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A) ORIGINAL INVESTIGATION LETTER

Aldosterone increases vascular calcification in *in vitro* studies

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To the editor: We would like to report the results of a recent work performed in our Laboratory about the hypothesis that aldosterone plays a relevant role in vascular calcification. Briefly, the method was an *in vitro* model of normal vascular smooth muscle cells (VSMC) in a primary culture, induced to get calcified in the presence of beta-glycerophosphate. The study shows that aldosterone increases calcification in VSMC. The effect is inhibited by spironolactone. Until recently, vascular calcification was considered a passive process, provoked by a non specific response of vascular tissues to different insults, with the deposition of calcium phosphate, mainly in the form of hydroxyapatite. Nowadays, it is known that calcification is due to a regulated process, in which several cell types are implicated, as well as a great deal of mediators, both pro-calcifying like alkaline phosphatase (AP), Cbfa-1, osteocalcin, and anti-calcifying agents such as matrix protein gliadin (MPG), osteoprotegerin (OPG) and fetuin-A[1-3]. The normal response of VSCM is anti-calcifying. However, in the uremic states this balance is altered favoring the pro-calcifying factors. The causes are multiple, such as increase of Ca and/or P in the milieu, the above-mentioned inflammatory and oxidative processes, or simply the stress associated to prolonged use accompanying the aging process.

Uremic vascular calcifications in the middle layer of the VSMC mainly involve areas of extracellular matrix and the elastic lamina. This kind of calcification is the most frequent and the earliest among uremic calcifications[2].

Aldosterone is currently considered a pro-fibrotic vascular factor. This concept has been widely accepted in the clinical practice, especially after the publication of the RALES study[4]. There are many studies supporting the presence of aldosterone receptors in VSMC, with different functional implications[5,6]. However, despite the obvious temporal relationship between the development of vascular fibrosis and stiffness, and the appearance of vascular calcifications, no studies have been conducted, exploring the possible relationship between both phenomena. Old reports in the literature suggested that aldosterone could participate in calcium transportation in VSMC[7]. According to our own results, the population on dialysis due to advanced renal disease presents significantly high aldosterone levels compared to normal subjects (Caramelo *et al.*, data not published).

The aim of this work was to examine the role of aldosterone in the induction of vascular calcifications in a well-known experimental model, that is the exposition of VSMC to beta-glycerophosphate. Our hypothesis is that aldosterone possibly participates in this process. The following methods were employed:

Primary culture of VSMC from bovine aorta, according to the method described by Campbell[8]. The VSMC were employed between steps 2 and 8.

Induction of calcification process: We have used a standardized calcification method, which is used in the Laboratory [9]. The calcification medium was changed every 48 hours during 12 days and it is based on beta-glycerophosphate (10 mM) (Sigma-Aldrich, Madrid). The beta-glycerophosphate acts as a PO_4^{-3} donor when it is hydrolyzed by alkaline phosphatase.

Quantification of the calcification by ^{45}Ca accumulation: The cells that are on the calcification process are incubated with 0.5 mCi/mL of ^{45}Ca (GE Healthcare, Barcelona). The detection of the accumulation is made by means of a scintillation fluid UltimaGold (Perkin Elmer, Waltham, MA) in a Beckman LS6000TA counter.

Von Kossa stain: To visualize the calcification process, VSMC were fixed with Merkcofix (Merck KGaA, Darmstadt, Germany), incubated with 5% silver nitrate for 30 minutes in the darkness and exposed to sun-light until the stain could be seen. The pictures were made with a digital camera Nikkon Co-

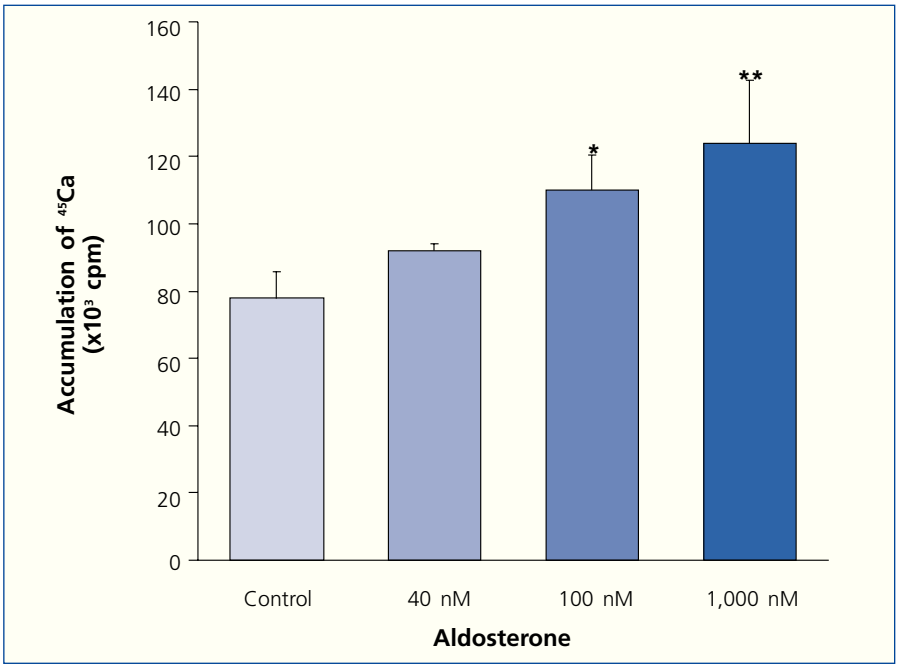

Figure 1. Significant increase in ^{45}Ca uptake by VSMC in the presence of increasing concentrations of aldosterone, determined 12 days after incubation (*p < 0.05; **p < 0.01).

olpix 995 with an illuminated background.

Incubation with aldosterone. During the whole calcifying process, VSMC were incubated with aldosterone or vehicle aggregated to the medium. In each medium change the adequate concentration of aldosterone was added.

Statistics: Data were expressed as mean ± standard deviation of the mean. Except when indicated, all values correspond to a minimum of 5 experiments with triplicate samples. Comparisons were made by means of the Student's t test with matched pairs or not, and ANOVA test with Scheffé's test. Significance was accepted with a P value < 0.05. All calculations were made with SPSS statistical software 10.0 for Windows.

The findings show the reliability of the model for the calcium accumulation in the form of crystals positive for von Kossa stain and ^{45}Ca. The increase was generally higher than 250%. With aldosterone ^{45}Ca uptake was significantly higher but with high hormone concentrations (fig. 1). The increase was massively inhibited (85 ± 4% inhibition, P < 0.01) in the presence of spironolactone (10^{-7} and 10^{-6} M).

Jaffe *et al.* have recently reported a similar finding but using an extremely selective model with calcifying cells and the results cannot be extrapolated to all VSMC[10]. Our results point out that aldosterone favors calcification in normal VSMC, and this fact widely spreads the possible calcifying action of the hormone. Of note, the strong inhibition in the presence of aldosterone indicates that the effect occurs through the mineralocorticoid receptor.

It is well known that aldosterone induces pro-fibrotic changes in the protein composition of the arterial wall[4]. But to our knowledge it had not been reported, except for the work of Jaffe *et al.*[10], that it also influences the calcification process. It is true that the concentrations at which we detected the calcifying effect were elevated. But many interactions with other pro-calcifying factors can be crucial *in vivo*, and lower aldosterone concentrations can act as co-adjuvant of other mediators.

The presence of extra-bone calcifications in patients with early onset hyperaldosteronism of the childhood, like the Bartter and Gitelman syndromes, are another example, in which the relationship between hyperaldosteronism and calcification can play a role, yet not suspected[11,12].

In summary, this study shows new data about the factors implicated in vascular calcification. The potential therapeutic interest of aldosterone antagonists cannot be disregarded. If it can be proved that they act on the vascular calcification process, they could be added as a new therapeutic tool to those currently available to control calcifications.

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B) CASE REPORTS AND CLINICAL EXPERIENCES

Small kidney in ANCA-positive renal vasculitis: a possible marker of subacute evolution

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To the Editor: The aim of this letter is to highlight a clinical phenomenon, insufficiently recognized: the ANCA-positive vasculitis with a subacute evolution. We present a series of five patients with ANCA-positive systemic vasculitis and small-sized kidneys on diagnosis.

All patients were women (mean age 67 years). Two of them had a history of arthralgias, epistaxis, severe anemia and repetitive miscarriages, and one of them had bronchial asthma. The clinical picture began with a constitutional syndrome. In two cases it was accompanied by nicturia and in one by he-