Parathyroid hormone and phosphorus overload in uremia: impact on cardiovascular system

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Parathyroid hormone and phosphorus overload in uremia: impact on cardiovascular system

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Abstract

Background. Cardiac remodeling in uremia is characterized by left ventricular hypertrophy, interstitial fibrosis and microvascular disease. Cardiovascular disease is the leading cause of death in uremic patients, but coronary events alone are not the prevalent cause, sudden death and heart failure are. We studied the cardiac remodeling in experimental uremia, evaluating the isolated effect of parathyroid hormone (PTH) and phosphorus.

Methods. Wistar rats were submitted to parathyroidectomy (PTx) and 5/6 nephrectomy (Nx); they also received vehicle (V) and PTH at normal (nPTH) or high (hPTH) doses. They were fed with a poor-phosphorus (pP) or rich-phosphorus (rP) diet and were divided into the following groups: ‘Sham’: G1 (V + normal-phosphorus diet (np)) and ‘Nx + PTx’: G2 (nPTH + pP), G3 (nPTH + rP), G4 (hPTH + pP) and G5 (hPTH + rP). After 8 weeks, biochemical analysis, myocardium morphometry and arteriolar morphological analysis were performed. In addition, using immunohistochemical analysis, we evaluated angiotensin II, α-actin, transforming growth factor-beta (TGF-β) and nitrotyrosine, as well as fibroblast growth factor-23 (FGF-23), fibroblast growth factor receptor-1 (FGFR-1) and runt-related transcription factor-2 (Runx-2) expression.

Results. Nx animals presented higher serum creatinine levels as well as arterial hypertension. Higher PTH levels were associated with myocardial hypertrophy and fibrosis as well as a higher coronary lesion score. High PTH animals also presented a higher myocardial expression of TGF-β, angiotensin II, FGF-23 and nitrotyrosine and a lower expression of α-actin. Phosphorus overload was associated with higher serum FGF-23 levels and Runx-2, as well as myocardial hypertrophy. FGFR-1 was positive in the cardiomyocytes of all groups as well as in calcified coronaries of G4 and G5 whereas Runx-2 was positive in G3, G4 and G5.

Conclusion. In uremia, PTH and phosphorus overload are both independently associated with major changes related to the cardiac remodeling process, emphasizing the need for a better control of these factors in chronic kidney disease.

Keywords: FGF-23; hyperphosphatemia; left ventricular hypertrophy; parathyroid hormone; vascular calcification

Introduction

Cardiovascular disease (CVD) is the leading cause of death in patients with chronic kidney disease (CKD) in all stages [1]. Data from the United States (United States Renal Data System) show that this complication accounts for 42.2% of 17.9 deaths by 100 at-risk patients [2] and sudden death and congestive heart failure are more frequent than acute myocardial infarction [3].

Pathological cardiac remodeling occurs in response to injuries such as volume overload and/or arterial pressure and the myocyte is the principal cardiac cell involved in remodeling. Left ventricular hypertrophy (LVH) is the result of early alteration characterized by the hypertrophy of the myocyte, increased fibrosis and microvascular alterations. Arteriosclerosis and atherosclerosis are closely related with endothelial dysfunction and acute coronary events. Atherosclerosis, present in the initial phases of CKD, is associated with high incidence of calcification of the coronary arteries, cardiac valves, myocardium and peripheral arteries [4, 5].

In addition to traditional risk factors to CVD (hypertension, diabetes, dyslipidemia, smoking, gender, menopause, advanced age and sedentary lifestyle), patients with CKD have ‘uremia-related’ risk factors such as anemia, volume overload and neurohormonal activation, as well as local factors, inflammation, ischemia, cellular necrosis, apoptosis, oxidative stress (OS) and disturbances of the mineral metabolism [6].

Increased OS has been documented in CKD patients, and it affects cardiovascular function, increases sympathetic nervous activity and enhances atherosclerosis primarily via endothelial cell dysfunction [7]. Nitrotyrosine is one of the OS markers and is related to cell death, reactive oxygen species accumulation and endothelial nitric oxide synthase expression [8].

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Disturbances of the mineral metabolism are early and frequent in CKD [9]. The progressive loss of renal function lead to the lower production of calcitriol and phosphorus retention leading to hypocalcemia, as well as increased parathyroid hormone (PTH) and fibroblast growth factor-23 [fibroblast growth factor-23 (FGF-23)] secretion. Moreover, recent studies have shown the association of FGF-23 and hyperphosphatemia with left ventricular mass, as well as with increased mortality in CKD patients [10]. PTH overload contributes to LVH, myocardial fibrosis and vascular calcification (VC), regardless of arterial pressure [11, 12]. VC is commonly seen in patients with CKD. It has been shown that high phosphate levels changes the phenotype of vascular smooth muscle cells (VSMCs) into calcifying cells with up-regulation of osteochondrogenic markers like runt-related transcription factor 2 (Runx-2) [13].

In previous studies of experimental uremia in rats, we had demonstrated that hyperphosphatemia was associated with myocardial hypertrophy (increased heart weight) and that high PTH (hPTH) levels induced VC [11, 14]. In this experimental study, we compared uremic parathyroidectomized groups submitted to diets with different phosphorus content and different PTH infusion rates. Therefore, the aim of this study was to evaluate the isolated effect of PTH and phosphorus on myocardial tissue and coronary arteries, as well as to quantify the expression of some proteins involved in these alterations.

Materials and methods

Experimental protocol
A total of 46 male Wistar rats, at initial weights of 280–320 g, were obtained from our local breeding colony for use in this 8-week study. All animals were housed in individual cages in a light-controlled (12 h on/12 h off) environment in which temperature (25°C) and humidity (55%) were constant. Weight measurement and tail cuff plethysmography (TCP) were performed weekly.

Some animals were submitted to parathyroidectomy (PTx) and 5/6 nephrectomy (Nx), as described below, whereas others were sham operated. All animals were submitted to implantation of an osmotic minipump for the infusion of 1–34 rat PTH (Sigma-Aldrich, St Louis, MO) and delivered at a physiological rate [0.022 μg/100 g/h, normal PTH (nPTH)] or a supraphysiological rate (0.11 μg/100 g/h, hPTH) or vehicle (2% cysteine; Sigma-Aldrich). Immediately after Nx or sham Nx, animals were fed one of three types of rodent chow (Harlan-Teklad, Indianapolis, IN), identical except for the phosphorus content, which was 0.2, 0.7 and 1.2%, representing poor, medium and high phosphorus (pP) diets, respectively. All diets were equal in their content of vitamin D, calcium (0.7%), protein (24%) and calories. A pair feeding protocol was used.

Five subgroups were created:

G1: Sham + pP ( sham-operated rats fed with pP diet and receiving infusion of vehicle only)
G2: Nx PTX + pP nPTH (nephrectomized rats fed with a pP diet and receiving nPTH infusion)
G3: Nx PTX + hP nPTH (nephrectomized rats fed with a hP diet and receiving nPTH infusion)
G4: Nx PTX + pP hPTH (nephrectomized rats fed with pP diet and receiving hPTH infusion)
G5: Nx PTX + hP hPTH (nephrectomized rats fed with hP diet and receiving hPTH infusion)

All experimental procedures were conducted in accordance with the guidelines of the Standing Committee on Animal Research of the University of São Paulo.

Surgical procedures. The rats were anesthetized with pentobarbital (50 mg/kg i.p.) and animals of G2, G3, G4 and G5 were then submitted to PTx. The PTx procedure involved microsurgical techniques using an electocautery. The animals, including those that were sham operated, were allowed to recover from surgery for 7 days. Those presenting a serum concentration of ionized calcium (iCa) <0.9 mmol/L (considered an indicator of successful PTx) were anesthetized as before and submitted to 5/6Nx, consisting of removal of the right kidney and infarction of approximately two-thirds of the left kidney. The sham-PTx animals were also anesthetized as before and were then submitted to sham Nx.

hPTH infusion. Simultaneous to the Nx procedure, an Alzet model 2mL osmotic minipump (Alza Corp., Palo Alto, CA) was implanted subcutaneously, providing continuous PTH infusion. On Day 28 (post-Nx), the animals were given light ether anesthesia and the osmotic minipump (pumping lifetime, 28 days) was replaced with another minipump set to the same infusion rate.

Serum sample collection. After 8 weeks of PTH infusion, the animals were anesthetized and sacrificed through aortic exsanguinations. Serum samples were frozen at −20°C for later biochemical evaluation.

Biochemical evaluation. At Nx procedure, whole blood was collected by retro-orbital puncture. A model AVL-9140 autoanalyzer (AVL Scientific Corporation, Roswell, GA) was used to measure iCa levels in this blood either immediately after collection or following sacrifice (in frozen samples). Serum creatinine was determined by using a colorimetric assay (modified Heinegard-Tidestrom). Phosphorus was determined using a different colorimetric assay (Labetest, Lagoa Santa, Brazil). PTH was measured using an immunoradiometric assay kit (Immunotops, San Clemente, CA) and FGF-23 by intact FGF-23 (Elisa assay; Kainos, Japan; reference range 8.2–54.3 pg/mL); hematocrit was also determined.

Morphological analysis
Myocardial. Hearts were excised, blotted and weighed. We also determined the heart weight to 100 g of body weight ratio. The hearts were fixed in buffered formalin.

Cross-sections of the heart, including both ventricles at the equatorial plane, were embedded in paraffin and cut into 4-μm sections. Tissue sections were stained with hematoxylin–eosin (HE), Picrosirius red, periodic acid–Schiff (PAS) and Von Kossa. A digital image analysis system (Leica Imaging Systems Ltd, Cambridge, UK) was used for morphometric measurements. A pathologist blinded to the origins of the samples performed a qualitative analysis.

To estimate cardiomyocyte hypertrophy, PAS-stained sections were examined under ×400 magnification. The myocyte diameter (μm) around oval and central nuclei of longitudinally displayed myocytes was measured. For each of the following measurements, a total of 10 myocytes per section of subendocardial region were examined.

Collagen volume fraction (%) was estimated in Picrosirius red-stained sections under ×200 magnification. Collagen volume fraction was determined as the percentage of red-stained connective tissue areas per total myocardial area, excluding perivascular areas. A total of 30 fields per section of left ventricle in the special subendocardial region were examined.

Arteries. The wall thickness and wall and lumen area of small intra-mycardial arteries were determined by planimetry using the automatic image analyzing system cited before. The contours of the arterial profiles were marked manually with a cursor and the minimal and maximal diameters, as well as the wall and lumen areas, were calculated. The wall thickness of these arteries was then determined as the mean of the measurements of the two opposite walls toward the minimal diameter (because this is the direction where measurements are least affected by sectioning angle). The wall:lumen ratio was calculated by dividing the mean minimal diameter by the mean lumen diameter.

Wall thickness of the medium layer: To evaluate the wall thickness of the medium layer, we used HE-stained sections and selected arteries of 50–200 and 200–400 μm diameters and a minimal and maximal diameter ratio of >0.5 μm. The images were analyzed under ×590 magnifications using the software IMAGE J (National Institutes of Health, Bethesda, MD). Four measures were taken, two of each diameter, minimal and maximal, and the results were expressed in micrometer.

Evaluation of the arterial wall: To evaluate all the different layers of arteries in a semi-quantity form, the following scores were used:
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(0) 'normal': an intact intima and media layer and without calcifications;
(1) light lesion: partial or total discontinuity of intima/elastic membrane, organized media layer and without calcifications;
(2) moderate lesion: partial or total discontinuity of intima/elastic membrane, disorganized media layer and without calcifications;
(3) severe lesion: partial or total discontinuity of intima/elastic membrane, disorganized media layer and calcifications.

About 3–5 arteries per section were analyzed and received a score and the comparison was done with the mean scores of each animal.

Calcification. The calcification of the arteries was stained in black color by Von Kossa and analyzed under ×200 magnification. The calcifications were estimated at the ratio of perceptual calculus between the area stained black and the total media layer area.

Immunohistochemical analysis. The immunohistochemical analyses were performed on transversal paraffin sections of the hearts using an antibody against angiotensin II (Peninsula, Belmont, CA) and α-actin (Sigma Chemical Co., St Louis, MO) by streptavidin–biotin/alkaline phosphatase technique and transforming growth factor-beta (TGF-β) (Santa Cruz Biotech, Santa Cruz, MN) and nitrotyrosine (Invitrogen, Carlsbad, CA) by avidin–biotin/peroxidase technique. Heart sections were also tested for anti-FGF-23 (kindly provided by Genzyme Co.), anti-fibroblast growth factor receptor-1 (FGF-R-1) (SC-121; Santa Cruz Biotech) and anti-Ruxn-2 (ab54868; Abcam Inc., MA), using streptavidin–biotin—LSAB technique (Dako, CA). Negative controls were performed by omitting the primary antibody.

To evaluate the angiotensin II and TGF-β expression, we utilized a point count technique, using a microscope with a 100-point ocular connected to a video monitor. We analyzed 25 fields under ×200 magnification. The results were expressed in percentage considering the total area. We evaluated the frequency of positive arteries for nitrotyrosine and FGF-23 in the cardiac tissue. The α-actin positivity was semi-quantitatively evaluated using the following scores: (i) absent or light scoring in few areas of media layer, (ii) moderate scoring in scattered and irregular areas of media layer and (iii) strong scoring with uniform distribution in media layer. FGF-R-1 and Runx-2 expressions were evaluated in the arteries and cardiac tissue; however, no quantification was performed.

Statistical analysis

All data are expressed as mean ± standard error. Comparisons of the biochemical parameters among the groups were made using one-way analysis of variance (ANOVA) and Newman–Keuls post hoc test. A two-way ANOVA was conducted in order to understand how biochemical, morphometric and immunohistochemical parameters could be affected by the following two factors: phosphorus content in the diet and PTH infusion rate. In the case of a significant interaction of phosphorus content and PTH infusion, Bonferroni post-test was performed. When no interaction was observed, data were rearranged and grouped according to the phosphorus content in the diet and the PTH infusion rate and Student’s t-test analysis was used. Fisher’s 2 × 5 exact test was performed to assess the association between nitrotyrosine and FGF-23 immunohistochemical positivity and the study groups. GraphPad Prism software, version 4.0 (GraphPad, San Diego, CA), was employed for all tests except for Fisher’s exact test when we applied StatXact version 9 (Cytel, Cambridge, MA). Values of P < 0.05 were considered to be statistically significant.

Results

TCP, heart weight, biochemical data and hematocrit

Table 1 shows that the arterial pressure and serum creatinine were higher in all Nx PTx groups compared to sham group (P < 0.05). Among Nx PTx groups, G2 showed the lowest serum creatinine. Heart weight/body weight ratio was higher in groups with hPTH (G4 and G5) compared to normal and sham groups (G2, G3 and G1), whereas G3 presented a heart weight higher than G1 and G2. Anemia was observed in all Nx groups. Nx PTx animals fed a rP diet developed hypocalcemia and hyperphosphatemia in both PTH concentrations (G3 and G5). Hypercalcemia was observed in the Nx PTx group with hPTH and fed with pP diet (G4). As expected, the groups with hPTH infusion showed the highest PTH serum levels (G4 and G5). Serum FGF-23 was higher in animals with hPTH (G4 and G5) compared to sham and animals with nPTH fed with pP diet (G2).

Myocardium

Myocardial hypertrophy evaluated through the myocyte diameter was higher in G3, G4 and G5 groups than in G1 and G2 groups (Table 2). Among the groups with hPTH, the animals fed with rP (G5) showed the highest myocardial hypertrophy. Myocardial fibrosis was higher in animals with hPTH and fed with rP (G5) compared to sham and G2 animals.

The thickness of medium layer was not different among all the groups (data not shown). The highest artery lesion score was found in hPTH groups.

Regarding immunohistochemistry findings, only G5 showed an increased expression of TGF-β compared to sham group. The highest angiotensin II expression was found in hPTH groups (Table 2). The coronaries of the animals that received hPTH showed significant expression of nitrotyrosine (G4 = 32%; G5 = 44%; Figure 1C) and FGF-23 (G4 = 20%; G5 = 28%; Figure 1D) compared to the other groups (P < 0.0001) (Figure 1A).

All groups showed positive FGF-R-1 expression in myocytes (Figure 2A) and groups that received hPTH infusion (G4 and G5) presented calcified coronaries, as shown in Figure 2B. Runx-2 expression was negative in G1 and G2 groups (Figure 3A) but was positive in coronary arteries and myocytes of the animals from groups G3, G4 and G5 (Figure 3B).

The expression of α-actin was significantly lower in hPTH groups compared to that of the sham group, giving a mirror image of coronary lesion score (Figure 4).

Analysis of isolated effects of PTH and phosphorus on structural, biochemical and immunohistochemical parameters

Serum creatinine was the only parameter where an interaction between phosphorus and PTH was found. Post-test analysis disclosed that serum creatinine was influenced by phosphorus content in the diet (pP = 0.80 ± 0.08; rP = 1.02 ± 0.10; P = 0.054).

Table 3 shows the parameters where the interaction between phosphorus and PTH was not found. Student’s t-test analysis revealed that hPTH infusion increased TCP, heart weight, coronary lesion score, myocyte diameter (Figure 5A), fibrosis (Figure 5B) and both TGF-β and angiotensin II expression (Figure 5C and D, respectively), whereas it decreased hematocrit and α-actin expression. Regarding the phosphate content in the diet, the rP groups presented an increase in serum FGF-23 (Figure 6A) and myocyte diameter (Figure 6B) and a decrease in hematocrit and ionized calcium. We also observed a tendency to increased fibrosis and decreased TCP in rP groups.

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### Table 1: Arterial pressure, heart weight, hematocrit and biochemical data

<table>
<thead>
<tr>
<th>Group</th>
<th>Arterial Pressure (TCP) (mmHg)</th>
<th>Heart Weight (body weight) (%)</th>
<th>Ht (%)</th>
<th>Cr (mg/dL)</th>
<th>iCa (mmol/L)</th>
<th>P (mg/dL)</th>
<th>iPTH (pg/mL)</th>
<th>FGF-23 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1—Sham (n = 10)</td>
<td>124.29 ± 3.95</td>
<td>46.22 ± 0.85</td>
<td>4.26 ± 0.6</td>
<td>0.38 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.26 ± 0.06</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td>G2—Nx PTx nPTH</td>
<td>141.64 ± 2.11</td>
<td>42.94 ± 1.69</td>
<td>0.59 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>G3—Nx PTx nPTH</td>
<td>137.49 ± 6.99</td>
<td>39.08 ± 1.30</td>
<td>1.09 ± 0.04</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.10 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>G4—Nx PTx hPTH</td>
<td>149.55 ± 2.58</td>
<td>30.12 ± 1.63</td>
<td>1.63 ± 0.03</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>G5—Nx PTx hPTH</td>
<td>143.49 ± 4.69</td>
<td>23.58 ± 1.47</td>
<td>1.47 ± 0.05</td>
<td>0.15 ± 0.03</td>
<td>0.15 ± 0.03</td>
<td>0.15 ± 0.03</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.02</td>
</tr>
</tbody>
</table>

aData are expressed as mean ± standard error; Ht = hematocrit; Cr = creatinine; iCa = intact parathyroid hormone; Nx = nephrectomized rat; nPTH = normal parathyroid hormone; hPTH = hormone; Sham = control animal; P = phosphorus.

**Discussion**

This study showed that high-serum PTH levels in uremic animals contributed to myocyte hypertrophy, interstitial fibrosis and vascular lesion, with a significant change in angiotensin II, α-actin, TGF-β, nitrotyrosine and FGF-23 expression. On the other hand, phosphorus overload contributed to myocardial hypertrophy and was probably involved in the myocardial fibrosis event. We also observed an expression of FGFR-1 in myocytes of all animals and in the coronaries of those that received hPTH. An interesting finding was the expression of Runx-2 in coronaries and myocytes of the animals with phosphorus overload (G3 and G5) and hPTH (G4 and G5).

The past concept that cardiac hypertrophy in uremia is the result of hypervolemia and hypertension has changed and currently the involvement of other factors such as anemia, sympathetic overactivity and hyperparathyroidism have grown in importance [15]. Recently, Siedlecki et al. [16], in an elegant study, showed that despite the control of arterial hypertension through the management of renin–angiotensin–aldosterone system and volume overload, uremic animals still develop myocardial hypertrophy. In the current study, all Nx PTx animals showed hypertension and anemia. However, we could evaluate the independent contribution of phosphorus and PTH in cardiac hypertrophy.

It is well known that PTH may contribute to the development of myocardial hypertrophy [17]. Clinical studies carried out in patients with secondary hyperparathyroidism and in general population have demonstrated an association between PTH serum levels with LVH and mortality [18, 19].

In vitro studies have shown that PTH appears to have chronotropic, inotropic, as well as hypertrophic effects on cardiomyocytes [20]. Our results confirm the effect of PTH in myocardial hypertrophy since the animals that received hPTH infusion showed increased heart weight and myocyte diameter.

In uremia, the isolated deleterious effect of hyperphosphatemia on cardiac hypertrophy is still a matter of debate. Our results showed that hyperphosphatemia was associated with the development of myocardial hypertrophy. We clearly observed the phosphorus influence when we compared two groups of animals receiving different contents of phosphorus in the diet (G4 and G5) and that animals fed with a P diet presented a higher myocyte diameter. This finding was similar to that showed by Amann et al. [21], which demonstrated the protective effect of a P diet on the development of myocardial hypertrophy in uremic animals. However, in the preceding study, the authors could not rule out the effects of PTH since these animals also presented lower PTH levels.

Recent studies revealed that phosphorus regulates the expression of a wide variety of genes involved in the regulation of enzymes and proteins related to OS [22]. One hypothesis of the deleterious effect of phosphorus overload would be an indirect action decreasing nuclear factor erythroid 2-related factor (Nrf2) expression and antioxidant proteins and increasing OS and stimulating hypertrophy as well as myocardial fibrosis. Therefore, there
might be distinct metabolic pathways leading to myocardium changes and VC induced by hyperphosphatemia in addition to phenotypic changes in VSMCs, which can be exemplified by the reduction of myocardial hypertrophy [23], but not of VC, when you reduce phosphorus content in the diet [24].
Fig. 3. Runx-2 immunohistochemical expression in coronaries and myocardium tissue of the animals. (A) Runx-2-negative expression in G1 and G2 groups (magnification, ×400); (B) Runx-2-positive expression in nucleus from the cells in coronaries and cardiomyocytes in G3, G4 and G5 groups (magnification, ×400; detail: ×1000).

Fig. 4. Analysis of arterial lesions score and α-actin expression of nephrectomized animals. (A) Normal artery (score 0); (B) a calcified artery (score 3); (C) a normal artery expressing α-actin; (D) a calcified artery expressing low α-actin (magnification, ×580).

Table 3. Analysis of the isolated effects of PTH and phosphorus on structural, biochemical and immunohistochemical parameters of nephrectomized animals

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PTH</th>
<th></th>
<th>Phosphorus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nPTH (n = 16)</td>
<td>hPTH (n = 16)</td>
<td>P</td>
<td>pP (n = 17)</td>
</tr>
<tr>
<td>TCP (mmHg)</td>
<td>139.6 ± 1.4</td>
<td>146.9 ± 2.5</td>
<td>&lt;0.05</td>
<td>145.8 ± 1.9</td>
</tr>
<tr>
<td>HW/100 g</td>
<td>0.33 ± 0.01</td>
<td>0.42 ± 0.02</td>
<td>&lt;0.0001</td>
<td>0.37 ± 0.02</td>
</tr>
<tr>
<td>Ht (%)</td>
<td>41.0 ± 0.9</td>
<td>38.1 ± 1.0</td>
<td>&lt;0.05</td>
<td>41.1 ± 1.0</td>
</tr>
<tr>
<td>iCa (mmol/L)</td>
<td>0.90 ± 0.08</td>
<td>1.09 ± 0.11</td>
<td>NS</td>
<td>1.32 ± 0.05</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>10.1 ± 1.7</td>
<td>8.5 ± 1.2</td>
<td>NS</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>iPTH (pg/mL)</td>
<td>100.9 ± 17.9</td>
<td>301.8 ± 40.3</td>
<td>&lt;0.0001</td>
<td>190.7 ± 32.7</td>
</tr>
<tr>
<td>FGF-23 (pg/mL)</td>
<td>418.8 ± 198.6</td>
<td>695.0 ± 115.6</td>
<td>NS</td>
<td>352.8 ± 101.9</td>
</tr>
<tr>
<td>Coronary lesion score</td>
<td>0.53 ± 0.18</td>
<td>2.28 ± 0.17</td>
<td>&lt;0.0001</td>
<td>1.52 ± 0.30</td>
</tr>
<tr>
<td>Myocyte diameter (µm)</td>
<td>11.03 ± 0.46</td>
<td>12.37 ± 0.31</td>
<td>&lt;0.05</td>
<td>10.92 ± 0.35</td>
</tr>
<tr>
<td>Fibrosis (%)</td>
<td>3.19 ± 1.14</td>
<td>7.55 ± 1.37</td>
<td>&lt;0.05</td>
<td>3.68 ± 1.00</td>
</tr>
<tr>
<td>Angiotensin II (%)</td>
<td>1.12 ± 0.22</td>
<td>2.74 ± 0.25</td>
<td>&lt;0.0001</td>
<td>2.12 ± 0.29</td>
</tr>
<tr>
<td>α-Actin (%)</td>
<td>2.80 ± 0.09</td>
<td>1.92 ± 0.22</td>
<td>&lt;0.01</td>
<td>2.34 ± 0.21</td>
</tr>
<tr>
<td>TGF-β (%)</td>
<td>2.29 ± 0.24</td>
<td>3.95 ± 0.50</td>
<td>&lt;0.01</td>
<td>2.90 ± 0.48</td>
</tr>
</tbody>
</table>

aData are expressed as mean ± standard error. hPTH = high parathyroid hormone; Ht = hematocrit; HW/100 g = heart weight/100 g of body weight; nPTH = normal parathyroid hormone; NS = statistically not significant; PTH = parathyroid hormone.
We observed a nitrotyrosine expression only in the coronaries of the animals that received hPTH infusion. This result demonstrated the important role of PTH in increasing the OS leading to VC.

Our animals developed myocardial hypertrophy with major participation of the process of fibrosis, and the animals submitted to hPTH infusion and rP diet showed more fibrosis than the others. It is known that PTH activates fibroblasts and regulates pro-fibrotic factors, such as aldosterone and angiotensin II [25]. In this study, we could demonstrate a dependency of angiotensin II expression on PTH serum level. Certainly, this would be an important factor on cardiac remodeling. Although the phosphorus-dependent fibrosis results have not achieved statistical significance, phosphorus may have also participated in the process of fibrosis as was demonstrated by Amann et al. [21].

Analyzing some of the proteins involved in cardiac remodeling, we found that the TGF-β expression was influenced by PTH levels. It is believed that PTH activates protein kinase C, which is hypertrophy inducing and in addition, activates other proteins, such as TGF-β, which, in turn, stimulates the proliferation of fibroblasts, collagen synthesis and fibrosis [26]. Regarding the angiotensin II expression in the heart, we could demonstrate a significant increase in high-PTH groups. It is known that PTH facilitates the effect of angiotensin II as a mediator of CV lesion [25].

One important finding was PTH as the major determining factor of coronary artery lesions, ranging from the discontinuity of the elastic lamina to the calcification of the medial layer, confirming the permissive action of PTH as previously demonstrated by Amann et al. [25]. These results were not dependent on the concentration of phosphorus in the diet, probably because high-PTH concentration was the strongest determinand of vessel lesion. Uzawa et al. [27] has previously shown that continuous administration of PTH at high-flow rates has a catabolic effect on bone, whereas intermittent PTH administration has an anabolic effect. Therefore, PTH may have exerted an indirect effect, increasing calcium and phosphorus load, thus favoring vascular lesion. On the other hand, the animals that received PTH in physiological concentrations and a rP diet did not present calcifications.

Another interesting finding was the expression of α-actin, which correlated negatively with the intensity of the vessels’ lesions. It was shown in literature that as smooth muscle cells of the vessels suffer phenotypic changes, as occurs in
hyperphosphatemia, they underexpress α-actin. Wang et al. [28] confirmed this hypothesis describing the finding of low α-actin expression in the medial of calcified arteries from uremic patients. We observed the same finding in the animals that received high concentration of PTH, unrelated to phosphorus overload. Therefore, as we described before, the effect of phosphorus is masked in high PTH concentration [11].

The animals that received high PTH infusion showed high FGF-23 expression in calcified coronaries and we hypothesized that maybe FGF-23 is linked to VC, as previously demonstrated by Inaba et al. [29]. Trying to verify if the cells suffered phenotypic modifications, we looked for the expression of Runx-2. Our results confirmed the previous observations that phosphorus overload leads to phenotypic changes in VSMCs. We also showed that high PTH infusion changes VSMCs phenotype and promotes coronary calcification [30]. Surprisingly, we also found Runx-2 expression in cardiomyocytes in the groups with phosphorus overload (G3 and G5) and high-PTH infusion (G4 and G5). We believe that both phosphorus and PTH per se promote modifications in these cells by unknown mechanisms.

FGF-1 and FGF-2 are involved in biological activities in adult myocardium and FGFR-1 is the prevalent receptor in cardiomyocytes [31, 32]. We have shown expression of FGFR-1 in cardiomyocytes in all groups, but only the groups with hPTH presented expression of FGFR-1 in calcified coronaries. High concentrations of FGF-23 may induce unspecific Klotho-independent FGFR signaling [33], justifying our findings of FGF-23 expression in coronaries in the same groups. Probably, the FGF-23 expression in coronaries results from the VSMCs that suffered phenotypic modification (Runx-2 expression) and also from unspecific binding to the FGFR-1 [13, 33].

Regarding serum FGF-23 levels, we only observed a correlation with phosphorus overload. It was previously shown that FGF-23 is independently associated with ventricular hypertrophy in CKD patients [34] and we could also hypothesize whether the phosphorus-induced myocardial hypertrophy that was found in our animals was mediated by elevated FGF-23.

Our study suffers the limitation of not evaluating the role of vitamin D in the cardiac remodeling process, but despite its importance, our design protocol could not cover all the involved variables.

Our results point to important aspects regarding the disturbances of the mineral metabolism and CVD in CKD. In recent years, numerous studies have assessed the participation of these disturbances in VC, but little attention has been given to their effect in uremic cardiomyopathy.

We showed that both PTH and phosphorus participated in hypertrophy and maybe in myocardial fibrosis. However, the mechanism of action of phosphorus in uremia was not clear, that is, whether hyperphosphatemia per se was cardiotoxic or whether it activated inflammatory pathways, promoting OS and endothelial dysfunction. As far as we know, our study is unique in the literature because we analysed all the cardiac remodeling processes at the same time. In summary, our data confirmed that, in addition to classical factors already described in uremia, disturbances of the mineral metabolism, such as phosphorus and PTH overload, play an important role in the cardiac remodeling processes. Nevertheless, further studies are still necessary to elucidate the molecular pathways of phosphorus and PTH in the myocardium and coronary vessels.

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