2.29 U/mg prot./h. PMN + BP: 3 h = 3.25 ± 0.64 U/mg prot./h; 6 h = 6.21 ± 2.34 U/mg prot./h; 24 h = 1.19 ± 0.87 U/mg prot./h.; $p < 0.001$; Fig. 2).

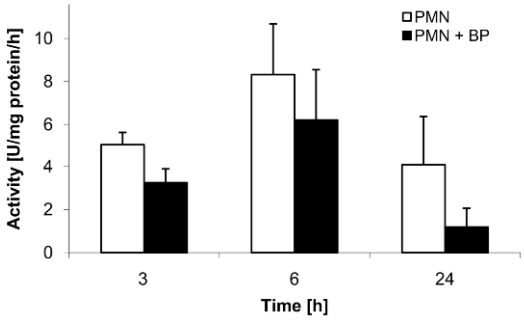

Fig. 2. The PMN MPO activity after 3, 6 and 24 h of incubation with disodium pamidronate (PMN + APD) in comparison with the untreated cells (PMN). The means ± S.D. are shown. $p < 0.001$.

DISCUSSION

Pamidronate is an aminosubstituted bisphosphonate which produces a potent inhibition of bone resorption [25]. Moreover, an anti-inflammatory response to this drug was previously suggested in a study of patients with rheumatoid arthritis [26].

Since there is increasing evidence that BP have effects beyond the osteoclasts [27], we investigated the action exerted by pamidronate on PMN and platelets, and in particular we studied the effects of the drug on the $[Ca^{2+}]_i$, the level of NO production, and the MPO activity, because its mode of action is not completely clear. Our results indicate that BP treatment provokes a significant increase in the $[Ca^{2+}]_i$, both in PMN and platelets, and that this increase was parallel to an enhancement in NO release, thus suggesting the possible activation of the constitutive nitric oxide synthase (cNOS), which is Ca^{2+}-dependent and present both in PMN and platelets [28-30]. The NO production by these cells in the bone microenvironment could regulate the osteoclast function, and, in addition, both PMN and platelets may also serve as targets for NO action.

In fact, NO can down-regulate neutrophil aggregation and secretion and may protect from the damage induced by the potent reactive oxygen metabolites produced by PMN themselves, as previously described, since it has been reported to be a free radical scavenger [31-33]. Moreover, NO might inhibit platelet aggregation and adhesion, thus having an anti-inflammatory action [34].

Concerning MPO, our results show a significant reduction of its activity at 3, 6, and 24 h of incubation with pamidronate in comparison with the controls, which

is in line with data previously obtained using sodium aledronate in animal studies [4]. Even if MPO is an important part of the antimicrobial defence, the increased formation of hypohalous acid may aggravate tissue damage during the inflammation processes. In this context, we could hypothesize that NO serves as a negative regulator of MPO activity, in agreement with previous data demonstrating that NO inhibits many iron-containing enzymes [32].

In conclusion, at this stage we can only speculate about the clinical relevance of our observations, even if our suggestion is that treatment with pamidronate could stimulate NO-production by cells present in or near the bone compartment, thus constituting a protective mechanism against bone resorption occurring during pathological conditions such as inflammation. In addition, PMN- and platelet-derived NO could act as a negative feed-back signal to restrict the inflammatory processes, confirming the anti-inflammatory properties of pamidronate.

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