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Mechanisms of calcification by vesicles isolated from atherosclerotic rabbit aortas

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Abstract

Although several lines of evidence support the role of calcifiable vesicles in dystrophic vascular calcification, the mechanisms whereby vesicles promote aortic calcification remain incompletely understood. Previous reports indicate that ATP promotes in vitro vesicle calcification. Whether ATP-initiated calcification is simply mediated through increased Pi concentrations or by other unknown mechanisms related to ATP hydrolysis is unclear. To determine whether high Pi levels resulting from ATP hydrolysis may cause Ca × P ion products to surpass the threshold for calcium phosphate precipitation, 3 mM Pi instead of 1 mM ATP was added to calcifying media. The inclusion of 1 mM ATP in calcifying media with an initial serum level of Ca^{2+} (1.45 mM) and Pi (2.3 mM) was much more effective in promoting calcification than the addition of 3 mM Pi. The higher effectiveness of ATP over Pi in promoting calcification was consistent throughout various incubation periods and vesicle protein ranges. To minimize the effect of Ca × Pi ion products on calcification, the ion product was kept within the physiological ranges throughout the incubation period by reducing initial Pi or ATP concentrations in calcifying media. At these low levels of ion products, ATP was still more effective than Pi in promoting calcification. Both ATP- and Pi-stimulated calcifications were found to increase with increasing levels of ion products whereas greater effectiveness of ATP over Pi remained unaltered. These observations indicate that ATP hydrolysis may initiate calcification through some mechanisms other than a simple provision of Pi in order to surpass the solubility products. Concanavalin A (Con A) was found to bind to vesicles and to enhance both ATP- and Pi-promoted calcification. Taken together, these observations suggest that ATP hydrolysis, Ca × P ion products, and vesicle-associated carbohydrates are implicated in vesicle-mediated calcification.

Keywords: Calcification; Membrane; ATP; Calcifiable vesicle; Lectin

1. Introduction

Aortic wall calcification associated with atherosclerosis may impose a serious rupturing effect on aortas and cause bioprosthetic valves to fail [1–3]. The mechanism of the dystrophic calcification process remains unknown although current studies suggest that a number of interacting factors are involved (for a review see Ref. [4]). Investigations derived from morphological evidence [5,6] and recent demonstrations of the ability of vesicles isolated from human and rabbit atherosclerotic aortas to calcify [7–9] strongly support the role of membrane vesicles in vascular calcification. However, the detailed mechanisms whereby these subcellular organelles initiate calcification have not been fully elucidated. Since ATP can initiate in vitro vesicle calcification, it remains to be established whether a specific event or the resulting increased levels of Pi related to ATP hydrolysis underlie the causal effect of ATP. We hereby compare the effect of Pi and ATP on vesicle calcification. Additionally, we demonstrate physical binding of concanavalin A (Con A) to calcifiable vesicles as a plausible prerequisite step for the stimulation of vesicle calcification by lectin, further supporting the role of vesicle-associated carbohydrates in vesicle calcification.

2. Experimental procedures

2.1. Induction of atherosclerotic calcification by a high-cholesterol diet

Four-month-old rabbits were fed a standard rabbit diet with 0.25% cholesterol and 1% peanut oil as supplements

Abbreviations: Con A, concanavalin A; FT-IR, Fourier transform infrared spectroscopy

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(supplied by Harland Teklad). After 6 months of chronic dietary interventions, rabbits developed conspicuous calcification in the intimal areas immediately adjacent to the media.

2.2. Isolation of calcifiable vesicles from aortas

To isolate calcifiable vesicles, the method of Hsu and Camacho [7] was used. Segments of ascending thoracic aortas (about 3-in. long) were removed, the attached fat on the adventitia removed, and immediately submerged in phosphate-buffered saline (PBS) at 4 °C. The tissues were minced into fine pieces, washed once with 10-ml PBS by centrifugation, and then digested for 3 h at 37 °C in a crude collagenase solution (15 ml/g of tissue) containing 0.1% of crude collagenase (Boehringer Mannheim, Type B), 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl, 100 U/ml of penicillin, 1 mg/ml of streptomycin, and 0.02 M Tes buffer (N-tris[hydroxmethyl]-methyl-2-amino-ethanesulfonic acid), pH 7.45. The digests were centrifuged at $800 \times g$ to spin down cells and cell debris. The supernatants were then centrifuged at $30{,}000 \times g$ for 10 min to spin down mitochondria and microsomes. The resultant supernatants were centrifuged at $300{,}000 \times g$ for 20 min. The pellets were resuspended in 10 mM Tris-buffered saline (TBS, pH 7.6)–0.25 M sucrose and centrifuged. The resultant precipitates were then resuspended in 1-ml TBS–0.25 M sucrose to yield a protein concentration of about 0.3 mg/ml.

2.2.1. Calcium deposition

The term "Ca deposition" is used to describe the process by which Ca is deposited either as undefined forms of calcium phosphate, or as ions that are taken up by or bound to calcifiable vesicles. To measure Ca deposition, the method of Hsu and Camacho [7] was used with a slight modification. Unless otherwise stated, the freshly prepared standard calcifying medium (100 μl) consisted of 50 mM Tris, pH 7.6 (pH was adjusted at 37 °C), 85 mM NaCl, 15 mM KCl, 1 mM $MgCl_2$, 30 mM $NaHCO_3$, 1.45 mM $CaCl_2$, 2.3 mM Pi, and ± 1 mM ATP. $^{45}Ca^{2+}$ (1×10^6 cpm) was used as tracer. The reaction was initiated by addition of aliquots of calcifiable vesicles (final protein concentration: 15 μg protein/ml) to calcifying media and then incubated for 5 h at 37 °C in a water vapor-saturated incubation chamber with atmosphere CO_2. At the end of incubation, the reaction mixture was filtered through 0.1 μm pore-size Durapore membranes (Millipore Inc.) attached to a Millipore vacuum trap device. The membrane filters were washed twice each with 1 ml of TBS, pH 7.6, and then transferred to vials containing scintillation fluids for radioactivity counting. The nonspecific $^{45}Ca^{2+}$ binding is defined as the radioactivity nonspecifically bound to the filters under the identical conditions in the absence of calcifiable vesicles (0.6 ± 0.2% of the total radioactivity). These nonspecific counts were then subtracted from the radioactivity in the presence of calcifiable vesicles with or without ATP under various experimental conditions. Ca deposition is expressed as "nmol Ca/μg vesicle proteins/5 h" and is calculated using the formula, [cpm with calcifiable vesicles minus nonspecific binding/total cpm], multiplied by the concentration of $CaCl_2$, and then divided by the concentration of calcifiable vesicle proteins. Except for time course study, Ca deposition activity after 5 h of incubations was used to maximize the difference between calcium deposited by vesicles and the background count on filters in case perturbing agents happen to be inhibitory to vesicle calcification. The total deposition activity after 5 h instead of the rate was expressed in figures since the activity was relatively nonlinear during the incubation period possibly as a result of the complex pattern of mineral proliferation. Transmission electron microscopy, calcifying activity, and the ATPase activity measurement indicate that no significant amounts of vesicles and mineral passed through filters although some size-ranges of mineral and vesicles can be smaller than the pore size of the filters (not shown).

2.2.2. Protein and phosphate assays

Inorganic orthophosphate was determined by the method of Martin and Doty [10]. The protein concentration was measured by the method of Lowry et al. [11] using bovine serum albumin as standard.

2.2.3. Assessment of binding of Con A to calcifiable vesicles

A 10-μl aliquot of vesicles at various concentrations was added to a 100-μl TBS containing 100 μg/ml biotinylated Con A and incubated for 30 min at 25 °C. Two types of controls were used; one included biotinylated Con A without vesicles and the other with vesicles but without biotinylated Con A as the control for endogenous alkaline phosphatase. The latter showed no endogenous alkaline phosphatase activity [7,8]. The mixtures were centrifuged at $250{,}000 \times g$ to remove the supernatants and then the precipitates were washed twice each with 1-ml TBS. The precipitates were resuspended in 100 μl of TBS, followed by the addition of a 5-μl aliquot of avidin-conjugated alkaline phosphatase, and incubated for 30 min. The mixtures were centrifuged to remove the supernatants and the resulting precipitates were washed twice each with 1-ml TBS. The precipitates were then resuspended in 100 μl of TBS and assayed for the presence of alkaline phosphatase activity, indicative of Con A binding.

3. Results

3.1. To determine whether increased Pi levels resulting from ATP hydrolysis solely underlie the mechanism of ATP-initiated calcification by isolated vesicles

Previous experiments using isolated vesicles from atherosclerotic rabbits and humans demonstrated that ATP induces in vitro calcification [7,8]. To determine whether

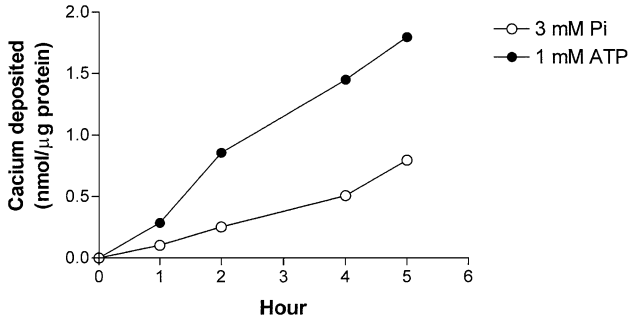

Fig. 1. Time course of Ca deposition by vesicles in the presence of 1 mM ATP or 3 mM Pi. In the presence of ATP, the initial Pi concentration was 2.3 mM whereas in its absence the addition of 3 mM Pi to calcifying media increased the initial Pi concentration to 5.3 mM. Under these circumstances, the addition of Pi to calcifying media created a higher initial Ca × P ion product than by adding ATP. In either case, the final ion products at the end of incubation were expected to be close to or within 7.7 mM^2. The purpose of these arrangements is to minimize the effect of Ca × P ion product on ATP-induced calcification, thereby allowing the comparison of the effectiveness of ATP versus Pi on calcification. Ca deposition is expressed as nmol Ca/μg protein deposited at the end of each incubation period.

high Pi levels resulting from ATP hydrolysis can cause Ca × P ion products to surpass the threshold for calcium phosphate precipitation, 3 mM Pi instead of 1 mM ATP was added to calcifying media. The effects of ATP versus Pi on calcification at various incubation periods and vesicle concentrations were then compared. The time-course of ATP- and Pi-induced Ca deposition indicates a gradual increase in activity over a period of up to 5 h (Fig. 1). ATP apparently was much more efficient than Pi in promoting Ca deposition throughout the entire incubation periods. A similar result was also obtained when the effect of ATP and Pi was compared at various concentrations of vesicles (Fig. 2). To minimize the effect of increased Ca × Pi ion products on calcification, the ion product was kept within the physiological ranges of 3.33 mM^2 throughout the incubation period. This was accomplished by reducing the initial concentrations of Pi in calcifying media to counter the increased levels of Pi resulting from ATP hydrolysis (the first data

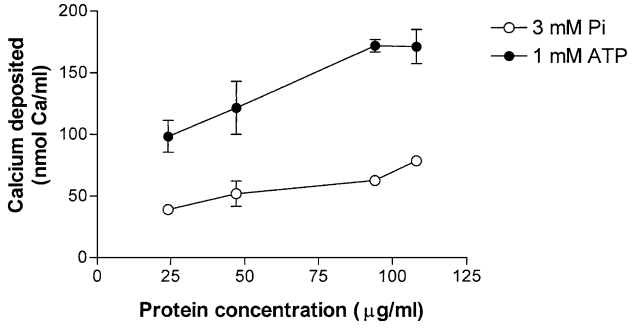

Fig. 2. Effect of vesicle concentrations on Ca deposition in the presence of 1 mM ATP or 3 mM Pi. In the presence of ATP, the initial Pi concentration was 2.3 mM whereas the addition of 3 mM Pi to calcifying media increased the initial Pi concentration to 5.3 mM. The expected initial and final ion products are described in the legend to Fig. 1. Ca deposition is expressed as nmol Ca/μg protein deposited at the end of 5-h incubation period.

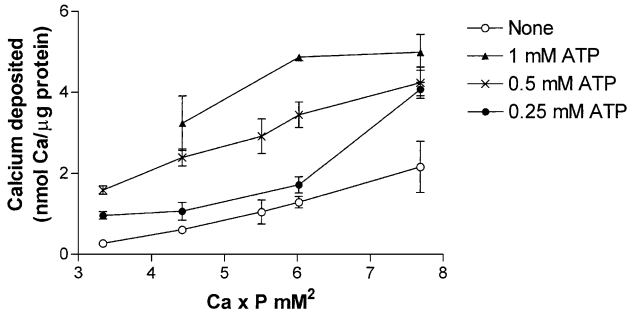

Fig. 3. Effects of final Ca × P ion products on ^{45}Ca deposition by vesicles. The concentration of $CaCl_2$ in calcifying media was fixed at 1.45 mM whereas the final Ca × P ion products were adjusted to a desired level by varying the initial concentrations of Pi or ATP, which led to high levels of Pi upon its hydrolysis. Accordingly, the initial Pi concentration in the medium for each ion product was adjusted to a lower level in proportion to an increase in Pi levels resulting from ATP hydrolysis. Since high levels of ionic products may spontaneously precipitate calcium phosphate, Ca deposition in the absence of vesicles was subtracted from that in their presence at each ion product. Ca deposition was measured as the amount of ^{45}Ca deposits trapped on Millipore filters. Values are given as means ± S.E. from three separate experiments. The vesicle preparation represents a pool of vesicles obtained separately from four rabbits. Details of the Ca deposition assay are described under Experimental procedures. Ca deposition is expressed as nmol Ca/μg protein deposited at the end of 5-h incubation period.

points at 3.33 mM^2 ion product in Fig. 3). At these low levels of ion products, ATP was still more effective than Pi in promoting calcification. To investigate the role of Ca × P ion products in calcification, the effects of increasing ion products on calcification was studied. Ca deposition was

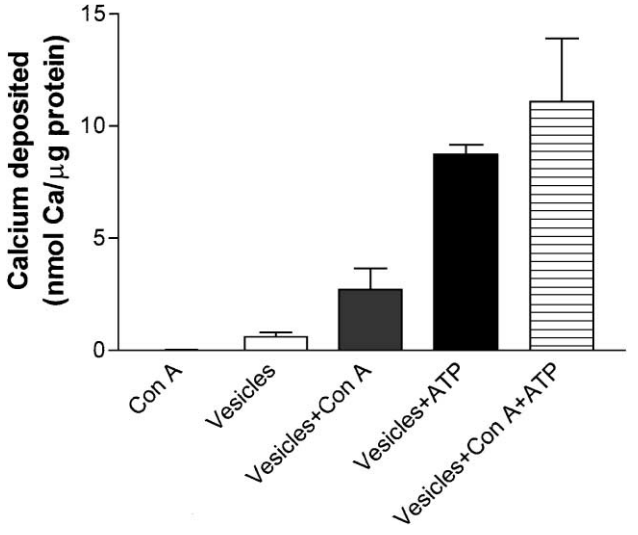

Fig. 4. Effects of ATP and Con A on calcification. Ca deposition was measured as the amount of ^{45}Ca deposits trapped on Millipore filters. $^{45}CaCl_2$ deposition on the filter in the absence of vesicles was used as a blank to subtract from all the experimental data. Values are given as means ± S.D. from three separate experiments. The vesicle preparation represents a pool of vesicles obtained separately from four rabbits. Details of the calcification assay are described under Experimental procedures. Ca deposition is expressed as nmol Ca/μg protein at the end of 5-h incubation period.

found to be enhanced with increasing levels of the ion products (Fig. 3). At each level of ion product, however, ATP was more effective than Pi in promoting calcification.

3.2. Effect of Con A on vesicle-mediated calcification

Previous studies demonstrated that Con A enhanced ATP-promoted calcification by isolated calcifying vesicles [12]. The present study showed that Con A at 12 μg /ml stimulated both Pi- and ATP-promoted Ca deposition (Fig. 4, 3rd and 5th columns, respectively). However, the magnitude of Pi-promoted calcification after stimulation by Con A (3rd column) was still much less than baseline ATP-initiated calcification without Con A stimulation (4th column). To determine whether the binding of Con A to vesicles is the prerequisite step for stimulation, a specific enzyme–ligand assay was performed (Fig. 5). The binding of Con A to vesicles was found to increase with vesicle protein concentrations. At nearly linear ranges of binding, it appears that about 1 μg of Con A was bound to 3 μg vesicle proteins.

Con A appeared to cause vesicles to swell and aggregate since the sediments after centrifugation resulting from Con A treatments were visibly much larger than those from untreated vesicles. The aggregation of vesicles may decrease the interstitial space among vesicles, thereby allowing close interactions between Ca^{2+} and Pi ions for the formation of mineral. To test this hypothesis, the effect of vesicle aggregation on calcification was studied by comparing calcifiability of pelleted vesicles to that of finely dispersed vesicles. An aliquot of vesicles was added to calcifying media and centrifuged immediately to pellet the vesicles, and then left as a pelleted form in calcifying media for 5 h to complete calcification. The result was then compared to that with finely resuspended vesicles. Under these conditions, there were no differences in the extent of calcification in finely dispersed and in pelleted forms of vesicles (data not shown).

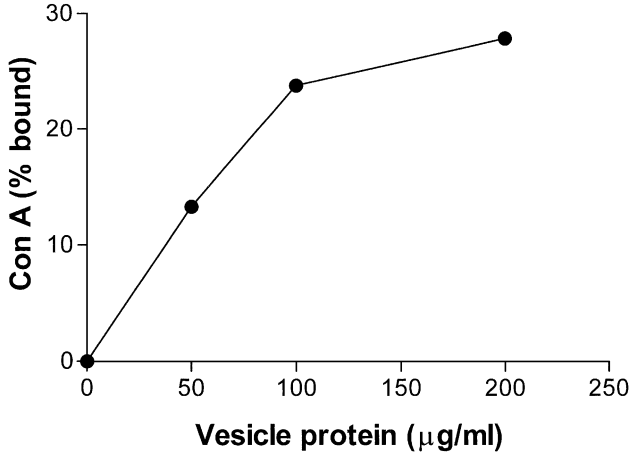

Fig. 5. Binding of Con A to calcifying vesicles. A specific ligand binding as described in detail under Experimental procedures was used to measure binding of Con A to calcifying vesicles.

4. Discussion

Earlier reports using vesicles isolated from human or rabbit atherosclerotic aortas indicated [7,8] that ATP can initiate vesicle-mediated calcification. The involvement of ATP in vesicle calcification has also been demonstrated using matrix vesicles isolated from epiphyseal and articular cartilage [13–15]. However, it remains to be established whether ATP through its hydrolysis by ATPase may simply generate sufficient Pi to exceed the solubility products. However, neither ATP nor high levels of Pi are required for Ca deposition when matrix vesicles isolated from chicken cartilage were used [16]. The observations reported herein suggest that some molecular events related to ATP hydrolysis may play an important role in calcification since ATP was much more effective than Pi in inducing calcification. Despite these observations, the identity of the factors related to the mechanisms whereby ATP promotes calcification remains to be established. Our previous studies [7,8] suggest the presence of a specific ATPase on the basis of several characteristics associated with ATPase including activation by Ca^{2+} or Mg^{2+}, and inhibition by either vanadate or Triton X-100. However, it is not known whether Ca transport-related ATPase is present in calcifiable vesicles to cause the accumulation of Ca^{2+} within vesicles as the prerequisite step prior to calcification.

The ability of high levels of Pi in inducing vesicle-mediated calcification may provide an alternative pathway for promoting plaque calcification. It has been shown that high-Pi diet can cure rickets in rats and that rachitic rat cartilage slices resume calcification in a high-phosphate medium [17]. Recently, Jono et al. [18] demonstrated that Pi concentration in excess of serum levels causes calcification to occur in human vascular smooth muscle cell culture. The high Pi level effect on mineralization appears to be mediated through an enhancement of osteoblastic expression markers including osteopontin and Cbfa-1. The enhanced phenotypic osteoblastic expressions offer a novel explanation for the stimulatory effect of Pi on vascular calcification. The source of Pi surrounding vesicles in vivo is not known but could well be ATP since the nucleotide is abundant in millimolar ranges within the cell. It is possible that ATP may somehow leak into extracellular matrices as a result of necrosis or apoptosis during atherosclerosis. The export of ATP from a variety of cells under physiological and pathological stimuli has been well documented (for review see Ref. [19]). Through ATP hydrolysis by various vesicle-associated enzymes, extracellular Pi levels can be elevated to create high-ion product conditions for mineralization. These ATP-hydrolyzing enzymes include ATPase and NTP-pyrophosphohydrolase, which produces AMP and pyrophosphate. AMP could be subsequently hydrolyzed by vesicle-associated AMPase to yield sufficient inorganic phosphate for mineral formation. Whether or not other types of phosphoesters can be a rich source of Pi has yet to be determined. β-Glycerophosphate has been widely used in

vitro as substrate for the induction of mineralized nodules in cultured osteoblasts or chondrocytes [20–22]. The induction of mineralization by β-glycerophosphate was thought to be mediated through increased levels of released Pi as a result of alkaline phosphatase activity. Despite these observations, the physiological function of β-glycerophosphate remains uncertain, as the substrate is not present in cells and the levels of the phosphoester required for calcification far exceed physiological concentrations [20,21]. It is interesting to note that minerals deposited by high levels of Pi are closer to the types of mineral in mineralized tissues in situ than those by β-glycerophosphate [21]. Additionally, the formation of mineralized nodules in a number of cell-lines are independent of β-glycerophosphate [22,23]. On the other hand, ATP could not only play a regulatory role in the calcification process through its intrinsic inhibitory effect on mineral formation but also an initiation role as a rich source of Pi upon its hydrolysis by ATPase, AMPase, and NTP-pyrophosphohydrolase. In contrast, β-glycerophosphate unlikely plays a role in aortic vesicle calcification in vitro as our previous observations indicated that its hydrolysis is virtually undetectable by vesicles isolated from human or rabbit aortas [7,8]. This is markedly contrary to high activity in matrix vesicles from cartilage [14]. Taken together, in spite of the in vitro ability of ATP and β-glycerophosphate to induce vesicle- and cell-mediated calcification, the functional substrates for biological calcification have yet to be identified.

In addition to the promoting effect of ATP and Pi on calcification, carbohydrates may also be involved in up-regulation of vesicle calcification since glucoside-specific Con A and galactoside-specific *Abrus precartorius* agglutinin were previously shown to stimulate Ca deposition by vesicles [12]. In contrast, the acetyl forms of glucosides or galactosides may not participate in up-regulation of vesicle calcification because their respective specific binding agglutinins did not exert an effect on vesicle calcification [12]. The stimulation of calcification by Con A appears to be mediated through binding of Con A to vesicles as evidenced by a specific ligand–enzyme binding assay (Fig. 5).

The mechanism by which Con A stimulated vesicle-mediated calcification remains unclear. Swelling of vesicles may also occur since the size of vesicle precipitate after centrifugation was visibly much larger than the precipitates from those vesicle without Con A pretreatment. The stimulation by Con A may be due to the aggregation of vesicles, thereby limiting the space among vesicles in favor of the interactions between Ca^{2+} and Pi ions for mineral formation. However, our attempts to aggregate vesicles by centrifugation failed to mimic Con A stimulation. Thus, an alternation at the molecular level as a result of its interactions with glycosyl residues on vesicles is more likely responsible for Con A stimulation.

Altogether, the observations that vesicle-mediated calcification can be enhanced by ATP, Pi, and membrane perturbation through glycosyl binding by lectins suggest that a complex calcification mechanism may operate within microenvironments surrounding and within vesicles involving various lipids, carbohydrates, and proteins.

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