

Natural antioxidants and vascular calcification: a possible benefit?

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ABSTRACT

Background: Several studies have demonstrated the impact of vascular calcification on morbidity and mortality both in the general and chronic kidney disease populations. The process of vascular calcification involves complex mechanisms including the overexpression of genes and proteins associated with mineralization and increments of reactive oxygen species (ROS). Taking into account previous findings, we decided to analyze in vitro the likely inhibitory effect of natural antioxidants in the process of vascular calcification.

Methods: Primary vascular smooth muscle cells (VSMCs) were cultured with either normal medium or normal medium supplemented with calcium and phosphorus (P + Ca) in combination with several antioxidants. Mineralization, intracellular reactive oxygen species levels and the protein expression of Cbfa1/RUNX2 and Mn-superoxide dismutase-2 (SOD-2) were investigated.

Results: Curcumin and silybin were the more effective, inhibiting both ROS increase and VSMC mineralization. Curcumin was able to prevent the increase in Cbfa1/RUNX2 expression, but did not modify SOD-2 expression in the VSMCs cultured with the P + Ca medium.

Conclusions: These findings support the importance of performing further studies in this field, as some antioxidants might have potential benefits in the management of vascular calcification.

Key words: Antioxidants, Chronic kidney disease, Natural compounds, Vascular calcification

INTRODUCTION

Several studies have demonstrated the impact of vascular calcification on morbidity and mortality in the general population and in chronic kidney disease (CKD) patients (1-4). The process of vascular calcification involves not only the physical deposition of calcium and phosphate but also a series of complex mechanisms including the overexpression of genes and proteins associated with mineralization (5). In addition, it has been shown that vascular calcification is also related to increments of reactive oxygen species (ROS) (6). In fact, hydrogen peroxide, one of the most common types of ROS, has been shown to be able to induce the expression of the osteoblastic transcription factor Cbfa1/RUNX2 (7).

Taking into account these previous findings, we decided to analyze in vitro the likely inhibitory effect of natural antioxidants in the process of vascular calcification.

METHODS

Primary vascular smooth muscle cells (VSMCs) from Wistar rats were cultured for 8 days with either normal medium (DMEM:F12 1:1 with 0.1% of bovine serum albumin) or normal medium supplemented with Ca and P (P+Ca medium) at concentrations of 2 and 3 mM, respectively. The latter were also cultured adding to the P+Ca medium the following natural antioxidants (all from Sigma): curcumin (C7727), silybin (02000585), resveratrol (R5010), alpha-tocopherol (258024), sodium L-ascorbate (A4034) and Trolox (238813). The antioxidant concentrations were se-

lected from previous publications (8). Cell cultures were studied always under subconfluency conditions.

Mineralization was investigated using alizarin red staining and quantified following procedures described elsewhere (9). Intracellular ROS levels were measured using flow cytometry and dihydrochlorofluorescein diacetate (DHCF-DA) (Molecular Probes). Briefly, cells were cultured in normal or P+Ca medium plus antioxidants for 8 days. Afterwards, the cells were starved and incubated for 60 minutes with the probe and immediately analyzed in a flow cytometer (Cytomics FC500). The results were expressed as percentage of fluorescent cells after the incubation with the different culture mediums.

The protein expression of Cbfa1/RUNX2 and Mn-superoxide dismutase-2 (SOD-2) was measured by Western blotting using a standard protocol. Antibody dilutions were 1:100 for Cbfa1/RUNX2 (ab54868; Abcam), 1:1,000 for SOD-2 (sc30080; Santa Cruz Biotechnology) and 1:30,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc25778; Santa Cruz Biotechnology).

RESULTS

A significant increase in mineralization was observed when VSMCs were cultured with P+Ca medium for 8 days. The effects of antioxidants were heterogeneous (Tab. I). Only curcumin (5 μ M) and silybin (50 μ M) were able to reduce mineralization, by 30% and 35%, respectively (Fig. 1) when compared with the VSMCs cultured with P+Ca alone.

Parallel to these changes, the levels of ROS increased 88% in the VSMCs which were cultured with the P+Ca medium. In fact, the addition of curcumin and silybin significantly decreased ROS, up to 82% and 64%, respectively. Interestingly, after the incubation with curcumin, the levels of ROS were close to the levels observed in the control group (Tab. II).

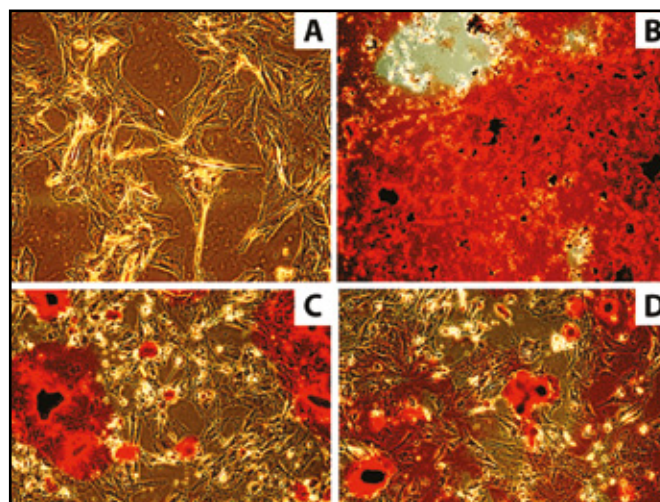


Fig. 1 - Microphotography of alizarin red staining (x100): A) control group; B) P+Ca group; C) P+Ca + silybin 50 μ M; and D) P+Ca + curcumin 5 μ M.

As curcumin was the more effective inhibitor of both ROS and VSMC mineralization, we investigated the effect of this antioxidant in Cbfa1/RUNX2 and SOD-2 expression. Curcumin was able to prevent the increase in Cbfa1/RUNX2 expression, but did not modify SOD-2 expression in the VSMCs cultured with the P+Ca medium (Fig. 2).

DISCUSSION

Vascular calcification is a frequent, threatening complication particularly in the scenario of CKD. In recent years, several studies have tried to better understand the mechanisms implicated in the genesis and regulation of this disorder.

Recently, it has been shown that ROS may play a role in

TABLE I

ALIZARIN RED QUANTIFICATION: CALCIFYING GROUP (P+Ca) WAS USED AS REFERENCE

	Control	P+Ca	P+Ca + Asc 100 μ M	P+Ca + Troll 100 μ M	P+Ca + Sily 50 μ M	P+Ca + Resv 50 μ M	P+Ca + Toco 500 μ M	P+Ca + Toco 100 μ M	P+Ca + Curc 5 μ M
Relative units	0.013	1	1.13	1.18	0.64	1.22	1.02	1.04	0.70
p Value, by <i>t</i> -test, vs. P+Ca	<0.0001	--	0.97	0.18	0.008	0.09	0.67	0.57	0.012

Asc = ascorbic acid; Curc = curcumin; Sily = silybin; Resv = resveratrol; Toco = tocopherol; Troll = trolox.

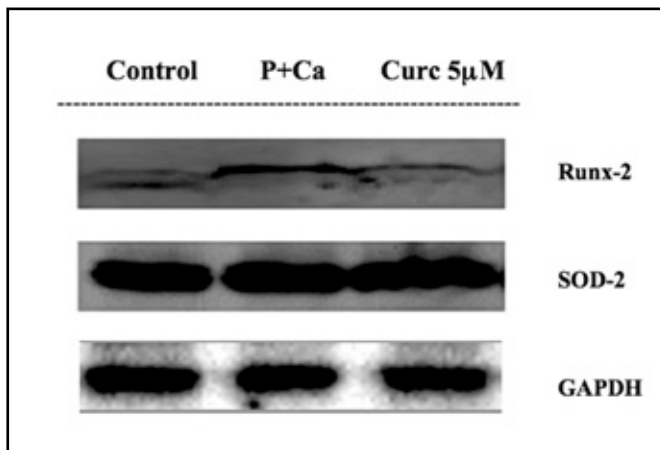


Fig. 2 - Western blot of Cbfa1/RUNX2 and SOD-2 of vascular smooth muscle cells (VSMCs) cultured with a calcifying media or a calcifying media supplemented with curcumin (Curc) 5 µM. GAPDH = glyceraldehyde-3-phosphate dehydrogenase; SOD-2 = Mn-superoxide dismutase-2.

the process of vascular calcification, showing that ROS signaling affects the expression of the osteoblastic proteins involved in the initiation of vascular calcification (10). In agreement with this effect, Miller et al (11) demonstrated the critical role of ROS in the pathogenesis of several forms of valvular calcification. Furthermore, in humans, increments in ROS in calcific aortic valvular stenosis have been associated with reductions in the defensive mechanisms responsible of removal of several ROS, including hydrogen peroxide (H_2O_2).

Several other works have also stressed the importance of H_2O_2 as a second messenger involved in oxidative stress, which in turn may increase VSMC mineralization at least partly by a direct up-regulation of Cbfa1/RUNX2 expression (4).

Our study shows that a medium supplemented with Ca

and P (3 and 2 mM, respectively) was able to induce both increase of ROS and mineralization in primary cultures of VSMCs. Even though these results and others show that oxidative stress is involved in the process of vascular calcification, the heterogeneous response to several antioxidants shows the complexity of this process, as only curcumin and silybin were able to reduce VSMC mineralization.

Curcumin is the active ingredient of the traditional herbal remedy and dietary spice turmeric (*Curcuma longa*) (12), and silybin is the major active constituent of the compound silymarin, a potent detoxifying agent. We showed that silybin, but especially curcumin, decreased ROS levels to those found in the control group. ROS was measured using fluorescent probes that are highly specific for H_2O_2 , so we can hypothesize that curcumin and silybin reduce specifically H_2O_2 levels. SOD-2 levels (responsible for the scavenging of superoxide ion into oxygen and H_2O_2) were increased in the P+Ca group, but there were no changes in the curcumin-treated group. This finding might indicate that curcumin could act specifically on H_2O_2 levels but not on other types of ROS located upstream in this cascade.

In conclusion, our results confirm that mineralization of VSMCs is associated with increments in oxidative stress. Curcumin and silybin, 2 well-known antioxidants of different origin with a variety of described actions (13), were able to decrease ROS. Curcumin decreased Cbfa1/RUNX2 without modifying SOD-2, suggesting that this antioxidant may directly reduce H_2O_2 levels. These findings, together with a recent study that suggested a potential role for ascorbic acid combined with phosphorus in worsening vascular calcification (14), support the importance of performing further studies in this field, as some antioxidants might have potential benefits in the management of vascular calcification.

TABLE II
INTRACELLULAR ROS QUANTIFICATION BY FLOW CYTOMETRY

	Control	P+Ca	P+Ca + Curc 5 µM	P+Ca + Sily 50 µM
Relative units	0.12	1.00	0.18	0.36
p Value, by <i>t</i> -test, vs. P+Ca	0.0034	--	0.001	0.001
p Value, by <i>t</i> -test, vs. control	--	0.003	0.73	0.06

Means of 3 measurements with dihydrochlorofluorescein diacetate (DHCF), expressed as percentage of fluorescent cells, and relative to phosphorus group (P), are depicted for each group.

Curc = curcumin; Sily = silybin; ROS = reactive oxygen species.

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Conflict of interest statement: None.

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