Phosphate overload directly induces systemic inflammation and malnutrition as well as vascular calcification in uremia

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1Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; 2Department of Integrated Therapy for Chronic Kidney Disease, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and 3Department of Internal Medicine, Fukuoka Dental College Medical and Dental Hospital, Fukuoka, Japan

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Yamada S, Tokumoto M, Tatsumoto N, Taniguchi M, Noguchi H, Nakano T, Masutani K, Ooboshi H, Tsuirtyu K, Kitazono T. Phosphate overload directly induces systemic inflammation and malnutrition as well as vascular calcification in uremia. Am J Physiol Renal Physiol 306: F1418–F1428, 2014. First published May 7, 2014; doi:10.1152/ajprenal.00633.2013.—Hyperphosphatemia contributes to increased cardiovascular mortality through vascular calcification (VC) in patients with chronic kidney disease (CKD). Malnutrition and inflammation are also closely linked to an increased risk of cardiovascular death in CKD. However, the effects of Pi overload on inflammation and malnutrition remain to be elucidated. The aim of the present study was to investigate the effects of dietary Pi loading on the interactions among inflammation, malnutrition, and VC in CKD. We used control rats fed normal diets and adenine-induced CKD rats fed diets with different Pi concentrations ranging from 0.3% to 1.2% for 8 wk. CKD rats showed dietary Pi concentration-dependent increases in serum and tissue levels of TNF-α and urinary and tissue levels of oxidative stress markers and developed malnutrition (decrease in body weight, serum albumin, and urinary creatinine excretion), VC, and premature death without affecting kidney function. Treatment with 6% lanthanum carbonate blunted almost all changes induced by Pi overload. Regression analysis showed that serum Pi levels closely correlated with the extent of inflammation, malnutrition, and VC. Also, in cultured human vascular smooth muscle cells, high-Pi medium directly increased the expression of TNF-α in advance of the increase in osteochondrogenic markers. Our data suggest that dietary Pi overload induces systemic inflammation and malnutrition, accompanied by VC and premature death in CKD, and that inhibition of Pi loading through dietary or pharmacological interventions or anti-inflammatory therapy may be a promising treatment for the prevention of malnutrition-inflammation-atherosclerosis syndrome.

chronic kidney disease; inflammation; malnutrition; phosphate; vascular calcification; malnutrition-inflammation-atherosclerosis syndrome

HYPERPHOSPHATEMIA, which is highly prevalent in patients with chronic kidney disease (CKD), is the manifestation of the relative Pi overload due to decreased Pi clearance in CKD (12). Mounting evidence has revealed that hyperphosphatemia contributes to increased cardiovascular and all-cause mortality in CKD patients, which is considered to be mediated by vascular calcification (VC) (4, 23). Both clinical and preclinical studies have shown that dietary Pi restriction and Pi binder use can prevent hyperphosphatemia and retard the progression of VC, thereby lowering the mortality rate in CKD (13, 16, 31), suggesting the importance of maintaining an appropriate Pi balance in the management of CKD patients.

Another important disorder of CKD is malnutrition-atherosclerosis (MIA) syndrome, a recently identified missing piece that partly explains the high cardiovascular mortality rate in CKD patients (25, 41, 42, 48). Clinical studies have reported that CKD is associated with elevated levels of inflammatory markers (3, 37) and that chronic inflammation in CKD promotes malnutrition and atherosclerosis (42). Furthermore, malnutrition has been reported to induce chronic inflammation (17). Accordingly, malnutrition and inflammation form a vicious cycle in CKD, leading to the development of cardiovascular disorders, VC, and subsequently increased mortality.

Recent experimental studies have revealed the underlying mechanisms by which Pi, directly impairs the cardiovascular system (15, 39, 43). Hyperphosphatemia induces VC via transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells and via apoptosis of VSMCs, which is triggered by Pi entry into cells through Pi transporter (PiT)-1 (15, 35, 43). Hyperphosphatemia also promotes endothelial dysfunction via impairment of nitric oxide synthesis (39). Given that PiT-1 is expressed ubiquitously (44), it is plausible to speculate that excessive Pi loading has harmful effects not only on the cardiovascular system but also on other tissues, probably via inflammation and malnutrition, that may account for the development of several CKD-related disorders. However, studies focused on the potential influence of Pi overload on the interactions among inflammation, malnutrition, and VC are lacking.

Based on the above background, it was hypothesized that Pi overload is located on the upstream of MIA syndrome and triggers multistep inflammation, oxidative stress, malnutrition, and atherosclerosis (arterial medial calcification) cascades, leading to premature death in uremic milieu. To investigate this hypothesis, the effects of Pi overload on inflammation and malnutrition as well as VC in adenine-fed uremic rats and on cultured human VSMCs and HepG2 cells were examined. Our main objective was to clarify whether Pi loading is directly involved in the development of chronic inflammation and malnutrition in uremia.

METHODS

Cell cultures. Human umbilical arterial smooth muscle cells (HUASMCs) and HepG2 cells (a frequently used human liver hepatocytoma cell line for examining hepatocyte biology) were grown in a humidified 5% CO₂ incubator at 37°C in DMEM containing 4.5 g/l...
glucose supplemented with 10% FBS, 10 mM sodium pyruvate, and 1% penicillin-streptomycin. Media were changed every 2 days. Cells were cultured on 12-well plates and grown until confluence. After reaching confluency, HUASMCs and HepG2 cells were cultured in DMEM with either of the following P concentrations: 0.9 mM (normal P concentration) or 2.9 mM (high P concentration). The P concentration was adjusted by the mixture of NaH2PO4 and Na2HPO4. On day 1 after exposure to each medium, total RNA was extracted and used for real-time PCR for the determination of relative mRNA expression.

Animals and materials. All protocols were reviewed and approved by the Committee on Ethics of Animal Experiments of Kyushu University Faculty of Medicine (A23-198-0). Male Sprague-Dawley rats (10 wk old) were purchased from Kyudo (Saga, Japan). Animals were housed in a climate-controlled space with a 12:12-h day-night cycle and allowed free access to food and water. Standard diet (1.0% Ca2⁺ and 1.2% P, Oriental Yeast, Tokyo, Japan) was used as the diet for control rats and for all rats during the acclimatization period.

It has been a challenge to create a uremic animal model that consistently develops extensive arterial medial calcification without genetic manipulation (2, 38). To create such a rat model, a new rodent diet was used that modified the conventional adenine-based diet in the following three ways. First, a 0.3% adenine diet was used to slow the progression of CKD; a 0.75% adenine diet induces severe and rapid renal failure, leading to high mortality in 4–6 wk (51). Second, a casein-based diet was selected for the protein source because it can promote Ca2⁺ and P absorption from the gastrointestinal tract (26). Finally, 20% lactose was added to the diet to further enhance Ca2⁺ and P absorption from the gastrointestinal tract (19). This synthetic rodent diet provided a rat model exhibiting extensive and robust arterial medial calcification with a relatively longer lifespan, enabling researchers to investigate the complex mechanisms of arterial medial calcification in uremia. All synthetic rodent diets were purchased from Oriental Yeast.

Experimental protocols. Male Sprague-Dawley rats (n = 66) were fed the standard diet for 7 days to acclimate. On day 1, rats were randomly subdivided into the following six groups, and each group was fed one of the specific diets for 8 wk (until day 56): control rats (CNT group; 1.2% P, n = 10), CKD rats fed a low-P diet (CKD-LP group; 0.3% P, n = 10), CKD rats fed a moderate-P diet (CKD-MP group; 0.6% P, n = 10), CKD rats fed a high-P diet (CKD-HP group; 0.9% P, n = 12), CKD rats fed an extremely high-P diet (CKD-EP group; 1.2% P, n = 14), and CKD rats fed an extremely high-P diet and 6% lanthanum carbonate (CKD-LaC group; 1.2% P, n = 10). Diets in the CKD groups contained 0.3% adenine to induce renal failure and 1.0% Ca2⁺, 20% lactose, and 19% casein-based protein to accelerate intestinal Ca2⁺ and P absorption. The diet in the CNT group contained 1.0% Ca2⁺, 1.2% P, and 19% protein-based protein but did not contain lactose. The content of each diet is shown in Table 1.

Table 1. Content of the animal diets

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca2⁺, %</th>
<th>P, %</th>
<th>Protein</th>
<th>Lactose, %</th>
<th>Adenine, %</th>
<th>Lanthanum Carbonate, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNT</td>
<td>1.0</td>
<td>1.2</td>
<td>19% (grain based)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CKD-LP</td>
<td>1.0</td>
<td>0.3</td>
<td>19% (casein based)</td>
<td>20</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>CKD-MP</td>
<td>1.0</td>
<td>0.6</td>
<td>19% (casein based)</td>
<td>20</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>CKD-HP</td>
<td>1.0</td>
<td>0.9</td>
<td>19% (casein based)</td>
<td>20</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>CKD-EP</td>
<td>1.0</td>
<td>1.2</td>
<td>19% (casein based)</td>
<td>20</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>CKD-LaC</td>
<td>1.0</td>
<td>1.2</td>
<td>19% (casein based)</td>
<td>20</td>
<td>0.3</td>
<td>6</td>
</tr>
</tbody>
</table>

On day 1, rats were randomly subdivided into the following six groups, and each group was fed one of the specific diets for 8 wk (until day 56): control rats (CNT group; 1.2% P, n = 10), CKD rats fed a low-P diet (CKD-LP group; 0.3% P, n = 10), CKD rats fed a moderate-P diet (CKD-MP group; 0.6% P, n = 10), CKD rats fed a high-P diet (CKD-HP group; 0.9% P, n = 12), CKD rats fed an extremely high-P diet (CKD-EP group; 1.2% P, n = 14), and CKD rats fed an extremely high-P diet and 6% lanthanum carbonate (CKD-LaC group; 1.2% P, n = 10). Diets in the CKD groups contained 0.3% adenine.
areas for 8-OHdG were quantitatively assessed and expressed as arbitrary units using National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/).

**Real-time PCR.** Total RNA was extracted from HUASMCs and HepG2 cells and frozen rat tissue in liquid nitrogen using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) using the guanidinium thiocyanate phenol-chloroform method according to the manufacturer’s instructions and used to prepare cDNA by reverse transcription using a PrimeScript RT reagent kit (Perfect Real Time, Takara Bio, Otsu, Japan). Real-time quantitative PCR was performed using SYBR Premix Ex Taq (Takara Bio), Applied Biosystems 7500 real-time PCR systems (Applied Biosystems), and the following primers purchased from Takara Bio: rat GAPDH, RA015380; rat TNF-α, RA043092; rat albumin, RA065413; rat fetuin-A, RA065433.

**Fig. 1.** Effects of dietary Pi loading on survival and vascular calcification. A: the Kaplan-Meier method and log-rank test were used to compare survival among groups. B: representative photomicrographs of the abdominal aorta stained with von Kossa. Original magnification: ×1100. C: quantification of Ca²⁺ content in the abdominal aorta. On day 1, rats were randomly subdivided into the following six groups, and each group was fed one of the specific diets for 8 wk (until day 56): control rats (CNT group; 1.2% Pi, n = 10), rats with chronic kidney disease (CKD) fed a low-Pi diet (CKD-LP group; 0.3% Pi, n = 10), CKD rats fed a moderate-Pi diet (CKD-MP group; 0.6% Pi, n = 10), CKD rats fed a high-Pi diet (CKD-HP group; 0.9% Pi, n = 12), CKD rats fed an extremely high-Pi diet (CKD-EP group; 1.2% Pi, n = 14), and CKD rats fed an extremely high-Pi diet and 6% lanthanum carbonate (CKD-LaC group; 1.2% Pi, n = 10). Diets in the CKD groups contained 0.3% adenine. Data are expressed as means ± SE. One-way ANOVA followed by the Tukey-Kramer test was performed. Two-tailed P values of <0.05 were considered statistically significant. *P < 0.05 vs. the CNT group; #P < 0.05 vs. the CKD-EP group.

**Table 2. Serum and urinary biochemical parameters at week 8.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CNT Group</th>
<th>CKD-LP Group</th>
<th>CKD-MP Group</th>
<th>CKD-HP Group</th>
<th>CKD-EP Group</th>
<th>CKD-LaC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Ca²⁺, mmol/l</td>
<td>2.50 ± 0.10</td>
<td>3.37 ± 0.15†</td>
<td>3.19 ± 0.08†</td>
<td>2.77 ± 0.20†</td>
<td>1.80 ± 0.55*</td>
<td>3.19 ± 0.10†</td>
</tr>
<tr>
<td>Serum P, mmol/l</td>
<td>2.64 ± 0.10</td>
<td>1.94 ± 0.13†</td>
<td>3.07 ± 0.13†</td>
<td>4.65 ± 0.39†</td>
<td>9.11 ± 0.32*</td>
<td>2.62 ± 0.13†</td>
</tr>
<tr>
<td>Serum intact parathyroid hormone, pg/ml</td>
<td>41.6 ± 4.7</td>
<td>35.8 ± 4.5†</td>
<td>202.1 ± 58.4*</td>
<td>463.1 ± 110.5*</td>
<td>778.5 ± 116.6*</td>
<td>82.2 ± 14.2†</td>
</tr>
<tr>
<td>Serum FGF23, pg/ml</td>
<td>438 ± 33</td>
<td>5186 ± 799†</td>
<td>30,188 ± 4,360*</td>
<td>87,462 ± 23,662*</td>
<td>132,754 ± 32,513*</td>
<td>21,485 ± 7,404*</td>
</tr>
<tr>
<td>Serum calcitriol, pg/ml</td>
<td>95.4 ± 25.2</td>
<td>5.2 ± 1.4†</td>
<td>5.1 ± 0.8*</td>
<td>18.2 ± 4.0*</td>
<td>18.5 ± 5.2*</td>
<td>6.0 ± 1.9†</td>
</tr>
<tr>
<td>Urinary P, excretion, mg/day</td>
<td>0.6 ± 0.1</td>
<td>1.0 ± 0.4†</td>
<td>15.3 ± 0.8†</td>
<td>49.3 ± 3.2*</td>
<td>101.8 ± 4.2*</td>
<td>6.6 ± 1.0†</td>
</tr>
</tbody>
</table>

Data are means ± SE. Diets in the CKD groups contained 0.3% adenine. One-way ANOVA followed by the Tukey-Kramer test was performed. P values of <0.05 were considered statistically significant. *P < 0.05 vs. the CNT group; #P < 0.05 vs. the CKD-EP group.
rat Slc20a1 (Pit-1), RA011281; human GAPDH, HA607812; human albumin, HA118070; human fetuin-A, HA149248; human TNF-α, HA198263; human Klotho, HA148243; human NADPH oxidase 4 (Nox4), HA184575; and human bone morphogenetic protein-2, HA193805. The cycling condition was 30 s at 95°C followed by 40 cycles of 5 s at 95°C for denaturation and 40 s of annealing at 60°C. The specificity of the PCR products was confirmed by analysis of the melting curves and additionally by agarose gel electrophoresis. All measurements were performed in duplicate, and mRNA fold changes were calculated using the 2\(^{-\Delta\Delta C_{t}}\) method (where C\(_t\) is threshold cycle) using GADPH as an internal reference.

Statistical analysis. Statistical analyses were performed using JMP (version 10.0, SAS institute, Tokyo, Japan). Data are presented as means ± SE. Differences among groups were analyzed by an unpaired t-test for two groups and one-way ANOVA followed by a Tukey-Kramer test for more than three groups. Univariable and multivariable linear regression analyses were performed to determine correlations among parameters. For regression analysis, data from CKD-LP, CKD-MP, CKD-HP, and CKD-EP rats were used (n = 40). Serum Cr was used as the covariate in multivariable analysis. For all tests, two-tailed P values of <0.05 were considered statistically significant.

Table 3. Blood pressure levels at each week

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CNT Group</th>
<th>CKD-LP Group</th>
<th>CKD-MP Group</th>
<th>CKD-HP Group</th>
<th>CKD-EP Group</th>
<th>CKD-LaC Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>105 ± 3</td>
<td>109 ± 7</td>
<td>102 ± 3</td>
<td>111 ± 5</td>
<td>103 ± 4</td>
<td>110 ± 4</td>
</tr>
<tr>
<td>Week 4</td>
<td>103 ± 3</td>
<td>118 ± 4</td>
<td>110 ± 4</td>
<td>115 ± 4</td>
<td>106 ± 2</td>
<td>106 ± 3</td>
</tr>
<tr>
<td>Week 6</td>
<td>102 ± 7</td>
<td>103 ± 5</td>
<td>112 ± 4</td>
<td>110 ± 5</td>
<td>110 ± 7</td>
<td>112 ± 4</td>
</tr>
</tbody>
</table>

Data are means ± SE. Diets in the CKD groups contained 0.3% adenine.

![Graphs and images](http://ajprenal.physiology.org/)
RESULTS

Dietary Pi loading induces a short lifespan. To determine the effects of Pi loading and Pi binder use, rats were divided into the following six groups and given different amounts of Pi: CNT, CKD-LP, CKD-MP, CKD-HP, CKD-EP, and CKD-LaC. The content of each diet is shown in Table 1. Four rats in the CKD-EP group and two rats in the CKD-HP group died during the study period (8 wk). All rats in the other four groups survived. Log-rank tests revealed a significant difference in survival curves (Fig. 1A).

Dietary Pi loading induces aortic medial calcification. Figure 1B shows representative photomicrographs of the abdominal aorta stained using the von Kossa method. CKD-HP and CKD-EP rats developed moderate to severe degrees of aortic calcification, respectively; the other four groups did not. Aortic Ca\(^{2+}\) content increased in a dietary Pi loading-dependent fashion in CKD rats. Lanthanum carbonate lowered the Ca\(^{2+}\) content, which had increased with Pi loading (Fig. 1C).

Dietary Pi loading induces biochemical disorders in a concentration-dependent manner. Table 2 shows the findings of serum and urinary biochemical analyses after 8 wk. Dietary Pi loading increased the amount of urinary Pi excretion, serum levels of Pi, intact parathyroid hormone, and serum FGF23 in a dose-dependent manner. Lanthanum carbonate significantly attenuated these Pi-related changes. The serum Ca\(^{2+}\) level was dependent on the Ca\(^{2+}\)-to-Pi ratio in an inverse relationship with dietary Pi content. Serum levels of calcitriol in all CKD rats were significantly lower than those levels in CNT rats. Calcitriol levels in CKD rats were comparable among groups. Serum levels of fetuin-A were comparable among CKD rats (data not shown).

Dietary Pi loading does not influence systolic blood pressure level, kidney function, and urinary protein excretion. The effects of diets on systolic blood pressure, kidney histology, kidney function, and proteinuria were determined (Table 3 and Fig. 2). Systolic blood pressure levels were comparable among groups at each time point, ruling out the effects of different diets on blood pressure level and blood pressure-related vascular changes, including VC. Histological evaluation of the kidney by Masson trichrome staining revealed that tubulointerstitial fibrosis, cellular infiltration, and deposition of adenine crystals in renal tubules and the renal interstitium were present in all CKD groups, and Ca\(^{2+}\)-Pi deposition was observed only in CKD-HP and CKD-EP rats, reflecting the relatively high Pi burden on the kidneys in these two groups (Fig. 2A). However, the extent of tissue injury was almost comparable among the five CKD groups. In fact, serum Cr levels in all CKD rats were significantly higher than those levels in CNT rats, and Cr clearances in all CKD rats were significantly lower than in CNT rats, whereas there was no significant difference among the five CKD groups (Fig. 2, B and C). There was no statistical difference in urinary protein excretion among the six groups (Fig. 2D).

Fig. 3. Effects of dietary Pi loading on nutritional status and systemic inflammation and oxidative stress markers. A: body weight. B: serum albumin level. C: urinary Cr excretion. D: mean daily food intake. E: serum TNF-α level. F: urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG)-to-Cr ratio (8-OHdG/Cr). Mean daily food intake was calculated using the arithmetic mean of food intake at weeks 2, 4, 6, and 8. Data are expressed as means ± SE. One-way ANOVA followed by the Tukey-Kramer test was performed. Two-tailed P values of <0.05 were considered statistically significant. *P < 0.05 vs. the CNT group; #P < 0.05 vs. the CKD-EP group.
Dietary Pi loading induces malnutrition in a concentration-dependent manner. To determine the effects of dietary Pi loading on nutritional status, body weights and serum albumin levels were measured. Urinary Cr excretion was used as the surrogate marker for total muscle mass. Body weight, serum albumin, and urinary Cr excretion at week 8 decreased in a Pi concentration-dependent manner. Lanthanum carbonate significantly inhibited the increase (Fig. 3, A–C). Food intake in CKD-EP rats was significantly higher compared with CKD-LP, CKD-MP, and CKD-LaC rats (Fig. 3D), indicating that the decrease in body weight was not caused by reduced food intake.

Dietary Pi loading promotes systemic inflammation and oxidative stress. To determine the effects of Pi loading on systemic inflammation and oxidative stress, serum TNF-α and urinary 8-OHdG/Cr levels were measured. Dietary Pi loading increased serum TNF-α levels in CKD rats dose dependently. Lanthanum carbonate significantly inhibited the increase (Fig. 3E). Similarly, dietary Pi loading dose dependently increased urinary 8-OHdG/Cr levels in CKD rats, which was ameliorated by lanthanum carbonate (Fig. 3F).

The level of serum Pi is strongly correlated with the extent of MIA syndrome. To determine the association between derangement in Pi metabolism and MIA components, simple linear regression analysis was performed. The serum Pi level was significantly correlated with each MIA component, including body weight, serum albumin, aortic Ca\(^{2+}\) content, and 8-OHdG/Cr (Fig. 5, A–D).

Multivariable analysis showed that the serum TNF-α level was significantly correlated with body weight, serum albumin level, urinary 8-OHdG level, and aortic Ca\(^{2+}\) content even after adjusting for Cr clearance, indicating that these associations were independent of kidney function [body weight (r = −0.353, \(P < 0.05\)), serum albumin (r = −0.523, \(P < 0.05\)), aortic Ca\(^{2+}\) content (r = 0.405, \(P < 0.05\)), and urinary 8-OHdG/Cr (r = 0.663, \(P < 0.05\)).

Pi loading increases oxidative stress and inflammation in the kidney, heart, and aorta. To determine whether Pi loading induces local inflammation, relative mRNA levels of TNF-α in the aorta, heart, and kidney in the CNT, CKD-LP, CKD-EP, and CKD-LaC groups were examined. mRNA levels of TNF-α in the aorta, heart, and kidney of CKD-EP rats were significantly higher than those levels in CNT rats. The increases were significantly attenuated with low dietary Pi loading and lanthanum treatment (Fig. 6, A–C).

Next, to determine local oxidative stress levels, immunohistochemistry for 8-OHdG in the aorta was performed (Fig. 6D). The 8-OHdG-positive number in CKD-EP rats was significantly increased compared with CNT rats. The positive numbers in CKD-LP and CKD-LaC rats were lower than CKD-EP rats, a finding that was confirmed by semiquantitative analysis (Fig. 6E). Immunohistochemistry of the aorta for Ser\(^{276}\) phosphorylated p65 was also performed. Strong staining of phosphorylated p65 was observed only in the calcified area of CKD-EP rats (Fig. 6F). The positive areas in CKD-LP and CKD-LaC rats were lower than CKD-EP rats, a finding that was confirmed by semiquantitative analysis (Fig. 6G). These

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Fig. 4. Correlations between serum Pi, and components of malnutrition-inflammation-atherosclerosis (MIA) syndrome. A–F: correlation of serum Pi, with body weight (A), serum albumin (B), urinary Cr excretion (C), aortic Ca\(^{2+}\) content (D), urinary 8-OHdG/Cr (E), and serum TNF-α (F). The correlation coefficient \((R^2)\) was determined using Pearson’s method. Two-tailed \(P\) values of <0.05 were considered statistically significant.

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results indicate that both inflammation and oxidative stress are activated in the calcified area of the aorta.

To determine the effect of Pi loading on the expression of Pit-1, the relative mRNA expression of Pit-1 in the aorta was examined. mRNA levels of Pit-1 in the aorta of CKD-EP rats were significantly higher than those levels in CNT rats. Additionally, the increase was significantly attenuated by low dietary Pi loading and lanthanum treatment (Fig. 6H).

Pi loading and uremia inhibit albumin and fetuin production in the rat liver. To determine the effects of Pi loading on negative acute-phase protein, mRNA levels of albumin and fetuin-A in the rat liver were determined. mRNA expression levels of albumin and fetuin-A significantly decreased in CKD-LP, CKD-EP, and CKD-LaC rats. The lowest level was observed in CKD-EP rats (Fig. 7, A and B). However, the difference in albumin and fetuin-A mRNA levels induced by Pi loading was relatively small compared with the effect of CKD.

Pi loading directly induces local inflammation in cultured HUASMCs but does not reduce negative acute-phase protein synthesis in HepG2 cells. To determine the direct effects of Pi loading on local inflammation, oxidative stress, and protein synthesis, an in vitro experiment using cultured HUASMCs and human HepG2 cells (a frequently used human liver hepatocytoma cell line for examining hepatocyte biology) was performed. High-Pi medium (2.9 mM) directly increased mRNA expression of TNF-α and Nox4 and decreased expression of Klotho in HUASMCs on day 1 compared with normal-Pi medium (0.9 mM; Fig. 8, A–C). Bone morphogenetic protein-2 (a bone-related marker) did not increase at this time point, indicating that inflammation and oxidative stress precede the transdifferentiation of HUASMCs into osteoblast-like cells (Fig. 8D). High-Pi medium (2.9 mM) did not influence the synthesis of either albumin or fetuin-A in HepG2 cells compared with normal-Pi medium (0.9 mM) for 1 day (Fig. 8, E and F).

**DISCUSSION**

MIA syndrome is a prominent feature in CKD patients and contributes to increased mortality (41, 42, 48). Growing evidence has revealed the harmful effects of Pi overload on the cardiovascular system (4, 15, 23, 39, 43). However, the effects of Pi overload on the interactions among inflammation, malnutrition, and VC have not been investigated. To the best of our knowledge, the present study is the first to report that dietary Pi loading dose dependently induces inflammation and malnutrition as well as VC and premature death in uremic rats without affecting kidney function. Furthermore, use of a Pi binder and dietary Pi restriction almost reversed all of the Pi overload-related changes described above. These findings suggest that MIA syndrome is partially mediated by Pi overload in CKD and that appropriate management of Pi through dietary or pharmacological intervention or anti-inflammatory therapy may be a promising therapeutic strategy for the prevention of MIA syndrome in CKD.

Chronic inflammation is a common feature and a major cause of cardiovascular and other complications of CKD (50). Clinical studies have reported that serum Pi, Ca\(^{2+}\)-Pi product, and FGF23 correlate well with serum inflammatory markers in CKD patients (22, 28, 30). In the present study, Pi loading dose dependently induced inflammation in the aorta, heart, and kidneys and increased serum TNF-α levels in uremic rats. Linear regression analysis showed that there was a close association between Pi overload and serum TNF-α levels. Furthermore, in the present in vitro study, Pi overload directly increased the expression of TNF-α in VSMCs. Zhao et al. (53)
reported that Pi loading induces the generation of mitochondrial ROS, leading to the activation of NF-κB signaling and VC in vivo and in vitro, an effect ameliorated by antioxidative drug treatment. In addition, TNF-α, which is induced by activation of the NF-κB pathway, in turn activates the NF-κB pathway (27). Collectively, these results strongly suggest that Pi loading directly induces local inflammation in uremia.

Pi inflow into the intracellular space by Pit-1 is one of the possible mechanisms of Pi-induced cellular inflammation. Recently, Voelkl et al. (47) reported that the expression of TNF-α was increased in the various tissues of Klotho knockout mice and that Pit-1 downregulation reversed the upregulation of TNF-α and calcification in VSMCs. Pi enters into cells via Pit-1, a Na⁺-Pi co-transporter that is ubiquitously expressed in the cell membrane. This suggests that Pit-1 is critical for triggering Pi-induced local inflammation and the resulting VC in Klotho knockout mice. In the present study, cultured VSMCs exposed to Pi overload directly increased their mRNA expression of TNF-α and Nox4 (one of the NADPH oxidase family members), which occurred in parallel with decreases in Klotho mRNA, in advance of the increase in bone morphogenetic protein-2 (a marker of osteoblastic transdifferentiation). Furthermore, Pit-1 expression increased in aortas of CKD-EP rats. Collectively, the results of the present study suggest that Pi overload directly induces local inflammation and oxidative stress in a Pit-1-dependent manner and subsequently causes the adverse outcomes, including VC, in CKD.

Experimental studies have reported that oxidative stress and inflammation promote VC in CKD (10, 49, 53). An animal study (52) reported that inhibition of the NF-κB pathway ameliorated VC. Because both oxidative stress and inflammatory responses share the NF-κB pathway, and because phosphorylation of p65 indicates the activation of NF-κB signaling, the activation of NF-κB pathway (27). Collectively, these results strongly suggest that Pi loading directly induces local inflammation in uremia.

Fig. 6. Effects of Pi loading on local inflammation and oxidative stress levels. A–C: relative mRNA levels of tissue TNF-α in the rat aorta (A), heart (B), and kidney (C). D: representative microphotographs of immunohistochemistry for 8-OHdG in the aorta. Original magnification: ×200. E: quantification of the positively stained area for 8-OHdG in the aorta. F: representative microphotographs of immunohistochemistry for phosphorylated p65 (Ser276, RelA) of the aorta. Original magnification: ×200. G: quantification of the positively stained area for phosphorylated p65 in the aorta. H: relative mRNA level of Pi transporter (Pit)-1 in the aorta. mRNA expression was corrected to the level of GAPDH. AU, arbitrary units. Data are expressed as means ± SE. One-way ANOVA followed by the Tukey-Kramer test was performed. Two-tailed P values of <0.05 were considered statistically significant. *P < 0.05 vs. the CNT group; #P < 0.05 vs. the CKD-EP group.
the results of the present study are consistent with the hypothesis that aortic medial calcification is mediated by oxidative stress and inflammation in CKD (1, 52). Given the tight link between Pi loading and oxidative stress, the cross-talk between ROS and the NF-κB signaling pathway, and the important role of the NF-κB pathway in the pathogenesis of inflammation, inhibition of NF-κB signaling by antioxidant treatment has the potential to prevent the progression of Pi-induced MIA syndrome.

Another potential mechanism of Pi-induced inflammation other than the Pit-1 pathway should be considered because Pi entry through Pit-1 has been reported to be already saturated at physiological Pi concentrations (46), although upregulation of Pit-1 can increase the total Pi inflow into the intracellular space under high-Pi conditions. The fetuin-mineral complex (also called the calciprotein particle, matrix vesicle, or basic calcium phosphate crystal) is emerging as a substance that is thought to induce cardiovascular diseases (11, 14, 40). This is because the fetuin-mineral complex is formed extracellularly under high-Pi conditions, and the complex is incorporated into VSMCs via endocytosis and induces various types of responses that can promote VC, for example, apoptosis of VSMCs and a Ca$^{2+}$ burst (34). Interestingly, VC was prevented by chelating the extracellular fetuin-mineral complex in an in vitro study of VSMCs exposed to high Pi. Furthermore, recent experimental studies have reported that the fetuin-mineral complex directly induces macrophages/monocytes to release an array of cytokines, including TNF-α (29, 32). Given that high Pi loading increases the levels of the fetuin-mineral complex in serum and tissue in an adenine-fed rat model (24), the fetuin-mineral complex may be a direct cause of local and systemic inflammation and malnutrition in uremic rats with Pi overload. Further studies are needed to clarify the precise pathogenesis of Pi-related inflammation and the progression of VC.

The mechanism of Pi overload-induced malnutrition is another important subject. In the present study, malnutrition may be partly explained by chronic inflammation and oxidative stress (7, 21). Proinflammatory cytokines (TNF-α, IL-1, and IL-6) are known to induce oxidative stress, decrease hepatic albumin synthesis, cause systemic catabolism (including muscle degradation), and increase energy expenditure, leading to a decrease in body weight and muscle atrophy (9, 33). Oxidative stress also produces inflammatory cytokines in the liver and muscle (8). Collectively, these results explain our present observation that Pi overload decreased serum albumin level, body weight, and muscle mass by Pi overload-related chronic inflammation. Furthermore, as albumin also acts as a very important serum antioxidant by exerting glutathion-linked thiol peroxidase activity (5), the reduction in serum albumin facili-

Fig. 8. Direct effects of Pi loading on the phenotype of human umbilical arterial smooth muscle cells (HUASMCs) and HepG2 cells. A–F: mRNA expression of α-Klotho (A), TNF-α (B), NADPH oxidase 4 (Nox4; C), and bone morphogenetic protein (BMP)-2 (D) in HUASMCs and albumin (E) and fetuin-A (F) in HepG2 cells. HUASMCs and HepG2 cells were exposed to culture media containing 0.9 or 2.9 mM Pi for 1 day. mRNA expression was corrected to the level of GAPDH. Data are expressed as means ± SE. An unpaired t-test was performed. Two-tailed P values of <0.05 were considered statistically significant. *P < 0.05 vs. the CNT group; #P < 0.05 vs. the CKD-EP group.

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tates oxidative stress and inflammation, leading to the vicious cycle between malnutrition and inflammation.

The P1-induced decrease in the serum albumin level is another interesting issue. In the present study, both decreased kidney function and P1 loading might be equally involved in decreased albumin synthesis. With regard to decreased albumin synthesis, P1-induced systemic inflammation might be possible, because P1 loading did not directly decrease albumin synthesis in HepG2 cells. Because the decrease in the serum albumin level was not proportional to the decrease in albumin synthesis in the rat liver, albumin degradation might be involved in the reduction in the serum albumin level; the serum albumin level is determined by the balance between albumin synthesis and albumin degradation. Experimental studies have shown that inflammation promotes albumin degradation through the neonatal Fc receptor pathway (6, 20, 45). These results indicate that P1-induced inflammation could have also accelerated albumin degradation in the present in vivo study. Collectively, the P1-induced reduction in the serum albumin level can be explained by both decreased albumin synthesis and enhanced albumin degradation. However, further studies are required to identify the precise mechanisms for how P1 overload induces hypoalbuminemia.

The effects of dietary P1 restriction and P1 binder use on inflammation and malnutrition were compared. Both dietary P1 restriction and P1 binder use equally decreased the levels of TNF-α and urinary 8-OHdG/Cr, ameliorated VC, and produced a survival advantage. These results indicate that the amount of P1 absorbed from the gut primarily determines the degree of MIA syndrome. In contrast, dietary P1 restriction is frequently accompanied by protein restriction (18), and clinical studies have suggested the potential harm of P1 restriction (36). However, no detrimental effects were observed in CKD-LP rats in the present study because P1 and protein restriction were successfully separated by providing synthetic diets. Given that P1 restriction inevitably induces protein restriction in clinical medicine, P1 binder use may be a more practical way of preventing P1-related MIA syndrome in CKD patients.

There are several limitations in the present study. First, urinary P1 excretion in CNT rats was extremely decreased compared with the CKD-EP rats, although the P1 concentrations in these two groups were identical. This is because the food for CNT rats contained grain-based protein and did not contain 20% lactose; these differences in the diet directly affected the absorption rate of P1 from the intestine. Second, the level of food intake in each group was slightly different. However, the amount of food intake in rats fed the extremely high-P1 diet was greater than that observed in rats fed the low-P1 diet, indicating that malnutrition was not induced by decreased food intake but rather by P1 overload in the setting of CKD. Third, we observed an association among P1 loading, inflammation, and malnutrition but did not examine the putative cause-effect relationship by directly intervening in the inflammatory process related to P1 loading. We did not determine the precise mechanism for how P1 induces inflammation via Na+·P1 cotransporters and/or via the formation and endocytosis of the P1-related fetuin-mineral complex. However, taking into account these limitations, we believe that P1 overload induces inflammation and malnutrition as well as VC in subjects with CKD and that the present study sheds light on another important harmful effect of P1 overload on CKD status.

In conclusion, this study demonstrated that dietary P1 overload directly induces chronic inflammation and malnutrition as well as VC in CKD rats. These data suggest that chronic inflammation induced by P1 overload plays a pivotal role in the pathogenesis of MIA syndrome in CKD and that inhibition of P1 loading through dietary or pharmacological intervention or anti-inflammatory therapy may be a promising treatment for the prevention of MIA syndrome.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


