Combined effects of ascorbic acid and phosphate on rat VSMC osteoblastic differentiation

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Abstract

Background. Ascorbic acid (AA) supplementation has been suggested to afford erythropoietin hyporesponsiveness and high levels of ferritin in haemodialysis (HD) patients. However, little is known about the possible side effects of this policy on vascular calcification (VC). VC, induced by a high-phosphate and uraemic milieu, is characterized by a passive deposition of calcium–phosphate (Ca-P) and an active transformation of vascular smooth muscle cells (VSMCs) in osteoblastic-like cells. The aim of these studies was to characterize the combined effects of AA and P on VC.

Materials and methods. Rat VSMCs were challenged with inorganic P (Pi) and AA, and Ca deposition analysis was performed to quantify VC. To investigate VSMC osteoblastic differentiation, we analysed α-actin protein content and core-binding factor alpha-1 (Cbfα1/RUNX2) messenger RNA (mRNA) expression.

Results. When incubated with 5 mM Pi, VSMCs showed a significant increase in Ca deposition compared to control cells. Interestingly, the addition of AA in the calcification medium resulted in a dose-dependent increase in Pi-induced Ca deposition. At the same time, the combined effect of AA and Pi on VSMCs resulted in the reduction of α-actin protein content and in a 4-fold increase of Cbfα1/RUNX2 mRNA expression.

Conclusions. We demonstrated that AA combined with Pi increases Ca deposition in rat VSMCs. The role of AA as
Ascorbate and phosphate-induced VC

cofactor in osteoblastic differentiation was demonstrated by phenotypic changes in VSMCs and enhanced bone mineralization key gene expression. These in vitro preliminary data suggest a potential role for AA combined with Pi in worsening VC.

**Keywords:** ascorbic acid; phosphate; vascular calcification; vascular smooth muscle cell

**Introduction**

Vascular calcification (VC) is a relevant complication in haemodialysis (HD) patients, leading to increased cardiovascular (CV) morbidity and mortality. In fact, uraemic subjects, in addition to accelerated atherosclerosis, develop diffuse medial calcification which, in turn, is associated with increased vascular stiffness, enhanced CV disease and higher mortality rates [1]. More specifically, two different pathogenetic pathways have been proposed to better understanding VC: a passive deposition of calcium–phosphate (Ca–P) and an active transformation of vascular smooth muscle cells (VSMCs) in osteoblast-like cells [2].

Over the last 10 years, many authors have investigated the pathogenesis of Pi-induced VC [3, 4]. The initial observations that high P levels in HD patients are associated with enhanced CV morbidity and mortality [5], suggested that inorganic P (Pi) may induce VSMC phenotypic transdifferentiation [6]. Indeed, this process in vitro appears to be mediated by a specific activation of the core-binding factor alpha-1 (Cbfα1/RUNX2), an osteoblast-specific gene, that regulates the expression of several bone morphogenic proteins [7, 8]. The cellular formation of a bone-like structure in calcified vessel walls indicates that the uraemic environment and elevations in serum P levels may be regulating factors.

Considering HD patients, together with mineral metabolism disorders, anaemia represents another major CV risk factor. Ascorbic acid (AA) supplementation has been suggested to afford erythropoietin (EPO) hyporesponsiveness and high levels of ferritin in HD patients [9]. However, little is known about the possible side effects of this policy on accelerated atherosclerosis and VC. A major concern regarding the safety of AA supplementation derives from its metabolism to oxalate, which may play a potential role in Ca oxalate extraskelatal deposition by exceeding the threshold of its solubility and eventually increasing CV risk in HD patients.

The biochemical role of AA has been described for several decades [10], and the majority of the studies investigating molecular mechanisms of VC have used AA as promoter of the pathogenesis of this process. Although the effects of AA in collagen synthesis and chondrocyte metabolism have been deeply investigated [11], on the contrary, its effect on VSMC calcification has not been yet elucidated.

In order to investigate the combined effects of AA and Pi on rat VSMCs, we evaluated VC by Ca deposition analysis and osteoblastic differentiation by phenotype changes and enhanced bone mineralization key gene expression.

**Materials and methods**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM) [high glucose, (4.5 g/L), fetal bovine serum (FBS) and BCA protein assay kit (Pierce)] were purchased from Euroclone (Milan, Italy), Na₂PO₄ was from Carlo Erba (Milan, Italy), calcium (Ca) kit was from Foker Italia (Salerno, Italy) and cell culture coverslip 13 mm diameter were from Nunc (Rochester, NY). Primary antibody for α-actin (A2547) and anti-mouse secondary antibody (A9917) were from Sigma (St Louis, MO). Polyvinilidene fluoride (PVDF) membrane, Taqman gene assay for Cbfα1/RUNX2 (Rn_01512298) and for β-actin (Rn_00667869) and all reagents for gene expression assays were from Invitrogen/Applied Biosystem (Milan, Italy). Unless otherwise mentioned, all the other reagents were obtained from Sigma.

**Cell culture**

Rat VSMCs were obtained by enzymatic digestion. Briefly, VSMCs were cultured according to Ross [12] from media layers of aorta of male Sprague-Dawley rats (200–250 g). Cells were grown in monolayers at 37°C in humidified atmosphere of CO₂ in DMEM supplemented with 10% (vol:vol) FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 20 mM trisodium buffer and 1% (vol:vol) non-essential amino acid solution. The medium was changed every third day. The cells grew out of explants after 12–16 days, piled up after confluency and contained numerous myofila-

**Induction of calcification**

VSMCs were routinely subcultured in growth medium (DMEM containing 10% FBS supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin). At 80% confluence, the cells were switched to calcification medium [DMEM containing 15% FBS, 10 mM sodium pyruvate and supplemented with 5 mM Pi and 50 μg/mL AA (unless otherwise stated), 100 U/mL penicillin and 0.1 mg/mL streptomycin] for up to 14 days. The medium was replaced every 2 or 3 days. For time course experiments, the first day of culture in calcification medium was defined as Day 0.

**Quantification of calcium deposition**

Cells were decalcified by incubation for 24 h with 0.6 M HCl, and Ca content in HCl supernatants was determined colorimetrically at 565 nm wavelength by the a-creophthalein complexone method. After decalcification, the cells were washed three times with phosphate-buffered saline and solubilized with 0.1 N NaOH/0.1% sodium dodecyl sulphate (SDS). The protein content was quantified by a BCA protein assay kit (Pierce). Alizarin Red staining was semi-quantitatively evaluated using a score system (0–4), related to the stained area (0 < 10%; 1 = 11–25%; 2 = 26–50%; 3 = 51–75%; 4 > 75%).

**RNA extraction and reverse transcription–polymerase chain reaction**

Total RNA was extracted using PurixLink RNA Mini Kit according to the manufacturer’s instructions. The concentration of RNA was measured using a UV spectrophotometer. Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit. All TaqMan PCR was performed using an StepOne Real-Time PCR System. Each reaction mixture (20 μL) contained 10 μL of 2X TaqMan Universal PCR Master Mix, 1 μL of Taqman gene assay, 9 μL of complementary DNA sample and water (50 ng). The thermal cycling conditions comprised the initial steps at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Amplification of the target genes was normalized to simultaneous amplification of an internal housekeeping gene, β-actin, and calibrated to a low expression normalized target sample.
Western blot analysis

Rat VSMC were harvested in ice-cold homogenization buffer (50 mM Tris pH 8 with 0.5% IGEPAL CA-630, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 1 mM NaF, 10 mM β-glycerophosphate and complete protease inhibitor), freeze thawed (2×) and probe sonicated 5×20 s, at 40% power. The samples were then centrifugated at 13 000 g for 15 min at 4 °C and protein concentration measured. Denatured samples (10 μg total protein) were separated by electrophoresis on a 12% SDS-polyacrylamide gel and transferred to PVDF membrane. The membranes were incubated 1 h with 1:15,000 β-actin primary antibody followed by 1 h incubation with 1:40 000 peroxidase-conjugated antimouse secondary antibody. Protein bands were visualized using an ECL detection kit and area intensity was measured.

Statistical analysis

Results were expressed as mean ± standard error of the mean. Each experiment was performed at least three times at least in triplicate. Differences between groups were analysed by two-way analysis of variance.

Results

Characterization of VSMC calcification

To induce VSMC calcification, we used two different Pi concentrations (3 and 5 mM), at two different time points (10 and 14 days). Figure 1 shows that the challenge with 5 mM Pi, but not with 3 mM Pi, significantly increases Ca levels from 0.9 ± 0.5 to 11.9 ± 1.0 μg Ca/mg protein (P < 0.01) at 10 days of incubation, whereas at 14 days from 0.9 ± 0.3 to 26.3 ± 4.3 μg Ca/mg protein (P < 0.01). Although 14 days of incubation resulted in enhanced Ca deposition, we used the time point of 10 days of incubation to avoid a massive cell death that was evident at 14 days (data not shown).

Combined effects of AA and phosphate on VC

In order to evaluate the effect of AA on the Pi-induced VC, we performed a concentration–response curve of AA. As shown in Figure 2, AA concentration dependently enhances the Ca deposition induced by Pi (P < 0.01). The concentration of 50 μg/mL of AA has been chosen for the following experiments since higher AA concentrations, such as ≥75 μg/mL, causes formation and deposition of calcium oxalate crystals. In mineralizing conditions of 5 mM Pi, VSMCs showed a positive staining with Alizarin Red compared to control cells, that is further augmented by the addition of 50 μg/mL of AA (Figure 3). The semiquantitative analysis confirmed a significant difference between VC in absence and presence of AA (2.34 ± 0.07 versus 3.23 ± 0.08, respectively; score 0–4; P < 0.05).

In order to verify if AA triggers or has a combined effect with Pi in VC, we performed two different Pi concentration–response curves, with or without AA (Figure 4). In the absence of AA, VC reached a plateau with a Pi concentration of 4 mM. In the presence of AA, there was not any additional effect at either 3 or 4 mM Pi on VC, but the effect of AA on increased amount of Ca deposition was evident at Pi concentration of 5 mM.

To confirm that also in presence of AA the VSMC calcification is mediated by a Pi-dependent mechanism, we used phosphonoformic acid (PFA), the inhibitor of the sodium-dependent Pi cotransporter Pit-1. As shown in Figure 5, 1 mM PFA inhibited Ca deposition after challenge with 5 mM Pi and AA (P < 0.01).

Combined effects of AA and phosphate on VSMCs osteoblastic differentiation

Since Pi challenge is able to induce an osteoblast-like transformation of VSMCs [10], we investigated the combined effects of AA and Pi on VSMC phenotype changes. In our experimental conditions, Pi-induced VC did not make any evident change in β-actin protein expression at 7 days of incubation (Figure 6, Lane 2), compared to control (Figure 6, Lane 1). On the contrary, the addition of 50 μg/mL of AA and 5 mM Pi in the calcification media, resulted in a significant reduction of β-actin protein expression (Figure 6, Lane 4), compared to AA alone (Figure 6, Lane 3) (from 86.8 ± 0.4% to 51.2 ± 2.7%, intensity area, P < 0.01). Interestingly, AA started to have a slight effect in decreasing β-actin expression at 7 days in control conditions (Figure 6, Lane 3 compared to Lane 1) (86.8 ± 0.4%; 97.9 ± 0.7%, intensity area, respectively; 0.9/C6 < 0.01).
not significant), with a more evident effect at Day 11 (Figure 6, Lane 7 compared to Lane 6) (73.8/0.3%; 99.9/7.1%, intensity area, respectively; not significant).

We next analysed the effect of AA on the gene expression of Cbfα1/RUNX2, a gene involved in the osteoblastic differentiation. The time course showed that, at 7 days of incubation, the challenge with 5 mM Pi and 50 μg/mL AA resulted in a peak with a 4.44/0.74-fold increase in Cbfα1/RUNX2 messenger RNA (mRNA), compared to the relative expression in normal VSMCs (t 0) (P < 0.01) (Figure 7A). Figure 7B showed that the combined effects of 5 mM Pi and 50 μg/mL AA on Cbfα1/RUNX2 mRNA expression was significantly higher than the effect of Pi alone (2.67/0.47 versus 1.54/0.03, P < 0.05). Interestingly, there was a slight increase in Cbfα1/RUNX2 mRNA expression in control VSMCs treated with AA, compared to those cultured in the absence of AA (1.25/0.14 versus 0.91/0.17, ns).

**Discussion**

In our study, we demonstrated that AA combined with inorganic phosphate (Pi) increases calcium (Ca) deposition in rat VSMCs. At the same time, the combined effect of AA and Pi resulted in phenotype changes in VSMCs and...
Data are presented as mean mRNA expression at 7 days. The effect of AA in increasing Pi-induced expression, compared to the relative expression in normal VSMCs (t0) mM Pi at 7 days induced a significant increase in Cbfala challenge with 5 mM Pi in presence of AA. About 50% Cbfala protein expression after 7 days was detected in AA-treated VSMCs compared to 25% in control VSMCs (Figure 6, Lane 4). Therefore, we found only a combined effect of AA and Pi in worsening VSMC phenotype, rather than an effect of high Pi alone.

In CKD patients, anaemia, together with hyperphosphataemia, represents another major CV risk factor impairing survival [16]. AA is believed to improve anaemia in patients with end-stage renal disease, but its overall effectiveness remains unclear [9]. It has been hypothesized that in HD patients with refractory anaemia and high levels of ferritin, the use of AA may improve EPO responsiveness, either by increasing iron mobilization from its tissue stores or by stimulating antioxidant effects [17].

In order to elucidate if the use of AA in HD patients may impact on the VC process, we investigated the combined effects of an AA and Pi in vitro model of VC. In different experimental models used to investigate Pi-induced VC, AA has been often added to calcification media, but this aspect has not been neither explained nor clarified. As already demonstrated by Trion et al. [18], we observed that AA concentration dependently increases the Ca deposition induced by Pi (Figure 2). Nevertheless, in our experiments, the maximal AA concentration used was 75 μg/mL since this and higher AA concentrations caused formation and deposition of calcium oxalate crystals.

Interestingly, it was possible to detect an effect of AA in worsening Ca deposition only at a Pi concentration of 5 mM, demonstrating that AA does not trigger Pi-induced VC, but it has a combined effect on calcification of VSMCs (Figure 4). To further support the hypothesis of a combined effect between AA and Pi on inducing Ca deposition, we showed that AA per se did not induce any calcification in VSMCs, but deserves high Pi as promoter, as demonstrated by the complete inhibition of Ca deposition by PFA, the inhibitor of the sodium-dependent Pi cotransporter Pit-1 (Figure 5).

Another interesting aspect in our study was to investigate the combined effects of AA and Pi on VSMCs osteoblastic differentiation. In fact, Pi alone did not make any evident change in α-actin protein expression at 7 days in calcified rat VSMCs (Figure 6, Lane 2), as demonstrated by other groups [19]. In contrast, Steitz et al. [20] demonstrated that high Pi can reduce the protein expression of VSMC markers, although these experiments were done in different species (bovine). Nevertheless, in our experimental model, the concurrent challenge with 5 mM Pi and 50 μg/mL AA resulted in an evident reduction of α-actin protein expression (Figure 6, Lane 4). Therefore, we found only a combined effect of AA on Pi induced in changing VSMC phenotype, rather than an effect of high Pi alone.

Furthermore, under similar experimental conditions, we observed a peak in Cbfala/RUNX2 mRNA expression at 7 days of calcification with >4-fold increase when compared to normal VSMCs (Figure 7A). In our model, also the mRNA expression of the well-known key regulator of osteoblastic activity, Cbfala/RUNX2, was significantly increased only under combined challenge with high Pi and AA (Figure 7B). This is the first demonstration of the combined effects of AA and Pi on osteoblastic differentiation of rat VSMCs.

Although AA per se did not have any effect on Ca deposition in our model at 11 days of incubation with 50 μg/mL of AA, there was a slight decrease in α-actin protein expression, suggesting a potential role of AA on phenotypic VSMC differentiation (Figure 6). This effect is supported by the trend towards an increase in Cbfala/RUNX2 expression after 7 days of Pi and AA treatment compared to 25% in control VSMCs (Figure 6, Lane 4). Therefore, we found only a combined effect of AA and Pi in worsening VSMC phenotype, rather than an effect of high Pi alone.
mRNA expression in VSMCs treated with AA alone, compared with untreated cells (Figure 7A and B).

The combined effect of AA and Pi on Ca deposition may be due to the role of AA as cofactor in intracellular collagen biosynthesis, alkaline phosphatase activity and extracellular Ca deposition in osteoblasts and osteoblastic-like cells [21]. In fact, it has been shown that AA plays an important role in different biological functions, such as mesenchymal [22] and chondrocyte differentiation [11]. It has been shown that the use of AA accelerates in a particular cell line (the mouse embryonal carcinoma-derived cell line ATDC5) the multistep chondrogenic differentiation observed during endochondral bone formation [11]. AA exposure was associated with increased expression of key genes implicated in both bone mineralization and VC, such as collagen II, Cbfβ1/RUNX2, Sox9 and collagen X. Furthermore, AA enhanced Erk activation, while Erk inhibition attenuated AA-induced differentiation [11]. In addition, it has been documented that ascorbate enhances 1,25-dihydroxyvitamin-D₃ synthesis, accompanied by upregulation of the vitamin D receptor, suggesting that it may be causing amplification of the vitamin D receptor-dependent genomic response to 1,25-dihydroxyvitamin D, resulting in promotion of terminal differentiation [21].

Pharmacokinetic studies in healthy people showed that intravenous administration of AA can achieve 70-fold higher blood levels than the highest tolerated oral dose [23]. Many clinicians use AA in HD patients affected by anaemia and the usual intravenous administered dosage is 500 mg thrice weekly [9]. This dosage is approximately equivalent to a peak, at 3 h after the administration, of 40–50 µg/mL AA, the same range of concentration used in our experimental studies to induce VSMCs osteoblastic differentiation and potentiate high Pi-induced VC. Moreover, AA is administered at the end of the HD session, when patients have metabolic alkalosis, have received calcium from dialysis bath, are treated with active vitamin D and have a pro-inflammatory status due to dialysis [24]. All these conditions predispose to VC and intravenously administration of AA may worsen this process.

In conclusion, a reproducible model of VC on rat VSMCs has been investigated. In our model, we found a combined effect of AA and Pi in inducing not only Ca deposition but also in osteoblastic differentiation of rat VSMCs. This effect is probably due to the role of AA as cofactor in intracellular collagen biosynthesis, bone mineralization key genes expression (Cbfβ1/RUNX2) and extracellular Ca deposition in osteoblastic-like cells. From our and other in vitro studies, it seems that AA can accelerate VC, and that, due to this effect on VC, the use of intravenous AA administration in HD patients should be reconsidered.

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References

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