Original Article

Up-regulation of Cbfa1 and Pit-1 in calcified artery of uraemic rats with severe hyperphosphataemia and secondary hyperparathyroidism

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Abstract

Background. Cardiovascular disease is the most frequent cause of death in patients with end-stage kidney disease (ESKD). Vascular calcification is a confirmed risk factor for cardiovascular events in the general population and has a high occurrence in patients with ESKD. Despite the high prevalence of vascular calcification in ESKD, the pathogenesis of the disorder is still obscure. The present study examined the expressions of bone-associated factors in calcified arteries in subtotally nephrectomized rats with severe secondary hyperparathyroidism (SHPT).

Methods. Seven-week-old male Sprague–Dawley rats were divided into five groups as follows: sham-operated rats that received a normal diet [0.8% of phosphorus (P), 1.1% of calcium (Ca)] (Sham), sham-operated rats that received a high-phosphorus and low-calcium (HPLCa) diet (1.2% P, 0.4% Ca) (Sham+HPLCa), 5/6 nephrectomized rats that received a normal diet as the uraemic control group (Nx), and 5/6 nephrectomized rats that received a HPLCa diet to induce the development of SHPT (Nx+HPLCa), and 5/6 nephrectomized and parathyroidectomized rats that received a HPLCa diet (Nx+PTx+HPLCa). The feeding period of each group was 10 weeks. The rats were then sacrificed and their serum was examined. The upper part of the abdominal aorta was used to investigate the expression of mRNAs of core-binding factor alpha-1 (Cbfa1) and sodium-dependent phosphate cotransporter (Pit-1) by real-time reverse transcriptase polymerase chain reaction (real-time PCR) analysis. The lower part was examined for calcification by von Kossa staining.

Results. Serum P level and Ca × P products increased significantly in the Nx+HPLCa group compared with those of any other groups. Severe hyperparathyroidism was also observed in the Nx+HPLCa group. Vascular calcification (medial layer) was observed in the Nx+HPLCa group only. There was a significant increase in Cbfa1 and Pit-1 mRNA expression levels in the aorta of the Nx+HPLCa group compared with that of any other groups.

Conclusions. These results suggest that medial layer vascular calcification in uraemic rats with severe hyper-phosphataemia and SHPT may be caused in part by Cbfa1 and Pit-1.

Keywords: core-binding factor alpha-1 (Cbfa1); hyperphosphataemia; secondary hyperparathyroidism (SHPT); sodium-dependent phosphate cotransporter (Pit-1); vascular calcification

Introduction

Cardiovascular disease is the most frequent cause of death in patients with end-stage kidney disease (ESKD) [1–3]. It has been reported that there is a high prevalence of cardiovascular calcification in patients with ESKD [4]. The most notable difference in vascular calcification compared with nonuraemic patients is the composition of the calcified vessel. Both plaque calcification and diffuse medial calcification (Mönckeberg type) are representative pathological manifestations in older subjects without renal impairment.

It has been demonstrated that hyperphosphataemia and increased calcium (Ca) × phosphorus (P) products play an important role in the pathogenesis of vascular calcification in the uraemic milieu [5]. The mechanism
through which vascular calcification is mediated by ionic P regulation has been revealed in recent years. An in vitro study demonstrated that the calcification of vascular smooth muscle cells (VSMCs) could be induced by culture media with high P concentration [6]. Jono et al. [6] reported that VSMCs incubated with high P concentration could up-regulate not only osteocalcin, which is an osteoblastic differentiation marker, but also core-binding factor alpha-1 (Cbfa1), presumably being the key regulator in the differentiation process. The osteoblastic differentiation was presumably shown to be mediated by the type III sodium-dependent phosphate cotransporter (Pit-1). This mechanism may have a close relationship with the development of vascular calcification in patients with ESKD.

The role of parathyroid hormone (PTH) as a risk factor for vascular calcification is discussed controversially. Shao et al. reported that 1–34 human PTH inhibits vascular calcification in diabetic low density lipoprotein receptor-deficient mice [7]. On the other hand, from the clinical aspect, the regression of medial calcification of small peripheral arteries was occasionally observed after parathyroidectomy in uremic patients with severe secondary hyperparathyroidism (SHPT) [8]. Taking into account this phenomenon, not physiological but the extremely high PTH concentration which induces Ca and P mobilization from bone may also be considered to be one of the contributors to vascular calcification.

Moe et al. [9] found that uraemic serum, in which P concentration was equal to that in control human serum, could induce Cbfa1 gene expression in bovine VSMCs. They also reported that expression of Cbfa1 was elevated in calcified arteries in patients with ESKD. It was suggested that Cbfa1 might play a crucial role in the development of vascular calcification and that non-P-mediated mechanisms, uraemic toxins, might be actively involved in the pathologic process.

Many findings regarding the pathogenesis of vascular calcification have been reported in recent years. However, most of them were obtained by in vitro studies. This in vivo investigation shows the novel evidence of the up-regulation of Cbfa1 mRNA and Pit-1 mRNA in calcified arteries of uraemic rats with severe hyperphosphataemia and SHPT.

Materials and methods

Animals

Seven-week-old male Sprague–Dawley rats weighing 200–250 g (SLC Japan, Tokyo, Japan) were housed in single cages at constant room temperature (25°C) and humidity (75%) under a controlled light/dark cycle. The Animal Studies Committee of Wakayama Medical University approved all experimental protocols.

Experimental animal groups

After a 7 day adaptation period, the rats were randomly allocated into five groups as follows: sham-operated control rats fed a standard chow diet for 10 weeks (Sham group, n = 8), sham-operated control rats fed a high phosphorus and low calcium (HPLCa) diet for 10 weeks (Sham+HPLCa group, n = 8), 5/6 nephrectomized rats fed a standard chow diet for 10 weeks (Nx group, n = 12), 5/6 nephrectomized rats fed a HPLCa diet for 10 weeks (Nx+HPLCa group, n = 12) and 5/6 nephrectomized and microsurgical parathyroidectomized rats that received a HPLCa diet for 10 weeks (n = 12, Nx+PTx+HPLCa group). The Nx+PTx+HPLCa group rats were subsequently given 5% calcium gluconate in the drinking water throughout the study to prevent development of hypocalcaemia [10]. The HPLCa diet (1.2% P, 0.4% Ca and <300 IU/kg vitamin D) was chosen to enhance the severity of hyperphosphataemia and hyperparathyroidism. The standard chow diet contains 0.8% of P, 1.1% of Ca and 1580 IU/kg of vitamin D. Both types of diet were obtained from Oriental Yeast Co, Ltd. (Tokyo, Japan). The rats were provided water ad libitum throughout the experiment.

In 5/6 nephrectomies, the right kidney was removed in an initial operation under anesthesia using pentobarbital (100 mg/kg body weight). The right kidney was weighed immediately after excision. Seven days after the unilateral nephrectomy, the cortical tissue of the hypertrophied remnant left kidney was removed so that the amount removed corresponded to the weight of the initially excised right kidney. Care was taken to remove the tissue preferentially from the upper and lower poles without damaging the large arteries. In the sham-operated rats, the kidneys were decapsulated in two consecutive operations.

Measurements

Serum samples were analyzed using standard laboratory methods with an automated multiparametric analyzer (DRI-CHEM 3030, Fuji Film, Tokyo, Japan). Serum PTH level was determined using a rat intact PTH ELISA kit (ImmunoTech, San Clemente, CA, USA).

Tissue preparation

Ten weeks after 5/6 nephrectomies, all the rats were anesthetized using pentobarbital, and the abdominal aorta was quickly removed. The upper part of the abdominal aorta was snap-frozen in liquid nitrogen, stored at −80°C for real-time polymerase chain reaction (PCR) analysis and the lower part was fixed in 4% paraformaldehyde for histologic examination by von Kossa staining.

Real-time PCR

Total RNA from each aorta was extracted using Trizol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed to first-strand cDNA using 2.5 μl reverse transcriptase, with random hexamers and TaqMan reverse transcription reagents (Applied Biosystems Japan, Tokyo). Quantitation was performed with real time quantitative reverse transcription polymerase chain reaction based on TaqMan fluorescence methodology. Taqman probe and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cbfa1 and Pit-1 were purchased from TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Assay ID of GAPDH primers and Taqman probe set is Rn99999916_s1. The assay ID of Cbfa1 and Pit-1 set is...
Sham (significantly higher in the Nx and the Nx+HPLCa groups. Ca level was significantly lower in the Sham and the other four groups (Table 1). The serum Ca level was significantly lower in the Sham and the groups. No significant difference was observed in those levels between the groups and the groups (Table 1). The serum Ca level was significantly lower in the groups than in the other groups. Ca × P product was significantly higher in the groups and this product was significantly lower in the groups than in the other groups, respectively (Table 1).

The serum PTH level was significantly higher in the groups than in the other groups, respectively (Table 1).

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<th>Albumin (g/dl)</th>
<th>SUN (mg/dl)</th>
<th>Cr (mg/dl)</th>
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<th>P (mg/dl)</th>
<th>Ca × P (mg²/dl²)</th>
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<tr>
<td>Sham (n=8)</td>
<td>4.2 ± 0.1</td>
<td>21.3 ± 0.7</td>
<td>0.4 ± 0.0</td>
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<td>8.0 ± 0.2</td>
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<td>Sham+HPLCa (n=8)</td>
<td>4.0 ± 0.0</td>
<td>41.6 ± 4.2</td>
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<td>Nx (n=10)</td>
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<td>46.9 ± 5.1</td>
<td>1.5 ± 0.4</td>
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<td>8.3 ± 0.3</td>
<td>91.5 ± 2.7</td>
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<tr>
<td>Nx+HPLCa (n=8)</td>
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<td>8.5 ± 0.3</td>
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<td>0.0958</td>
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All data are mean ± SE. SUN, serum urea nitrogen; Cr, creatinine; Ca, calcium; P, phosphorus; Nx, nephrectomized; HPLCa, high phosphorus and low calcium diet. 

\[ P < 0.05 \text{ vs } \text{Nx+HPLCa.} \]

\[ P < 0.05 \text{ vs } \text{Sham.} \]

\[ P < 0.05 \text{ vs } \text{Sham+HPLCa.} \]

\[ P < 0.05 \text{ vs } \text{Nx+PTx+HPLCa (Fisher’s PLSD).} \]

Rn01512296_ml and Rn00579811_ml, respectively. Taqman PCR reactions were performed in 50 µl volume. Taqman PCR reaction mix was 25 µl 2X Taqman Universal PCR Master Mix (Applied Biosystems 4304437), 2.5 µl primers and TaqMan probe set, and 2 µl cDNA. Amplification and detection were performed with an ABI 7500 system (Applied Biosystems). The thermal profile for PCR was 50°C for 2 min, 95°C for 10 s and 60°C for 1 min. Measurements on each sample were performed in duplicate, and the expression of the tested gene was normalized to a GAPDH standard curve run in duplicate on the same plate.

**Statistical analysis**

All results were expressed as mean ± SE. Statistical differences between the groups were assessed by one-way ANOVA (post-hoc test, Fisher’s protected least significant difference; Fisher’s PLSD was used for the comparison of the groups). A P value <0.05 was considered statistically significant.

**Results**

**Biochemical data**

There was no difference in serum albumin level among the five groups (Table 1). The serum creatinine and urea levels were significantly higher, both in the Nx+HPLCa and Nx+PTx+HPLCa groups than those in other three groups (the Sham, the Sham+HPLCa and the Nx groups). No significant difference was observed in those levels between the groups and the groups (Table 1). The serum Ca level was significantly lower in the Nx+HPLCa group than that in the Sham, the Nx and the Nx+PTx+HPLCa groups, but comparable to that in the Sham+HPLCa group. Serum P level was significantly higher in the Nx+HPLCa group than in any of the other four groups. Ca × P product was significantly higher in the groups and this parameter was significantly lower in the groups than in the other groups, respectively (Table 1).

The serum PTH level was significantly higher in the groups (5793.3 ± 577.8 pg/ml) than in the other groups (Sham; 24.8 ± 6.1 pg/ml, P < 0.01, Sham+HPLCa; 2432.1 ± 422.6 pg/ml, P < 0.01, Nx; 158.4 ± 28.9 pg/ml, P < 0.01, Nx+PTx+HPLCa; 22.5 ± 3.3 pg/ml, P < 0.01). There was no significant difference in serum PTH level among the groups, respectively, was 16.7% in the Nx group. No rats died in the other groups.

**Calcification of abdominal aorta**

The calcification of the abdominal aorta was histopathologically examined by von Kossa staining. In the histopathologic examination of the lower abdominal aorta, the medial layer was calcified in the Nx+HPLCa group (5 of 8 rats). On the other hand, no calcified arteries were observed in any of the other groups (Figure 2a and b).

**Cbfa1 gene and Pit-1 gene expression in abdominal aorta**

The quantitative analysis by the real-time PCR method revealed an approximately 6.74-fold increase in Cbfa1...
mRNA expression level in the Nx+HPLCa group as compared with the Sham group (Figure 3). No significant difference was noted in Cbfa1 mRNA expression levels among the four groups (the Sham, the Sham+HPLCa, the Nx and the Nx+PTx+HPLCa groups) (Figure 3). The Pit-1 mRNA level was 2.63-fold higher in the Nx+HP group as compared with the Sham group (Figure 4). No significant difference was noted in the Pit-1 mRNA expression level among the four groups (the Sham, the Sham+HPLCa, the Nx and the Nx+PTx+HPLCa groups) (Figure 4).

**Discussion**

Recently, vascular calcification was shown to involve not a passive deposition of calcium-phosphate crystals, but an active process in which vascular cells elicit osteoblastic-like cell characteristics [11]. Extracellular P concentration has a dramatic effect on vascular calcification through a type III Pit-1 in vitro [6]. In this study, we investigated the mRNA expression levels of the osteoblastic differentiation regulator, Cbfa1, and Pit-1 in the calcified arteries of uraemic rats. The results showed that medial layer aortic calcification could be induced in only the Nx+HPLCa group, and that calcified artery examined by von Kossa staining was not noted in any other group. The up-regulation of Cbfa1 mRNA and Pit-1 mRNA was simultaneously observed in the calcified arteries of uraemic rats. Subtotal nephrectomy fed an HPLCa diet evoked a much more severe renal impairment, hyperphosphataemia, and a higher level of serum PTH.

Hyperphosphataemia contributes to the pathogenesis of uraemia-induced soft-tissue calcification. In fact, it was reported that a Ca-free P binder, sevelamer hydrochloride, could inhibit the development of vascular calcification in uraemic animal models, through amelioration of hyperphosphataemia without Ca overload [12,13]. However, the precise mechanism of hyperphosphataemia-mediated vascular calcification remains unknown. Based on several in vitro studies, P has been considered to be an important factor for mineralization in osseous tissues [14,15]. Hyperphosphataemic disorders are frequently associated with disturbed bone mineralization, such as osteomalacia in patients with ESKD. Similarly, extrasosseous calcification occurs in the P-burden state. Jono et al. [6] reported that inorganic P could promote human VSMCs calcification, with expressed osteoblastic-like cell markers, in a time- and dose-dependent manner and that the response could be inhibited by phosphonoformic acid, a sodium-dependent phosphate cotransporter inhibitor. They also demonstrated that
P supplementation could up-regulate the Cbfa1 gene expression in the human VSMCs culture. Thus, it suggested that P entry into VSMCs mediated by Pit-1 plays an important role in the pathologic process of vascular calcification. However, the in vivo relationship between vascular calcification and Cbfa1 or Pit-1 activation has not been completely elucidated. The present study demonstrated that both Cbfa1 mRNA and Pit-1 mRNA were up-regulated only in the Nx+HPLCa group with severe hyperphosphataemia. The results strongly supported a hypothesis that high P concentration can be a predisposing factor and can promote transformation of VSMCs to osteoblastic-like cells with Cbfa1 expression. Pit-1-mediated P entry into cells might play an important role in this pathologic process. The precise mechanism of the regulation of Pit-1 expression remains obscure in uraemia; however the up-regulation of the Pit-1 gene might contribute to the cellular process of vascular calcification.

STPT has been demonstrated to be a representative risk factor in cardiovascular morbidity and mortality in patients with ESKD, as well as hyperphosphataemia [16]. High serum PTH concentrations induce mineral mobilization from bone tissue. Thus, excess PTH promotes Ca and P efflux from bone into extraosseous tissues. Extremely high PTH concentrations have the potential to cause molecular and functional changes in constitutive cells of the cardiovascular system, ultimately to cause their calcification in association with other factors in ESKD. In the present study, the Nx+HPLCa group presented severe hyperparathyroidism, which is responsible for influencing the elevation of serum P levels and Ca × P products. In the study, extremely increased P and PTH concentrations were observed in the Nx+HPLCa rats, as compared with those in standard 5/6 nephrectomized rats. Marked high P and low Ca diet caused mineral disarrangement, in part similar to that seen in ESKD. Not only a high P diet but also an extremely high PTH concentration was necessary to maintain severe hyperphosphataemia during this experimental period. The Nx+HPLCa group, in which calcified arteries were observed, demonstrated severe hyperparathyroidism as well as hyperphosphataemia, while arterial calcification was not observed in the Sham+HPLCa group. Also, no calcified arteries were observed in the Nx+PTx+HPLCa group. The Nx+PTx+HPLCa group had mild hyperphosphataemia; however, serum P level in this group was significantly lower than that in the Nx+HPLCa group. Severe elevation of serum P level in the Nx+HPLCa group probably resulted from not only dietary P intake but also mobilization from the bone by extremely high PTH. Thus, there was a limitation of P elevation in the Nx+PTx+HPLCa group. We could not exclude the single effect of PTH on vascular calcification because the serum P level was not equal between the Nx+HPLCa group and Nx+PTx+HPLCa group. These findings, however, indicated that much elevated PTH level as well as severe hyperphosphataemia plays a potential role in the progression of vascular calcification, directly or indirectly.

It has been reported that unidentified uraemic toxins, other than P or PTH, contribute to the pathogenesis of vascular calcification in patients with ESKD [17]. Moe et al. [9] demonstrated that Cbfa1 is expressed in human calcified vessels of dialysis patients and uraemic serum induced the up-regulation of Cbfa1 mRNA in bovine VSMCs. In the present study, the Nx+HPLCa rats with arterial calcification, that were observed had severe uraemia. On the other hand, no calcified arteries were observed in the Nx+PTx+HPLCa rats, and uraemia was comparable to that Nx+HPLCa rats. This result indicates that uraemia might play a permissive role in the pathogenesis of vascular calcification. The exposure period of uraemic toxin and severity of uraemia may also be important to induce calcification. Further studies are necessary to clarify the involvement of uraemic toxins on vascular calcification.

Giachelli’s group reported that extracellular Ca level induced vascular smooth muscle cell (VSMC) calcification in vitro [18]. They concluded that the effect of elevated extracellular Ca concentration on the mineralization was partly mediated by elevating Ca × P products. In the present study, although the serum Ca level was lower in the Nx+HPLCa group than that in other groups, Ca × P products in the group was markedly higher than that in any other groups. Thus elevated Ca × P products might cover up the effect of extracellular Ca level. It was necessary to use a low Ca diet to induce severe hyperparathyroidism so we could not test the effect of high serum Ca level on vascular calcification. Matrix vesicles from VSMCs have also been recognized as a key factor in vascular calcification [19]. We could not detect any vesicles or apoptotic bodies in this study. Further studies,
including electron microscopy, are necessary to completely rule out a possible involvement of apoptosis in the calcified process of arteries in the uraemic milieu.

In summary, we demonstrated that medial layer vascular calcification could be induced in uraemic rats with severe hyperphosphataemia and SHPT, in which the up-regulation of Cbfa1 and Pit-1 might be implicated. It is assumed that the up-regulation of Cbfa1 and Pit-1 mRNA levels may contribute to vascular calcification by two possible mechanisms. First, severe hyperphosphataemia has an important role for the pathogenesis of vascular calcification. P influx into the VSMCs through Pit-1 might promote transformation of VSMCs to osteoblastic-like cells which have mineralization activity that contributes to medial layer vascular calcification. Second, Cbfa1 up-regulation of VSMCs might be predisposed to develop calcification, particularly under uraemic conditions with burdened Ca and P. Further studies are required to determine the major contributor for vascular calcification.

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Conflict of interest statement. None declared.

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