[Frontiers in Bioscience 11, 136-142, January 1, 2006]

Phytate (*Myo*-inositol hexakisphosphate) inhibits cardiovascular calcifications in rats

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1. ABSTRACT

Calcification is an undesirable disorder, which frequently occurs in the heart vessels. In general, the formation of calcific vascular lesions involves complex physicochemical and molecular events. Calcification (hydroxyapatite) is initiated by injury and is progressed by promoter factors and/or the deficit of inhibitory signals. *Myo*-inositol hexakisphosphate (phytate, $InsP_6$) is found in organs, tissues and fluids of all mammals and exhibits an important capacity as a crystallization inhibitor of calcium salts in urine and soft tissues. The levels found clearly depend on the dietary intake but it can also be absorbed topically. In this paper, the capacity of $InsP_6$ as a potential inhibitor of cardiovascular calcifications was assessed in Wistar rats. Three groups were included, a control group, an $InsP_6$ treated group (subjected to calcinosis induction by Vitamin D and nicotine and treated with standard cream with a 2% of $InsP_6$ as potassium salt) and an $InsP_6$ non-treated group (only subjected to calcinosis induction). All rats were fed AIN 76-A diet (a purified diet in which $InsP_6$ is undetectable). Animals were monitorized every 12 hours. After 60 hours of calcinosis treatment, all rats of the $InsP_6$ non-treated group died and the rest were sacrificed. Aortas and hearts were removed. A highly significant increase in the calcium content of aorta and heart tissue was observed in the $InsP_6$ non-treated rats (21 +/- 1 mg calcium / g dry aorta tissue, 10 +/- 1 mg calcium / g dry heart tissue) when compared with controls (1.3 +/- 0.1 mg calcium / g dry aorta tissue, 0.023 +/- 0.004 mg calcium / g heart dry tissue) and $InsP_6$ treated (0.9 +/- 0.2 mg calcium / g dry aorta tissue, 0.30 +/- 0.03 mg calcium / g dry heart tissue) animals. Only $InsP_6$ non-treated rats displayed important mineral deposits in aorta and heart. These findings are consistent with the action of $InsP_6$, as an inhibitor of calcification of cardiovascular system.

2. INTRODUCTION

Calcification is an undesirable disorder frequently observed in the cardiovascular system, where it alters blood-vessel flexibility and promotes thrombosis and arterial rupture (1-3). When it appears in cardiac valves it is associated to several disorders that, if uncorrected, can lead to heart failure and death (4).

At present many identified risk factors for coronary artery calcifications as end-stage renal disease (5), advanced age (6), elevated plasma cholesterol (7), diminished high-density lipoprotein cholesterol (7), cigarette smoking (8), elevated blood pressure (9), obesity (7), diabetes (8) and elevated triglicerides (7) are known. Nevertheless, although vascular calcification may appear to be a uniform response to different types of vascular injury, it is a complicated disorder, with overlapping yet distinct unknown mechanisms of initiation and progression. In general, the formation of calcific vascular lessions appears to involve different complex physicochemical and molecular biological principles. Thus, it seems that the preexistence of an injury acting as inductor (heterogeneous nucleant) of the calcification (hydroxyapatite) is necessary and its progression would depend on the presence of other promoter factors (as hypercalcemia, hyperphosphatemia, etc.) and/or the deficit of inhibitory factors.
Some inhibitors of vascular tissue mineralization have been described, thus a candidate molecule seems to be a matrix gamma-carboxyglutamic acid (Gla) protein, a mineral binding protein (9), although the action of this protein appears to be also related to other complex cellular process linked to ossification processes (10). Phytate (*myo*-inositol hexakisphosphate, $InsP_6$) is a molecule found in abundance in plant seeds and also found in all mammalian organs, tissues and fluids but at significantly low concentrations (11,12). The levels found in blood and mammalian tissues clearly depend on its dietary intake (11,12), furthermore it has been demonstrated that this molecule is also topically absorbed (13) and it exhibits an important capacity as a crystallization inhibitor of calcium salts in urine and soft tissues (14,15). For this reason the aim of the present paper is to evaluate the capacity of phytate as a feasible inhibitor of cardiovascular calcifications.

3. MATERIALS AND METHODS

3.1. Animals, diets and treatments

Eighteen male Wistar rats of approximately 250 g from Harlan Iberica s.l. (Barcelona, Spain) were acclimated in the course of 7 days to our animal house. Animals were kept in Plexiglas cages (three animals per cage) at a temperature of 21 +/- 1 ºC and relative humidity of 60 +/- 5 % with a 12-h on-off light cycle. After this period, animals were randomly assigned into three groups of six rats respectively. Control group (it was subjected to placebo calcinosis induction), $InsP_6$ treated group (it was subjected to calcinosis induction and pre-treated with $InsP_6$ cream) and $InsP_6$ non-treated group (it was only subjected to calcinosis induction). All rats were fed with AIN 76-A diet (Ssniff Especialdiatën GmbH, Soest, Germany), a purified diet in which $InsP_6$ is undetectable. The procedures used in this experiment were carried out according to the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes and official permission to perform this animal experiment was obtained from the Bioethical Committee of our University.

3.2. $InsP_6$ cream pre-treatment

After a period of 16 days consuming AIN 76-A diet, the urinary $InsP_6$ became undetectable. Then $InsP_6$ treated group was topically subjected once a day until the end of the experiment (20 days) with 4 g of a standard cream with a supplement 2.0 % of $InsP_6$ as potassium salt used in previous work (13). The surface of treatment was about 50 cm^2. The application area was located on the back skin of the animal which was previously shaved using an *electric shaver* (each 4 days).

3.3. Calcinosis induction

After a period of 16 days with cream pre-treatment, the $InsP_6$ treated group and non-treated group were subjected to calcinosis induction and control group only to placebo calcinosis induction. Calcifications were induced according to P. Kieffer et al. (16) by injecting 300.000 IU/kg i.m. of vitamin D_3 (supplied by Fort Dodge Veterinaria, S.A., Girona, Spain) and 25 mg/kg p.o. of nicotine (as a solution 5 g/L of (-)-nicotine hydrogen tartrate salt supplied by Sigma Aldrich, Steinheim, Germany). The nicotine administration was repeated ten hours later. Control group received instead vitamin D_3 and nicotine, 0.15 M NaCl i.m. and destilled water respectively.

3.4. Monitorization and sample intake

The animals were monitorized every 12 hours. After 60 hours of calcinosis treatment, all rats of the $InsP_6$ non-treated group died and the rest of rats were sacrificed. Aortas and hearts were removed.

3.5. Histological analysis

Histological analysis of the aorta and heart calcifications was carried out on 10% buffered formalin fixed tissues. Tissues were first placed in 10% buffered formalin and fixed for 24 h at room temperature. Tissues were embedded, sectioned (sections of 4µm) and stained by hematoxiline-eosine. For histological analysis the section of all tissues was examined by an experimented pathologist. The calcium deposits presence was estimated semiquantitatively (in the transverse section) by absent, low, moderate or high notation.

3.6. $InsP_6$ determination

Samples of 24-h urine were collected at day 15 of cream pre-treatment to evaluate $InsP_6$ excretion by using a metabolic cage (Tecniplast, Gazzada, s.a.r.l., Italy).

The determination of InsP_6 levels in urine samples was performed using an analytical methodology based on column separation and following total phosphorus determination by inductively coupled plasma atomic emission spectrometry (ICP-AES) (17). This methodology allows a measurement of total InsP_6 with a detection limit of 60 µg/l.

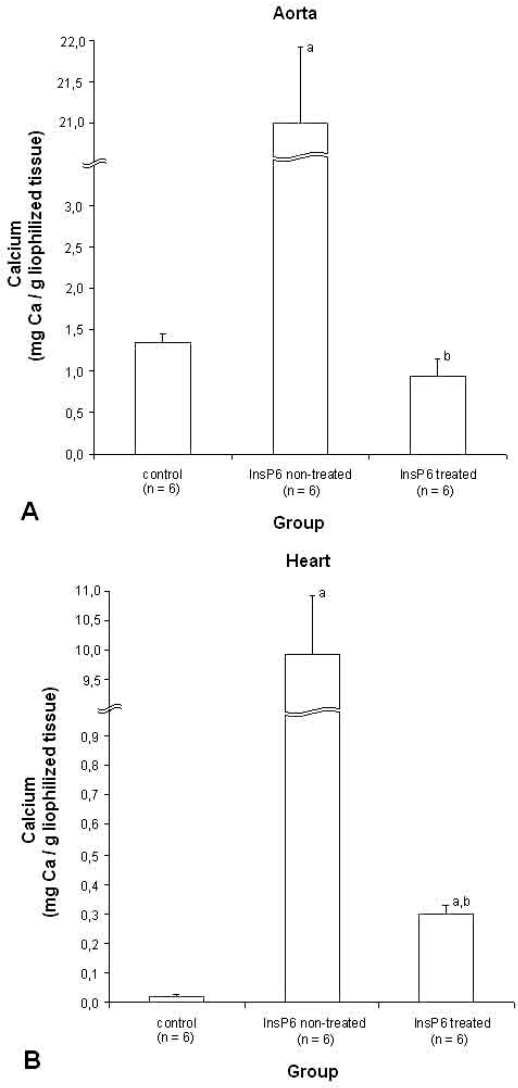

Figure 1. Calcium content in different tissues by the three studied groups (control, $InsP_6$ non-treated, $InsP_6$ treated): **a.** Aorta; **b.** Heart. a: $p < 0.001$ vs corresponding control group, b: $p < 0.001$ vs corresponding $InsP_6$ non-treated group

5.0 ml of urine (acidified with HCl 1:1 until pH = 3-4) was transferred to a column containing 0.2 g of anion exchange resin (the inner diameter was 4 mm). The first eluate was discarded, then the column was washed with 50 ml of HCl 50 mM. The second eluate was discarded. Then, the column was washed with 3.0 ml of HNO_3 2 M. The determination of InsP_6 was carried out through direct phosphorus analysis of this last eluate by ICP-AES using the corresponding calibration curve.

The ICP-AES conditions used were the following: outer argon flow 15 l/min, auxiliar argon flow 1 l/min, inner argon flow 1 l/min, nebulizer uptake rate 1 ml/min and wavelenght 213.618 nm.

3.7. Calcium determination

The samples of aortas and hearts were lyophilised and weighted. The residues were digested with 1:1 HNO_3:$HClO_4$ mixture in a sand bath until the solution was clear. For calcium determination, digested samples were diluted with distilled water until a final volume of 10 ml. The concentration of calcium was determined by inductively coupled plasma atomic emission spectrometry (Perkin-Elmer S.L., Spain) using the corresponding calibration curve.

3.8. Statistics

Values in the table and figures are expressed as mean +/- (SE). One-way ANOVA was used to calculate significance of difference between groups. The Student *t*-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p < 0.05$ was considered to assess statistical significance.

4. RESULTS

The calcium accumulation in aorta and heart tissues by the three studied groups (control, $InsP_6$ treated and $InsP_6$ non-treated) is shown in Figure 1 and phytate concentration in urine after 15 days of cream pre-treatment is shown in Table 1. As can be observed in Figure 1, a highly significant increase in the calcium content of the two studied tissues was observed in the $InsP_6$ non-treated rats (21 +/- 1 mg calcium / g dry aorta tissue, 10 +/- 1 mg calcium / g dry heart tissue) when compared with controls (1.3 +/- 0.1 mg calcium / g dry aorta tissue, 0.023 +/- 0.004 mg calcium / g dry heart tissue) and $InsP_6$ treated (0.9 +/- 0.2 mg calcium / g dry aorta tissue, 0.30 +/- 0.03 mg calcium / g dry heart tissue) animals. No significant differences were observed in aorta calcium content between the $InsP_6$ treated rats and controls. Significant differences between heart calcium content of $InsP_6$ treated rats and control rats were also observed. In Table 1 it can be appreciated that highly significant urinary excretion of $InsP_6$ was observed in the $InsP_6$ treated rats when compared with controls and $InsP_6$ non-treated rats.

As it is shown in Figures 2 and 3, only non-treated $InsP_6$ rats displayed important mineral deposits in aorta and heart. The histological analysis of calcium deposits of aortas and hearts were estimated as absent in control and $InsP_6$ treated tissues and as severe in $InsP_6$ non-treated tissues. The calcium deposits in aorta were observed predominantly in the intima and the intern vessel layers. The heart calcium deposits were observed in the walls of coronary vessels and in the adjacent myocardium. Also they presented myocardium necrosis and inflammation (heart attack).

5. DISCUSSION

As it is commented in the introduction section, the development of undesirable hydroxyapatite deposits (calcifications) in the cardiovascular system is a consequence of unbalance between promoter factors (injuries, hypercalcemia, hyperphosphatemia) and inhibitory factors (default of cellular defences, deficit of crystallization inhibitors).

Table 1. Phytate ($InsP_6$) concentration in urine for the different groups after 15 days of cream pre-treatment

	Control	**$InsP_6$ non-treated**	**$InsP_6$ treated**
$InsP_6$ concentration in urine (mg/L)	0.08 +/- 0.03 (n = 6)	0.09 +/- 0.03 (n = 6)	36.15 +/- 7.26 [1,2] (n = 6)

1: $p < 0.001$ vs control group, 2: $p < 0.001$ vs $InsP_6$ non-treated group

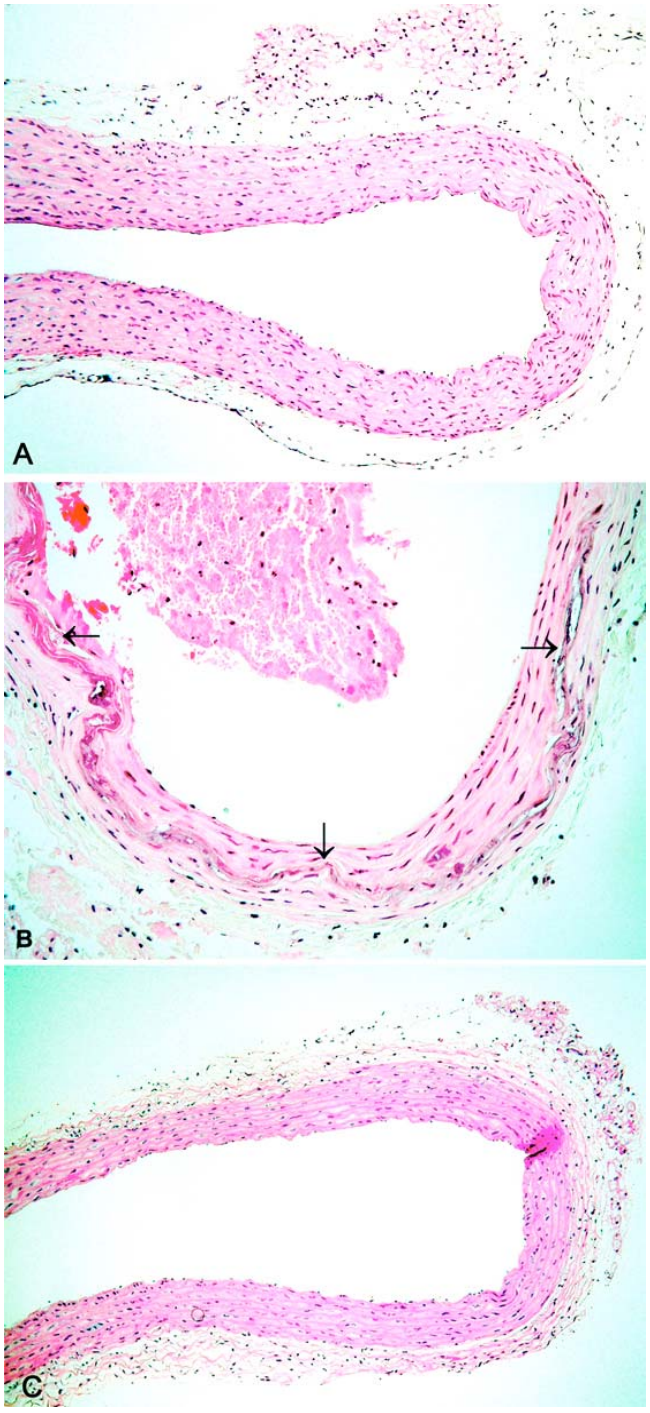

Figure 2. Images of sections of aortas by the three studied groups: **a.** control (original magnification x 10); **b.** $InsP_6$ non-treated (original magnification x 20); **c.** $InsP_6$ treated (original magnification x 10). The arrows show extensive calcifications.

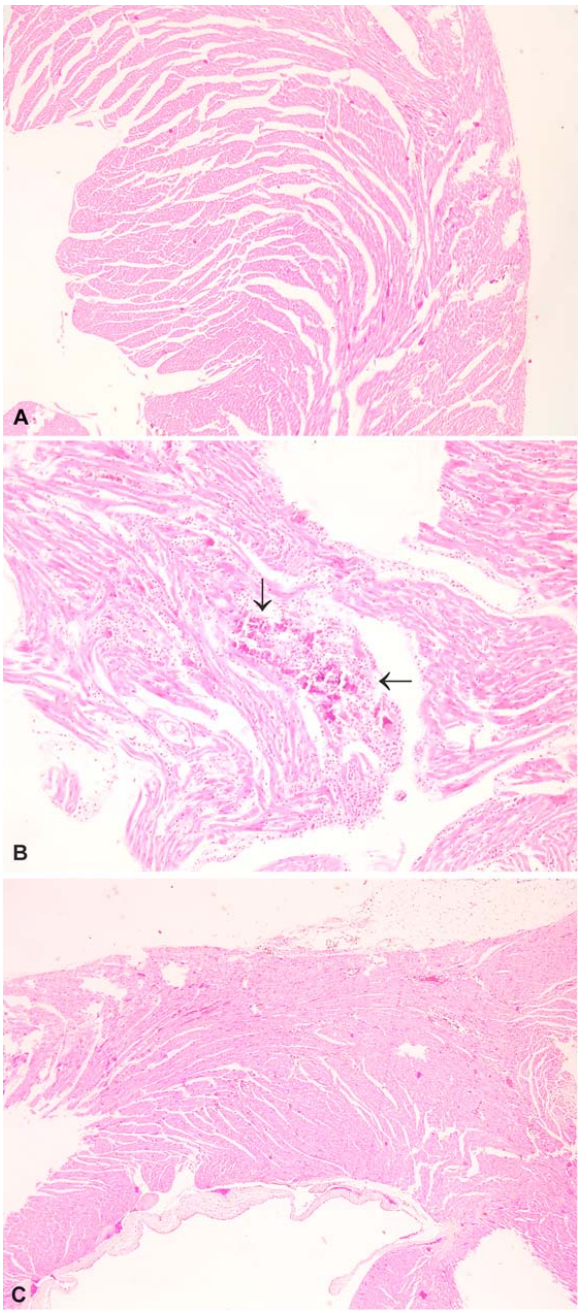

Figure 3. Images of sections of hearts by the three studied groups: **a.** control (original magnification x 4); **b.** $InsP_6$ non-treated (original magnification x 10); **c.** $InsP_6$ treated (original magnification x 4). The arrows show extensive calcifications.

A number of extracellular matrix proteins have been reported to inhibit mineralization, including osteocalcin, albumin, osteopontin and matrix gamma-carboxyglutamic acid (Gla) protein (9,16,18-20). *In vitro* experiments demonstrate some activity of these proteins as crystallization inhibitors. Thus, osteopontin has been reported to inhibit *de novo* mineral formation by blocking crystal growth rather than hydroxyapatite nucleation (19,21,22) and can also promote cell adhesion and migration (23). Otherwise, also promoter activity of hydroxyapatite development has been assigned to these proteins or their analogs due to their nucleating activity (24-26). Nevertheless, a clear action of some of these proteins regulating the cellular activity on calcification processes has been described. Hence, osteocalcin is a Gla-containing protein with a potential function as an inhibitor of osteoblast activity (27). In the rapidly calcifying bone of newborn rats matrix Gla protein levels are about 130% higher than those in adult rats (28). Hence it seems that extracellular protein matrix has an important role as signaling agents in the control of cellular processes associated to the tissue calcification, rather than a typical action as crystallization inhibitors.

$InsP_6$ (phytate) has demonstrated a powerful capacity as crystallization inhibitor of hydroxyapatite in *in vitro* experiments (29) and in no cases it has demonstrated a promoting capacity. The levels found in mammals clearly depend on its dietary intake (11,12) being this molecule also topically absorbed (13) as it is also demonstrated by the presented results, correlating the amounts found in blood and tissues with the urinary values (11,12). On the other hand, this is a molecule of low molecular weight to which no signaling capacity of cells implied in calcification processes has been assigned. Thus, considering the results obtained in this paper, the clear action of $InsP_6$ avoiding the calcification of cardiovascular system must be attributed to its capacity as crystallization inhibitor. Consequently, this could be another important factor linked to cardiovascular calcifications that need further studies to be able to evaluate its authentic dimension. In this aspect, it is interesting to comment that culture of normal aortas with alkaline phosphatase resulted in hydroxyapatite calcification of the elastic lamina. This was not due to dephosphorylation of osteopontin and calcification was not increased in aortas from osteopontin-deficient mice. The inhibitor was identified as pyrophosphate (30), which is another well known crystallization inhibitor of hydroxyapatite in *in vitro* experiments (29,31). Consequently, when hydroxyapatite inhibitory factors are considered, mechanisms of cellular defence to such processes and authentic crystallization inhibitory activity of some molecules in front of hydroxyapatite development should probably be distinguished.

6. ACKNOWLEDGEMENTS

J.P. and P.S express their appreciation to the Spanish Ministry of Education, Culture and Sport for a fellowship of the FPU program. Also, B.I. expresses his appreciation to the Conselleria d'Innovació i Energia del Govern de les Illes Balears for a fellowship. This work was supported by the Conselleria d'Innovació i Energia del Govern de les Illes Balears (Grant PROIB-2002GC1-04) and by the project BQU 2003-01659 of the Spanish Ministry of Science and Technology.

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Key Words: Phytate, Crystallization Inhibitor, Cardiovascular Calcifications, Heart, Vessel

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