Fetuin-A decrease induced by a low-protein diet enhances vascular calcification in uremic rats with hyperphosphatemia

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Calcification in uremic rats with hyperphosphatemia

Fetuin-A decrease induced by a low-protein diet enhances vascular calcification in uremic rats with hyperphosphatemia. Am J Physiol Renal Physiol 309: F744–F754, 2015. First published July 15, 2015; doi:10.1152/ajprenal.00017.2015.—Although dietary phosphate restriction is important for preventing hyperphosphatemia in patients with chronic kidney disease, it remains unclear whether a low-protein diet (LPD), which contains low phosphate, has beneficial effects on malnutrition, inflammation, and vascular calcification. The effects of LPD on inflammation, malnutrition, and vascular calcification were therefore assessed in rats. Rats were fed a normal diet or diets containing 0.3% adenine and low/norma protein and low/high phosphate. After 6 wk, serum and urinary biochemical parameters, systemic inflammation, and vascular calcification were examined. The protective effect of fetuin-A and albumin were assessed in cultured vascular smooth muscle cells. Rats fed the diet containing 0.3% adenine developed severe azotemia. LPD in rats fed high phosphate induced malnutrition (decreases in body weight, food intake, serum albumin and fetuin-A levels, and urinary creatinine excretion) and systemic inflammation (increases in serum tumor necrosis factor-α and urinary oxidative stress marker). LPD decreased the serum fetuin-A level and fetuin-A synthesis in the liver and increased serum calcium-phosphate precipitates. A high-phosphate diet increased aortic calcium content, which was enhanced by LPD. Reduced fetal calf serum in the medium of cultured vascular smooth muscle cells enhanced phosphate-induced formation of calcium-phosphate precipitates in the media and calcification of vascular smooth muscle cells, both of which were prevented by fetuin-A administration. Our results suggest that phosphate restriction by restricting dietary protein promotes vascular calcification by lowering the systemic fetuin-A level and increasing serum calcium-phosphate precipitates and induces inflammation and malnutrition in uremic rats fed a high-phosphate diet.

Fetuin-A; inflammation; low-protein diet; malnutrition; vascular calcification

Hyperphosphatemia is a serious complication that can lead to high cardiovascular morbidity and mortality rates in patients with chronic kidney disease (CKD) (3, 4). Phosphate (P(i)) overload in CKD, which usually presents as hyperphosphatemia, has been found to induce vascular calcification (VC) and endothelial dysfunction, leading to cardiomegaly and heart failure (2, 39, 42, 48). Among several treatment modalities available to prevent P(i) overload, dietary P(i) restriction and administration of a P(i) binder, are the two major approaches (7, 29). Clinical studies have shown that controlling serum P(i) level within the target range improves all-cause mortality in dialysis patients (15). However, it still remains unclear whether these two treatment modalities are clinically identical or have different effects on CKD-related outcomes, including inflammation, malnutrition, and VC (10, 25).

The most important difference between the two approaches is the amount of protein intake. Dietary P(i) restriction is usually achieved by restricting protein intake but may result in malnutrition (18), which is closely associated with inflammation and atherosclerosis/calcification, a condition known as malnutrition inflammation atherosclerosis (MIA) syndrome (17, 41, 45). Thus avoiding malnutrition is critical in managing patients with CKD (7). Indeed, CKD patients on a very low-protein diet were found to have a higher mortality rate (26), indicating the potential harmfulness of strict low dietary protein intake. Furthermore, an observational study showed that hemodialysis patients with lower protein intake were at increased risk of mortality compared with patients with higher protein intake (41). Thus it may be more desirable to control P(i) balance using a P(i) binder than by restricting dietary P(i). To date, however, it remains undetermined whether a low-protein diet actually causes MIA in patients with CKD. Moreover, even if dietary protein restriction causes MIA, its underlying mechanism remains unclear.

Calciprotein particles are recently identified microparticles and are a complex of various minerals and proteins, including calcium (Ca), P(i), albumin, and fetuin-A (16). Studies have shown that calciprotein particles play critical roles in the pathogenesis of P(i)-related inflammation and vascular calcification (28, 31, 36, 43). Because fetuin-A is a negative acute-phase protein produced in the liver and decreases in malnutrition (8, 22, 49), it is probable that the fetuin-A decrease related to malnutrition is involved in the pathogenesis of vascular calcification in uremic rats on a low-protein diet.

This study was designed to determine whether a low-protein diet has harmful effects on CKD-related complications, especially on inflammation, nutritional status, and VC, using a rat model of adenine-induced uremia. Groups of rats were fed synthetic diets containing different P(i) and protein concentrations, and the effects of dietary protein concentration on inflammation, malnutrition, and VC were evaluated. We also evaluated the involvement of calciprotein particles with the VC enhancement of a low-protein diet. Because no ideal method has yet been established to quantify the amount of calciprotein particles, we estimated calciprotein particles by measuring Ca and P(i) precipitates in
the pellet of serum and cultured media after supercentrifugation (32, 43).

METHODS

Cell cultures. Vascular smooth muscle cells (VSMCs) from umbilical cord arteries (Toyobo, Osaka, Japan) were grown in a humidified 5% CO2 incubator at 37°C in DMEM containing 4.5 g/l glucose supplemented with 10% fetal calf serum (FCS), 10 mM sodium pyruvate, and 1% penicillin-streptomycin. Cells at passages 6–8 were used in all experiments, with media changed every 2 days. Cells were cultured on 12- or 24-well plates and were grown to confluence. After reaching confluence, the VSMCs were cultured in DMEM containing 0.9 or 2.9 mM Pi, and in 2.5, 5, or 10% FCS. To assess the effects of fetuin-A and albumin on calcification, 10 or 50 μM fetuin-A from fetal bovine serum (F3385-1G, Sigma-Aldrich Japan, Tokyo, Japan) and 50 or 250 μM albumin from fetal bovine serum (015–23871, Wako, Tokyo, Japan) were added. After 2 days in culture, total RNA was extracted from VSMCs using TRI Reagent (TR118, MRC) and used for real-time PCR determination of relative mRNA expression. On day 5, the content of Ca corrected by protein was measured. Calcification was visualized by Alizarin red staining using a calcified nodule staining kit (AK21, PMC, Tokyo, Japan) and digital micrographs captured by inverted microscopy (CKX31, Olympus, Tokyo, Japan). Medium (500 μl) from each well on day 3 were collected and centrifuged at 16,000 g for 2 h. The resultant pellets were dissolved in 300 μl of 6N hydrochloric acid, and their Ca and P content were analyzed to quantify Ca-Pi precipitates as described, with some modifications (32, 36). We regarded these Ca-Pi precipitates as a surrogate of calcification, 10 or 50 μM fetuin-A from fetal bovine serum (F3385-1G, Sigma-Aldrich Japan, Tokyo, Japan) and 50 or 250 μM albumin from fetal bovine serum (015–23871, Wako, Tokyo, Japan) were added.

Serum levels of Ca-Pi precipitates were measured by centrifuging 200 μl of rat serum at 16,000 g for 2 h. The pellets obtained after centrifugation of serum were treated with 100 μl of 6N hydrochloric acid, and the Ca and P content of the supernatant was measured as Ca-Pi precipitates in the serum (32, 36).

Examination of arterial medial calcification. Four-micrometer sections from paraffin-embedded aortas were deparaffinized and processed for von Kossa staining using the standard method. To quantitatively evaluate the degree of aortic medial calcification, frozen aortic tissue was weighed and hydrolyzed in 1 ml of 6N hydrochloric acid for 24 h. The Ca and P content of the supernatant were determined using commercially available Calcium E-test and Phospha C-test kits (Wako, Osaka, Japan) and normalized to wet tissue weight (μg/mg wet weight).

Real-time PCR. Real-time PCR was performed as previously described (47). Total RNA was extracted from frozen rat tissue stored in liquid nitrogen and from cultured VSMCs using TRIzol reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Complementary DNA was synthesized 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 (Life Technologies, Applied Biosystems), and primers for rat GAPDH (RA015380), runt-related gene 2 (RA045439), Pit-1 (RA011281), rat TNF-α (RA043092), albumin (RA065413), fetuin-A (RA065433), and IL-1β (RA063423), all from Takara Bio. The amplification protocol consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 40 s. The specificity of the PCR products was confirmed by analysis of the melting curves and by agarose gel electrophoresis. All measurements were performed in duplicate. Fold-changes in mRNA were calculated using the 2−ΔΔCt method using GAPDH as an internal reference.

Statistical analyses. Statistical analyses were performed using JMP version 10.0 (SAS Institute, Tokyo, Japan). Data are presented as means ± SE. Differences between two groups were analyzed by unpaired t-tests and differences among more than two groups by one-way ANOVA, followed by the Tukey-Kramer test. Linear regression analyses were performed to determine the correlation between parameters. For all tests, a two-tailed P < 0.05 was considered statistically significant.
RESULTS

A low-protein diet did not shorten lifespan or affect blood pressure level or progression of CKD. To separately determine the effect of protein and Pi content on inflammation, malnutrition, and VC in CKD, rats were divided into five groups and fed various diets for 6 wk. The study protocol and content of each diet are shown in Fig. 1 and Table 1. No rats died during the 6-wk observational period, and the systolic blood pressure levels in the five groups were similar (data not shown).

Figure 2A shows representative microphotographs of Masson trichrome-stained kidneys in each group. The four uremic groups showed severe tubular atrophy and dilatation and interstitial fibrosis with cellular infiltration. Serum Cr levels were significantly higher (Fig. 2B), and Cr clearances significantly lower (Fig. 2C), in all four uremic groups than in control rats. However, there were no significant differences in Cr clearance levels among the four uremic groups. Furthermore, there were no significant differences among the five groups in proteinuria (Fig. 2D).

A low-protein diet did not affect Pi homeostasis. A high-Pi diet significantly increased fractional excretion of Pi, urinary Pi excretion, and serum levels of Pi, and fibroblast growth factor 23 in the U-HPi-LPr and U-HPi-NPr groups (Fig. 3, A–D). There were no significant differences in fractional excretion of Pi, urinary Pi excretion, and serum levels of Pi, and fibroblast growth factor 23 between the U-LPi-LPr and U-LPi-NPr groups. Serum calcitriol concentrations were significantly lower in the four uremic groups than in control rats, but were similar among the four uremic groups (Fig. 3E).

A low-protein diet induced malnutrition independently of dietary Pi content. To determine the effects of a high-Pi and low-protein diet on nutritional status, body weights and serum albumin levels were measured. Urinary Cr excretion was also measured as a surrogate marker for total muscle mass. Body weight, urinary Cr excretion level, and serum albumin levels were lower after 6 wk in uremic rats fed low-protein diets (U-LPi-LPr and U-HPi-LPr) than in uremic rats fed normal-protein diets (U-LPi-NPr and U-HPi-NPr) (Fig. 4, A–C). The amounts of food and water intake at week 6 are shown in Table 2. Food intake was lower in the four uremic groups than in the control group. A low-protein diet further significantly decreased food intake in the U-LPi-LPr and U-HPi-LPr groups. The expression of albumin in the liver was decreased in the uremic groups, which was accentuated in the rats fed the low-protein diet (U-LPi-LPr and U-HPi-LPr) (Fig. 4E). The serum fetuin-A level and the expression of fetuin-A in the liver were both decreased in the four uremic groups, decreases exacerbated by the low-protein diet (Fig. 4, D and F).

Low-protein diets promoted systemic inflammation and oxidative stress. To determine the effects of a low-protein diet on systemic inflammation and oxidative stress, serum TNF-α and urinary 8-OHdG/Cr levels were measured. Low-protein diets significantly increased serum TNF-α (Fig. 5A) and the urinary 8-OHdG/Cr ratio (Fig. 5B) in rats fed high dietary Pi content. Among the four uremic groups, the U-LPi-NPr group showed the lowest levels of serum TNF-α (Fig. 5A) and urinary 8-OHdG/Cr (Fig. 5B).

A low-protein diet enhanced Pi-induced VC in uremic rats. Figure 6A shows representative photomicrographs of calcification of the abdominal aorta determined by the von Kossa method. Aortic calcification was determined by measuring Ca and Pi contents in the abdominal aorta (Fig. 6, B and C). Ca and Pi content in the aorta were similar in the control and U-LPi-NPr groups. Both U-HPi-LPr rats and U-HPi-NPr rats developed extensive arterial medial calcification. Notably, Ca content was higher in U-HPi-LPr than in U-HPi-NPr rats. However, the low-protein diet plus low Pi did not induce VC, indicating that a low-protein diet alone did not induce VC in uremic rats. The aortic levels of expressions of mRNAs encoding runt-related gene 2, TNF-α, and IL-1β were higher in the U-HPi-LPr than in the other four groups (Fig. 6, D–F).

To determine calcification propensity, we measured serum levels of Ca-Pi precipitates in the five groups. Serum levels of Ca-Pi precipitates were higher in rats fed a high-Pi diet than in control rats (Fig. 6, G and H). Of note, the serum level of Ca-Pi precipitates was higher in the U-HPi-LPr than in the U-HPi-NPr group, with the serum level of Ca-Pi precipitates significantly correlated with Ca content in the aorta (Fig. 6I).

Table 1. Content of each diet

<table>
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<th>CNT</th>
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<th>U-LPi-NPr</th>
<th>U-HPi-LPr</th>
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To induce renal failure, 0.3% adenine was fed to rats in the four uremic groups. Casein-based protein was the source of protein in all diets. CNT; control, U, uremic; HPi, high Pi; LPi, low P; LPr, low protein; NPr, normal protein; Ca, calcium; P, phosphate.

Fig. 1. Study protocol for animal study. The diets are described in detail in Table 1. Diets in the uremic groups contained 0.3% adenine to induce renal failure.

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Fetuin-A prevented low FCS-mediated enhancement of calcification in VSMCs treated with high Pi medium. To clarify the mechanism by which a low-protein diet enhances VC, human umbilical VSMCs were treated with different FCS concentrations. To mimic clinical conditions in patients with malnutrition, VSMCs were exposed to low FCS. Figure 7A shows representative photographs of Alizarin red-stained cells. Ca content of VSMCs was increased by Pi loading, which was dose dependently enhanced by reducing FCS concentration in the medium (Fig. 7B). Ca-Pi precipitates in the media were increased by reducing FCS concentration in the medium (Fig. 7, C and D). The ability of fetuin-A and albumin to protect against calcification was analyzed by adding different concentrations of fetuin-A and albumin to the medium. Both fetuin-A and albumin decreased Ca-Pi precipitates dose dependently (Fig. 7, G and H). However, only fetuin-A dose dependently inhibited Ca precipitation in VSMCs treated with high Pi and low FCS (Fig. 7, E and F).

**DISCUSSION**

The present study clearly demonstrated that a low-protein diet enhanced malnutrition and systemic inflammation in adenine-induced uremic rats with hyperphosphatemia. Moreover, the combination of a low-protein and high-Pi diet increased Ca content in the aorta, reduced serum fetuin-A concentration, and increased serum levels of Ca-Pi precipitates. Furthermore, our in vitro study showed that low FCS in the media enhanced Pi-induced calcification of VSMCs and Ca-Pi precipitate formation, which was inhibited by fetuin-A administration. These results suggest that a low-protein diet promotes MIA syndrome in CKD accompanied by hyperphosphatemia, and enhances VC by decreasing systemic fetuin-A concentration and enhancing systemic Ca-Pi precipitate formation.

Previous studies have assessed the intriguing mechanism by which a low-protein diet promotes VC in uremic rats (33, 40). Price et al. (33) reported that a low-protein diet enhances Pi-induced VC by increasing bone resorption and serum factors that initiate ectopic mineralization. Deficiency in l-lysine, induced by a low-protein diet, was found to promote VC, because l-lysine inhibited the apoptosis of VSMCs and the precipitation of Ca-Pi crystals (40). We hypothesize that the systemic decrease in fetuin-A induced by a low-protein diet increases calciprotein particles, alters the procalcific properties of calciprotein particles, and promotes VC.

Fetuin-A plays important roles in preventing VSMC-related calcification processes (5, 34, 36). For example, fetuin-A prevents the apoptosis of VSMCs and decreases apoptotic bodies, which become a core of calcification. Neighboring intact VSMCs, influenced by fetuin-A, promote the clearance of apoptotic bodies released by VSMCs. Furthermore, fetuin-A prevents intracellular mineralization of matrix vesicles of VSMCs. More recently, fetuin-A was found to decrease exosomes from VSMCs (19). Because exosomes are closely related to the release of procalcific matrix vesicles by VSMCs, a decrease in fetuin-A increases the number of matrix vesicles in the extracellular space, leading to an increase in extracellular Ca-Pi precipitates. These combined activities of local fetuin-A suggest the mechanisms by which local fetuin-A deficiency increased Ca-Pi precipitates in our in vitro study.

Clinical and animal studies have shown that low serum fetuin-A and high serum levels of calciprotein particles are closely associated with the extent of VC, making them useful markers of VC stress in patients with CKD (2, 11, 27, 35). Calciprotein particles are an important inducer of VC and are composed of various minerals and proteins, includ-
ing Ca, Pi, magnesium, albumin, and fetuin-A (13, 14, 16), with the latter inhibiting calcification (16, 38). Experimental studies have shown that Ca-Pi crystals without fetuin-A exhibit strong cellular toxicity in VSMCs and other type of cells (14, 43). Abundant fetuin-A is also shown to mitigate the toxic and procalcific characteristic of calciprotein particles (6). Furthermore, fetuin-A is closely involved in the macrophage-dependent clearance of calciprotein particles from the circulation (14). These results collectively suggest that a decrease in systemic fetuin-A increases systemic calciprotein particles and influences the procalcific properties of calciprotein particles, enhancing VC in CKD.

Calciprotein particles, regarded as a potential cause of the harmful effects of Pi overload (21), were found not only to induce VC but also to directly provoke inflammation in immune cells and VSMCs (28, 31, 36, 43). We have also shown that a high-Pi diet directly induces inflammation in the aorta, heart, and kidney of uremic rats, although we did not directly show the involvement of calciprotein particles in Pi-induced inflammatory responses (47). In addition, the present study showed that a low-protein diet promoted systemic inflammation in uremic rats with Pi overload. Because inflammation itself inhibits fetuin-A synthesis in the liver (8), these results collectively suggest that a low-protein diet reduces the systemic fetuin-A level and that this decrease in fetuin-A promotes calciprotein particle formation and resultant inflammation. Furthermore, inflammatory cytokines have been shown to directly promote the transdifferentiation of VSMCs into osteoblast-like cells, to mediate the apoptosis of VSMCs, and to suppress the synthesis of fetuin-A in the liver (8, 44, 46), thereby resulting in a vicious cycle of malnutrition, inflammation, and atherosclerosis/calcification syndrome (17, 41, 45, 49). Reductions in fetuin-A and the resultant formation of toxic calciprotein particles likely occur upstream of MIA syndrome in uremic rats. These findings suggest that toxic calciprotein particles, whose formation is influenced by a low-protein diet, could be a potential therapeutic target in the management of MIA syndrome in patients with uremia.

Both albumin and fetuin-A, which are produced in the liver, are important serum proteins with multiple functions (5, 6, 12). These proteins decrease under conditions of inflammation and malnutrition. We observed inverse relationships between the extent of VC and the serum concentrations of albumin and fetuin-A. Because Ca-Pi crystals are entrapped and stabilized by these two proteins in blood and tissues (13, 16), the decreases in these two proteins may have been the causes of VC and may have been enhanced by the low-protein diet. Interestingly, only fetuin-A prevented calcification, although both albumin and fetuin-A prevented death of VSMCs induced by Ca-Pi crystals (6). In addition, fetuin-A had an unknown but
more potent effect in stabilizing secondary calciprotein particles (6). These results indicate that the features of calciprotein particles, not the amount, is of primary importance. However, calciprotein particles, measured as described, may not directly reflect calcification propensity, namely, the inability to separate primary and secondary calciprotein particles (1, 16). Because secondary, not primary, calciprotein particles are the direct cause of the calciprotein particle-related cascade (1), the present method of calciprotein particle quantification may be unsuitable in differentiating between these two forms. Transmission electron microscopy may be a more direct method of evaluating calciprotein particles. Hence, further studies are needed to determine the differential roles of fetuin-A and albumin in the prevention of VC and in the development of calciprotein particles in CKD.

Malnutrition, recently integrated into MIA syndrome, is an important complication closely associated with increased morbidity and mortality in CKD patients (10, 17, 45). Our study showed that a low-protein diet enhanced the decreases in body weight, serum albumin level, and muscle mass induced by uremia. Several mechanisms are possible. First, a low-protein diet reduced food intake, leading to low calorie intake. Actually, a low-protein diet affects taste sensitivity in rats (30). Second, a low-protein diet directly induced inflammation and oxidative stress, which in turn promoted protein degradation in various organs and potentiate VC (22, 23, 44, 46). In clinical settings, a deficiency in protein, typical of patients with kwashiorkor, has been found to induce inflammation and oxidative stress (9, 37). Third, under conditions of PI loading, low protein may promote inflammation by increasing calciprotein particles (28, 31, 36, 43). Taken together, these results suggest that a

Table 2. Daily food and water intake and urine volume at week 6

<table>
<thead>
<tr>
<th></th>
<th>CNT</th>
<th>U-LPi-LPr</th>
<th>U-LPi-NPr</th>
<th>U-HPi-LPr</th>
<th>U-HPi-NPr</th>
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<tbody>
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<td>Food intake, g/day</td>
<td>21.5 ± 1.8</td>
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<td>Urine volume, ml/day</td>
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<td>57.8 ± 4.8</td>
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Values are means ± SE and compared using 1-way ANOVA followed by the Tukey-Kramer test. Uremic rats were fed diets containing 0.3% adenine to induce renal failure. A 2-tailed \( P \) value < 0.05 was considered statistically significant. *\( P < 0.05 \) vs. CNT. #\( P < 0.05 \) vs. U-LPi-NPr. ¶\( P < 0.05 \) vs. U-HPi-NPr.
EFFECTS OF A LOW-PROTEIN DIET ON VASCULAR CALCIFICATION

low-protein diet potentiates malnutrition in uremia. However, in our study, the negative effect of a low-protein diet on malnutrition was limited to conditions of hyperphosphatemia. Hence, further studies are needed to clarify the mechanism by which a low-protein diet potentiates inflammation and malnutrition in uremia.

We found that a low-protein diet without P_i loading, which is achieved in CKD patients under dietary protein restriction, neither promoted VC nor accelerated formation of Ca-P_i precipitates in our uremic rats. Actually, serum levels of calciprotein particles were similar in our control, U-LPi-LPr, and U-LPi-NPr groups, suggesting that P_i loading is essential in the formation of Ca-P_i precipitates and that a low-protein diet alone does not enhance VC in CKD.

Rats administered a diet containing low P_i and normal protein remained relatively well-nourished and did not develop VC. These results indicate that a diet containing normal protein and low P_i, may be ideal in the treatment of CKD, and that this ideal situation can be clinically achieved by appropriate treatment with a P_i binder. Our finding, that a low-protein diet did not prevent the progression of CKD, may have been due, at least in part, to the severe renal failure in adenine-treated rats.

This study had several limitations. First, rats were not pair-fed. Therefore, we could not completely separate the effects on MIA of a low-protein and low-calorie intake, although CKD patients given a low-protein diet often experience inadequate calorie intake (17, 24). Second, the level of uremia was relatively high. The effect of a low-protein diet on MIA may be different in 5/6 nephrectomized rats, which develop milder uremia than adenine-treated rats. Third, we used urinary Cr excretion as a marker of muscle mass. Although clinical studies have shown that urinary Cr excretion is a surrogate marker of muscle mass (50), the effects of a low-protein diet on muscle mass should be confirmed by more direct and accurate methods. Fourth, we did not clarify whether Ca-P_i precipitates in our study were identical to calciprotein particles, as confirmed by a transmission electron microscope, although no method has yet been developed to quantitatively measure calciprotein particles. Thus further studies are needed to clearly determine how a low-protein diet actually induces malnutrition and inflammation, which was followed by enhancement of VC, and the association of calciprotein particles with MIA syndrome in CKD with P_i overload.

In conclusion, the results presented here demonstrated that a low-protein diet promoted systemic inflammation and malnutrition and potentiated high P_i-induced VC by reducing systemic fetuin-A levels and increasing systemic Ca-P_i precipitates in uremic rats with hyperphosphatemia. These findings suggest that care be taken in administering strict dietary protein restriction to manage P_i homeostasis, at least in patients with advanced CKD and inadequately controlled P_i, because a low-protein diet can enhance MIA syndrome.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: S.Y. and N.T. provided conception and design of research; S.Y., N.T., and H.N. performed experiments; S.Y., K.T., and H.O. analyzed data; S.Y., M.T., K.T., H.N., and H.O. interpreted results of experiments; S.Y. and H.N. prepared figures; S.Y. and H.O. drafted manuscript; S.Y., M.T., K.T., N.T., T.K., and H.O. edited and revised manuscript; S.Y., M.T., K.T., H.N., T.K., and H.O. approved final version of manuscript.

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Fig. 6. Effects of a low-protein diet on aortic calcification. A: representative photomicrographs of abdominal aortas stained using the von Kossa method. Also shown are quantification of calcium (Ca; B) and Pi (C) content in abdominal aortas; levels of mRNA expression of runt-related gene 2 (Runx2; D), TNF-α (E), and IL-1β (F) in aortas; Ca (G) and Pi (H) precipitates obtained from the serum after supercentrifugation; and correlation between serum Ca precipitate level and Ca content level (I) in abdominal aortas. The level of each mRNA was normalized to that of GAPDH mRNA. Diets in the uremic groups contained 0.3% adenine to induce renal failure. Ca and Pi precipitates were determined by the total Ca and Pi content in the pellet obtained from rat serum following centrifugation at 16,000 g for 2 h. Values are means ± SE and compared by 1-way ANOVA followed by the Tukey-Kramer test. A 2-tailed \( P < 0.05 \) was considered statistically significant. * \( P < 0.05 \) vs. CNT. ¶ \( P < 0.05 \) vs. U-HPi-NPr.
Fig. 7. Direct effects of fetuin-A and albumin on the calcification of vascular smooth muscle cells (VSMCs) treated with medium containing low FCS and high Pi. A: representative microphotographs of calcification in VSMCs treated with culture medium containing different FCS concentrations. Also shown are quantification of Ca content (B) of VSMCs and Ca (C) and Pi (D) precipitates obtained from the cultured media after supercentrifugation; representative microphotographs of calcification in the VSMCs treated with culture media containing fetuin-A or albumin (E); and quantification of Ca content in VSMCs (F) and Ca (G) and Pi (H) precipitates in the culture media. Ca and Pi precipitates were determined by the total Ca and Pi content in the pellet obtained from cultured medium following centrifugation at 16,000 g for 2 h. Values are means ± SE and compared by 1-way ANOVA followed by the Tukey-Kramer test. A 2-tailed $P < 0.05$ was considered statistically significant. *$P < 0.05$ vs. 0.9 mM Pi and 10% FCS. #$P < 0.05$ vs. 2.9 mM Pi and 2.5% FCS.


