Phosphorus overload and PTH induce aortic expression of Runx2 in experimental uraemia


Nephrology Division, Department of Internal Medicine, University of São Paulo, São Paulo, Brazil

Abstract

Background. Vascular calcification (VC) is commonly seen in patients with chronic kidney disease (CKD). Elevated levels of phosphate and parathormone (PTH) are considered nontraditional risk factors for VC. It has been shown that, in vitro, phosphate transforms vascular smooth muscle cells (VSMCs) into calcifying cells, evidenced by upregulated expression of runt-related transcription factor 2 (Runx2), whereas PTH is protective against VC. In addition, Runx2 has been detected in calcified arteries of CKD patients. However, the in vivo effect of phosphate and PTH on Runx2 expression remains unknown.

Methods. Wistar rats were submitted to parathyroidectomy, 5/6 nephrectomy (Nx) and continuous infusion of 1–34 rat PTH (at physiological or supraphysiological rates) or were sham-operated. Diets varied only in phosphate content, which was low (0.2%) or high (1.2%). Biochemical, histological, immunohistochemistry and immunofluorescence analyses were performed.

Results. Nephrectomized animals receiving high-PTH infusion presented VC, regardless of the phosphate intake level. However, phosphate overload and normal PTH infusion induced phenotypic changes in VSMCs, as evidenced by upregulated aortic expression of Runx2. High-PTH infusion promoted histological changes in the expression of osteoprotegerin and type I collagen in calcified arteries.

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Conclusions. Phosphate, by itself is a potential pathogenic factor for VC. It is of note that phosphate overload, even without VC, was associated with overexpression of Runx2 in VSMCs. The mineral imbalance often seen in patients with CKD should be corrected.

Keywords: chronic kidney disease; hyperphosphataemia; immunohistochemistry; parathyroid hormone; vascular calcification

Introduction

The risk of cardiovascular disease (CVD) is ~20–30 times higher in patients with chronic kidney disease (CKD) than in individuals of the same age with normal renal function. In addition, CVD accounts for ~50% of deaths and one-third of all hospitalizations among CKD patients [1,2]. Most patients with CKD, especially those on dialysis, develop bone disease secondary to disturbances in calcium and phosphate metabolism, with a consequent increase in parathyroid hormone (PTH) levels, leading to the development of secondary hyperparathyroidism, skeletal demineralization and loss of structural stability, as well as to the occurrence of fractures.

Population-based studies involving normal individuals and individuals with CKD have demonstrated that CVD, vascular calcification (VC) and bone disease are interrelated. The interaction between bone disease and CVD has not been fully clarified. Nevertheless, epidemiological data have demonstrated that elevated calcium, phosphate and PTH levels are associated with higher mortality in patients with CKD. In particular, hyperphosphataemia has come to be considered one of the leading nontraditional risk factors for CVD in this population [3].

Currently, VC is defined as an active process regulated by cells that can differentiate through certain stimuli, acquiring the phenotype of osteoblasts that can synthesize mineralization-regulating proteins (e.g. osteocalcin, osteopontin) [4]. In a recent study, Moe et al. demonstrated that there is a phenotypic change in the arteries of patients with CKD, and that, in those patients, calcified arteries present expression of bone matrix proteins [5]. Similarly, in vitro studies have demonstrated that high phosphate concentrations promote a phenotypic change in vascular smooth muscle cells (VSMCs), which transform into osteoblast-like cells, as evidenced by overexpression of runt-related transcription factor 2 (Runx2) [4]. However, there have been no in vivo studies showing that isolated hyperphosphataemia induces Runx2 expression in vascular cells. Regarding PTH, in vitro and epidemiologic studies have produced conflicting results. Although in vitro studies have shown that exogenous 1–34 PTH inhibits bovine VSMC calcification in a dose-dependent manner [6], population-based studies have demonstrated that hyperparathyroidism is associated with poor survival in CKD patients on dialysis [3].

In a pair of studies recently performed in our laboratory, we analysed the in vivo role of phosphate and PTH, in isolation, in a rat model of experimental uraemia. In the first study, nephrectomized rats were infused with PTH at a physiological rate and were fed a diet high in phosphate. Although the animals did not develop VC, they presented decreased bone volume [7]. In the second study, nephrectomized rats were infused with PTH at a supraphysiological rate and were fed the same high-phosphate diet. Those animals developed hyperparathyroidism and presented calcification of the aortic media [8].

These experimental models gave us the unique opportunity to determine the in vivo effects of phosphate overload and of elevated serum PTH levels (with and without phosphate overload) in CKD rats. In the present study, we evaluated the factors involved in the phenotypic transformation of VSMCs in uraemia by analysing histological changes in the vascular tissue of nephrectomized rats with normal or high PTH levels and fed a high- or low-phosphate diet.

Methods

Experimental protocol

A total of 47 male Wistar rats, with initial weights of 300–350 g, were obtained from our local breeding colony for use in this study. The animals were housed in individual cages and maintained on a 12/12-h light/dark cycle in a temperature- and humidity-controlled environment (25°C and 25% humidity). Animals were anaesthetized with pentobarbital (50 mg/kg i.p.) and then submitted to parathyroidectomy (PTx) involving microsurgical techniques and electrocautery. The animals were allowed to recover from surgery for 7 days, after which they were anaesthetized as before. Those presenting ionized calcium levels < 0.9 µmol/L were submitted to 5/6 nephrectomy (Nx), consisting of removal of the right kidney and infarction of approximately two-thirds of the left kidney. Simultaneous to the Nx procedure, Alzet Model 2 mL osmotic mini-pumps (Alza Corp., Palo Alto, CA, USA) were implanted, and PTH activity was restored by continuous infusion of 1–34 rat PTH (Sigma-Aldrich, St Louis, MO, USA) at a physiological (normal) rate (nPTH, 0.022 µg/100 g/h) or at a supraphysiological (high) rate (hPTH, 0.11 µg/100 g/h). On post-Nx day 28, the same animals were given light ether anaesthesia and each osmotic minipump was replaced (pumping lifetime of 28 days) with another minipump set to the same infusion rate. The control group animals were submitted to sham PTx and sham Nx, as well as subcutaneous implantation of identical osmotic mini-pumps, delivering only vehicle (2% cysteine; Sigma-Aldrich). Immediately following Nx, the animals were fed diets of rodent chow (Harlan-Teklad, Indianapolis, IN, USA) that were identical except for phosphorus content, which was either low (LP, 0.2%) or high (HP, 1.2%). The animals were then divided into six groups: sham-HP (n = 8), Nx-nPTH-HP (n = 8), Nx-hPTH-HP (n = 7), sham-LP (n = 8), Nx-nPTH-LP (n = 8) and Nx-hPTH-LP (n = 9). All diets were equal in their content of vitamin D (2200 IU of vitamin D₃/kg of diet), calcium (0.7%), protein (24%) and calories. A pair-feeding protocol was used.

After 8 weeks of treatment, the animals were anaesthetized and killed through aortic puncture exsanguination.
All experimental procedures were conducted in accordance with the guidelines of the Standing Committee on Animal Research of the University of São Paulo (CAPesq no. 931/02).

**Biochemistry**

On the day of Nx surgery, whole blood was collected by retro-orbital puncture. A model AVL-9140 Autoanalyzer (AVL Scientific Corporation, Roswell, GA, USA) was used to measure serum levels of ionized calcium either immediately after collection or following killing (samples were stored at −20°C). Serum creatinine was determined by using a modified Heinegård-Tiderström colorimetric assay. Phosphorus was determined using a colorimetric assay (Labtest, Lagoa Santa, Brazil). A rat PTH IRMA kit (Immunotopics, San Clemente, CA, USA) was used to measure PTH.

**Histology**

Cross-sections of the thoracic aorta were embedded in paraffin and sectioned at 4 µm, after which they were stained with haematoxylin–eosin and Von Kossa. A pathologist blinded to the origin of the samples performed a qualitative analysis in order to identify calcification.

**Immunohistochemistry**

Following removal, tissue (thoracic aorta) samples were cut down to small (<0.5 cm³) pieces, embedded in Jung tissue freezing medium (Leica Instruments GmbH, Nussloch, Germany) and placed on cork discs in an appropriate orientation, after which they were frozen in liquid nitrogen and stored at −80°C. The tissue sections (5 µm) were obtained in a Leica CM 3000 cryostat (Leica Instruments GmbH), fixed in acetone at 4°C for 10 min, air-dried and stored at −80°C for later use in the immunohistochemical analysis.

Immunostaining for Runx2, type I collagen, osteoprotegerin, osteocalcin and osteopontin was performed using the avidin–biotin peroxidase method. Endogenous peroxidase activity was quenched by incubating slides in 3% H₂O₂ in methanol for 30 min. Slides were washed in phosphate-buffer saline (PBS) before abiding-biotin blocking steps. To block nonspecific sites, slides were loaded with normal horse serum (1:20) for 30 min at 4°C. Slides were incubated overnight at 4°C in a humidified chamber using the following primary antibodies: polyclonal goat anti-rat type I collagen (C-18, 1:50); polyclonal goat anti-rat osteoprotegerin (N-2, 1:20) and polyclonal goat anti-rat PEBP2αA (S-19, 1:50). All primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

After incubation with the primary antibody, slides were rinsed in PBS and then incubated with a biotinylated secondary antibody—horse anti-goat IgG (Vector, Burlingame, CA, USA)—to identify all antibodies. After rising in PBS avidin–biotin, the HRP complex was used and, finally, chromogenic substrate 3′,3′-diaminobenzidine (DAB, Vector, Burlingame, CA, USA) or 3-amino-9-ethyl-carbazol (AEC, Sigma-Aldrich). The sections were rinsed in distilled water and counterstained with Hemalum (Merck, Darmstadt, Germany).

Negative controls were carried out simultaneously with all experiments by omitting incubation with primary antibodies.

**Immunofluorescence/confocal microscopy**

Aorta samples were washed three times with PBS, for 5 min each time, permeabilized for 15 min with 0.1% Nonidet P40 (Roche, Mannheim, Germany) in PBS at 37°C, washed with 1% BSA in PBS for 10 min and blocked for 1 h at room temperature with Protein Block (DAKO Corporation, Carpinteria, CA, USA). Immunofluorescence was performed by incubating tissue for 1 h at 37°C with the primary anti-rat and anti-human monoclonal mouse antibody to Runx2 (ab54868; Abcam Inc., Cambridge, MA, USA) diluted 1:120 in 1% BSA in PBS. After three washes with 1% BSA in PBS for 10 min, the bound primary antibody was visualized by the addition of a specific Alexa Fluor 488-conjugated goat anti-mouse antibody (A11001; Invitrogen, Carlsbad, CA, USA) for 90 min at 4°C. In the final wash, 4′,6-diamidino-2-phenylindole (DAPI, Sigma) was included in order to stain nuclear DNA. The slides were then washed in PBS three times for 10 min and mounted in an aqueous mounting medium (Glycergel; Merck). Images were collected with a Zeiss LSM 510 UV META laser-scanning confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY, USA).

**Statistical analysis**

Comparisons among the groups were made using one-way analysis of variance and Tukey’s post hoc test. The GraphPad Prism program, version 3.03 (GraphPad Software, Inc., San Diego, CA, USA) was used. Results are presented as mean ± SE. Values of P < 0.05 were considered statistically significant.

**Results**

**Biochemical analysis**

The animals in the Nx-nPTH-HP and Nx-hPTH-HP groups presented significant hypocalcaemia and hyperphosphataemia when compared with animals in the other groups. Serum creatinine was higher in the nephrectomized groups. Levels of PTH were restored in the nPTH groups, and a supraphysiological level was attained in the hPTH groups. In the sham groups, PTH levels were lower in the Sham-LP animals than in the sham-HP animals (Table 1).

**Aortic calcification**

At the time of the killing, macroscopic examination revealed aortic stiffness in Nx-hPTH-LP and Nx-hPTH-HP group animals, in which Von Kossa staining of histological sections revealed calcification of the aortic media (Figure 1). Staining was negative in all other groups.
Phosphorus overload and PTH induce aortic expression of Runx2 in experimental uraemia

Table 1. Biochemical parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>iCa (mmol/L)</th>
<th>P (mg/dL)</th>
<th>Cr (mg/dL)</th>
<th>PTH (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-HP (a)</td>
<td>1.23 ± 0.07</td>
<td>4.95 ± 0.33</td>
<td>0.46 ± 0.03</td>
<td>135.9 ± 28.6</td>
</tr>
<tr>
<td>Nx-nPTH-HP (b)</td>
<td>0.61 ± 0.05</td>
<td>15.71 ± 2.56</td>
<td>1.09 ± 0.15</td>
<td>86.8 ± 20.3</td>
</tr>
<tr>
<td>Nx-hPTH-LP (c)</td>
<td>0.58 ± 0.09</td>
<td>13.56 ± 2.03</td>
<td>1.08 ± 0.25</td>
<td>380.0 ± 66.6</td>
</tr>
<tr>
<td>Sham-LP (d)</td>
<td>1.22 ± 0.05</td>
<td>4.43 ± 0.39</td>
<td>0.32 ± 0.01</td>
<td>10.5 ± 2.8</td>
</tr>
<tr>
<td>Nx-nPTH-LP (e)</td>
<td>1.19 ± 0.05</td>
<td>5.59 ± 0.48</td>
<td>0.59 ± 0.03</td>
<td>114.9 ± 28.1</td>
</tr>
<tr>
<td>Nx-hPTH-LP (f)</td>
<td>1.44 ± 0.08</td>
<td>5.04 ± 0.58</td>
<td>1.02 ± 0.11</td>
<td>249.64 ± 45.2</td>
</tr>
</tbody>
</table>

P \( < 0.05 \): b and c vs. a, d and f; \( ^{*} \) b, c and f vs. a, d and e; \( ^{\text{#}} \) c and f vs. a, b, d and e; \( ^{\text{Y}} \) d vs. all.

iCa, ionized calcium; P, phosphate; Cr, creatinine.

Osteogenic proteins in the aortic media

Table 2 summarizes the results of the qualitative analysis of the expression of type I collagen proteins, osteoprotegerin and Runx2.

Type I collagen was detected in the aorta adventitia of all of the animals evaluated. However, in the groups in which there was intense calcification (Nx-hPTH-HP and Nx-hPTH-LP), expression of type I collagen was seen in the aortic media.

Osteoprotegerin was observed in the cells of the aorta adventitia of all of the animals evaluated. However, in the Nx-hPTH-HP and Nx-hPTH-LP groups, this protein was also observed in calcified areas.

Runx2 staining was intense in the calcified areas of Nx-hPTH-HP and Nx-hPTH-LP animals. However, even in the Nx-nPTH-HP group animals, which did not develop calcification, Runx2 staining was positive in internal limiting elastic cells and in the media next to the vessel lumen (Figure 2).

Intracellular colocalization of Runx2 was analysed by confocal microscopy in the aortic artery. Fluorescent immunolabelling demonstrated expression of Runx2 at the cytosolic and nuclear levels (Figure 3).

Discussion

The present study evaluated histological disorders in CKD rats fed diets with different phosphate contents and in which serum PTH was maintained at different levels (normal or high). Our most significant finding was that animals fed a high-phosphate diet and not developing VC presented Runx2 expression in the aortic media. Jono et al. stated that phosphate overload could induce a phenotypic change in VSMCs that would lead to VC [9]. However, their in vitro findings could not be confirmed until now since there was as yet no in vivo model to determine the isolated effects of phosphate on VC. To our knowledge, this is the first in vivo study confirming the findings of those authors.

Renal insufficiency, in in vivo animal models or in patients with CKD, leads to disturbances of the mineral metabolism and consequent impaired balance of serum calcium, phosphate and other circulating complexes. Therefore, in addition to other complications, two types of calcification are found in patients with CKD: calcification of the tunica intima, which represents an advanced stage of the atherosclerosis process and is associated with the development of occluding plaques and lesions, and calcification of the media, which mainly affects haemodynamic function of the vessels, increasing rigidity and decreasing compliance, thereby resulting in increased pulse pressure.

In recent years, elevated phosphate, resulting from higher dietary content or from changes in bone remodelling, has been reported to be a soft tissue calcification-stimulating factor. Jono et al. demonstrated that, in an inorganic phosphate-rich in vitro medium, vascular cells present calcification and Runx2 expression. The authors observed phosphate-induced phenotypic changes in these cells, which became osteoblast-like and therefore capable of mineralization [9]. Runx2 is considered the earliest marker of osteoblastic differentiation into cells that initiate the production of distinct type I collagen markers: alkaline phosphatase; osteopontin; and osteocalcin, according to the stage [10]. Since Runx2 is a specific osteoblast transcription factor, its presence in tissues other than bone suggests the occurrence of cell transdifferentiation. In 2003, Moe et al. studied patients on dialysis and demonstrated Runx2 expression in VSMCs of the tunica intima and tunica media of calcified arteries, as well as expression of osteogenic proteins, such as type I collagen and osteopontin [5]. The authors also showed that VSMCs of patients with CKD presented positive staining for Runx2 in a serum culture medium [11]. Our results confirm these findings.

In the present study, the Nx-nPTH-HP group animals did not present calcification. However, through immunohistochemical testing, we detected strong Runx2 expression in the VSMCs of the aortas of those animals, which was also observed in animals that presented VC, suggesting that the phenotypic change occurs prior to the development of VC. One question that arises is why the animals that received normal PTH infusion and were fed with an HP diet presented Runx2 expression and yet did not present VC.
Table 2. Immunohistochemistry findings

<table>
<thead>
<tr>
<th></th>
<th>Sham-HP</th>
<th>NxC-PTH-HP</th>
<th>Nx-hPTH-HP</th>
<th>Sham-LP</th>
<th>Nx-nPTH-LP</th>
<th>Nx-hPTH-LP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col I</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OPG</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Runx2</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Col I, type I collagen; +, positive staining; −, negative staining.

Fig. 2. Immunohistochemical analysis of bone proteins in aorta: (A and B) the Col I and OPG staining pattern in the animals of the sham groups and with normal PTH infusion; (C) Runx2 expression of the NxC nPTH-HP group; (D, E, F) the staining pattern for Col I, OPG and Runx2 in the animals that presented vascular calcification (magnification, 40×).

Fig. 3. Confocal microscopy (magnification, ×400) for Runx2 in a fragment of the aorta: (A) NxC-nPTH-LP; (B) NxC-nPTH-HP; (C) NxC-hPTH-HP and NxC-hPTH-LP. Higher intensity Runx2 expression is represented by green labelling, nuclei are labelled in blue, and elastic fibres are labelled in red.

However, it is known that rats do not easily develop calcification. Most studies evaluating VC in rats employed a combination of a dietary phosphate overload and hyperparathyroidism, together with a long observation period or higher doses of calcitriol [12–14]. Therefore, our hypothesis is that, over time, our animals would have developed VC.

In the present study, we observed overt VC in the animals that received PTH infusion at a supraphysiological rate. Our results are in conflict with those of previous in vitro studies, in which stimulation of PTH1R with this PTH fragment was found to inhibit calcification in bovine VSMCs [6], as well as with those of some experimental studies showing that pharmacological administration of 1–34 PTH prevents and regulates osteogenic VC [15]. Conversely, clinical studies have demonstrated that high PTH levels are associated with VC and with higher mortality [3]. The most feasible explanation is that PTH has a direct beneficial effect on VSMCs, which can be demonstrated in in vitro models or in animals without CKD submitted to intermittent PTH infusions at low doses. However, in experimental models that mimic hyperparathyroidism, PTH infusion at supraphysiological rates causes VC. These contradictory findings might be better explained by an indirect, deleterious effect of PTH. It must be borne in mind that hyperparathyroidism is associated with an increase in bone remodelling, which increases calcium and phosphate load. Some in vitro studies have demonstrated that calcium and phosphorus induce VC independently and synergistically [16].

In addition to the presence of Runx2, we found intense expression of type I collagen and osteoprotegerin in the high PTH groups. It is of note that this staining was performed exclusively in the aortic media where, under proper physiological conditions, these proteins are not constitutively present [17]. In this model, osteoprotegerin was observed in aortic adventitial cells in all animals. Nevertheless, in the NxC-hPTH-HP and NxC-hPTH-LP group animals, this
We thank Rosimeire Ap. Bezerra Costa and Acknowledgements. We demonstrate that even a supraphysiological rate caused VC by increasing bone recal rate. Finally, we cannot be certain that PTH infusion at phosphate diet and receiving PTH infusion at a physiolog-

We did not measure the calcium content in the aortic tissue, which could have helped us detect VC in the animals fed a high phosphate diet and receiving PTH infusion at a physiological rate. Finally, we cannot be certain that PTH infusion at a supraphysiological rate caused VC by increasing bone remodeling, leading to ‘endogenous’ calcium and phosphate overload. However, despite all limitations, we could clearly demonstrate that even in vivo, phosphate overload leads to the phenotypic change in VSMCs. We also showed that a high PTH level (through a direct or indirect mechanism) is associated with overt VC, confirming the findings of earlier clinical studies of patients with primary or secondary hyperparathyroidism [19,20].

In conclusion, VC itself was observed only in nephrectomized animals submitted to high-PTH infusion, regardless of dietary phosphate content, underscoring the role of PTH in the occurrence of VC, which is associated with a phenotypic change in VSMCs. However, phosphate overload, even under physiological PTH conditions and without apparent calcification, induced phenotypic change in VSMCs, as evidenced by Runx2 overexpression in the aortic media of these animals, which leads us to conclude that the phenotypic change precedes the appearance of VC in uraemic animals. The expression of Runx2, as well as that of other proteins involved in VC, confirms the role played by hyperphosphataemia and high PTH, in isolation or in conjunction, in inducing VC. Our findings also indicate that hyperphosphataemia, with or without high PTH levels, plays a role in the pathogenesis of VC, which is so common and has such adverse effects in patients with CKD.

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Conflict of interest statement. None declared.

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