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Very low protein diet enhances inflammation, malnutrition, and vascular calcification in uremic rats

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ABSTRACT

Aims: Clinical studies have shown that very low protein diet (VLPD) has negative effects on long-term survival. It remains unclear why VLPD induces premature death. The present study determined the underlying mechanism whereby VLPD exerts its harmful effects on uremic rats.
Main methods: Rats were divided into four groups and fed a normal diet or diets containing 0.3% adenine and low/normal protein with high/low phosphate. After 6 weeks, body weight, urinary biochemistry (creatinine and phosphate), serum biochemical parameters (urea, creatinine, fibroblast growth factor 23, albumin, and fetuin-A), systemic inflammatory markers (serum tumor necrosis factor-alpha and urinary 8-hydroxy-2′-deoxyguanosine), calcium content in the aorta, and serum calcium-phosphate precipitates were evaluated. Hepatic mRNA levels were also determined.
Key findings: Rats fed the diet containing 0.3% adenine developed severe azotemia. Rats fed VLPD developed malnutrition (decreases in body weight, serum albumin and fetuin-A levels, and urinary creatinine excretion) and systemic inflammation (increases in serum tumor necrosis factor-α and urinary 8-hydroxy-2′-deoxyguanosine) independent of phosphate status. VLPD decreased the serum fetuin-A level and hepatic fetuin-A synthesis and increased serum calcium-phosphate precipitates, a marker of calciprotein particle. A high-phosphate diet induced arterial medial calcification, which was enhanced by VLPD. Serum calcium-phosphate precipitate levels were correlated with the degree of inflammation, malnutrition, and aortic calcium content. Dietary phosphate restriction prevented VLPD-enhanced vascular calcification, but could not halt inflammation and malnutrition induced by VLPD.
Significance: VLPD enhances inflammation, malnutrition, and vascular calcification in uremic rats, among which only vascular calcification is prevented by dietary phosphate restriction.

1. Introduction

Phosphate overload, often presented as hyperphosphatemia, increases cardiovascular morbidity and mortality in patients with chronic kidney disease (CKD), by directly inducing endothelial dysfunction, vascular calcification, and myocardial hypertrophy [1–5]. Recent studies have shown that the negative effect of phosphate overload emerges in early CKD, triggering a complex cascade of derangement in mineral metabolism [6]. Strict phosphate control from an early stage of CKD has been regarded as the cornerstone for preventing cardiovascular disorders in CKD patients [7].

Dietary protein restriction is one of the treatment strategies for controlling phosphate balance in predialysis CKD patients, because protein is the main source of dietary phosphate [8]. Clinically, protein restriction is divided into low protein diet and very low protein diet (LVPD); the former is a milder and the latter is a more extreme form of protein restriction. Studies have shown that low protein diet can ameliorate the progression of CKD and improve short-term survival by decreasing phosphate-related nephrotoxicity and uremic toxins induced by protein catabolism [9–11]. So far, it is unknown which form of protein restriction is better overall in CKD patients, although some clinicians insist that daily protein intake need to be as low as possible. Importantly, protein restriction has the potential risk of developing malnutrition in CKD [12,13]. In a recent clinical trial, CKD patients on VLPD showed a higher mortality than those on low protein diet [12]. Judging from the clinical observations, apart from low protein diet, VLPD appears to be harmful from the viewpoint of long-term survival,

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in spite of its potential benefit on kidney function. However, the underlying mechanism whereby VLPD increases mortality in such patients remains unclear.

Clinicians have long been aware of a close link among malnutrition, inflammatory status, and vascular diseases in CKD patients. This concept is now integrated into malnutrition–inflammation–atherosclerosis syndrome [14]. As evidence of malnutrition–inflammation–atherosclerosis syndrome in CKD, we have recently shown that low protein diet promotes vascular calcification in uremic rats by decreasing fetuin-A synthesis in the liver [15]. In that study, moderate levels of protein restriction induced inflammation and malnutrition only under a high-phosphate diet, but not under a low-phosphate diet. We hypothesize that VLPD, an extreme form of dietary protein restriction, promotes malnutrition and inflammation in CKD independent of phosphate loading, thereby overriding the benefit of VLPD on kidney function and accelerating premature death. However, to date, the effects of VLPD on inflammation and malnutrition have not been determined in CKD.

The purpose of the present study was to clarify the mechanism whereby VLPD exerts its harmful effects on CKD. To this end, we determined the effects of VLPD on inflammation, malnutrition, and vascular calcification in adenine-induced uremic rats by feeding diets with different concentrations of protein and phosphate. We found that VLPD enhanced inflammation, malnutrition, and vascular calcification in uremic rats.

2. Materials and methods

2.1. Animals and materials

All protocols were reviewed and approved by the Committee on Ethics of Animal Experiments at Fukuoka Dental College Faculty of Medicine (11,020). Male Sprague–Dawley rats (10 weeks old), purchased from Kyudo Co. (Saga, Japan), were housed in a climate-controlled space with 12 h day/night cycles and allowed free access to food and water. Rats were fed a standard diet (calcium 1.0%, phosphate 1.2%, casein-based protein 19%, lactose 20%; Oriental Yeast Co., Ltd., Tokyo, Japan) during the acclimatization period. All synthetic rodent diets were purchased from Oriental Yeast Co., Ltd.

2.2. Experimental protocols

Male Sprague–Dawley rats ($n = 32$) were fed a standard diet during the acclimatization period. On day 1, they were randomly subdivided into four groups of eight rats each, with each group fed one of the specific diets for 6 weeks (until day 42): Control rats were fed the standard diet (CNT, phosphate 1.2%, protein 19%). Uremic rats were fed diets containing 0.3% adenine and high phosphate and normal protein (U-HPiNPr group, phosphate 1.2%, protein 19%), high phosphate and very low protein (U-HPiLPr group, phosphate1.2%, protein 2.5%), and low phosphate and very low protein (U-LPiLPr group, phosphate 0.3%, protein 2.5%). In our previous study, 8 weeks of adenine feeding induced high mortality [16]. We had two options to improve mortality; to shorten observational period or to lower adenine concentration in the diet. However, our preliminary data showed that diets containing 0.15%–2.5% of adenine could not induce vascular calcification within 10 weeks (data are not shown). Hence, we selected the observation period of 6 weeks to avoid premature death and obtain robust vascular calcification in uremic groups, instead of lowering adenine concentration in the diet. Diets in all four groups contained 1.0% calcium and 20% lactose. Proteins in the diets were casein-based to enhance absorption of calcium and phosphate from the intestine. The concentration of protein selected for very low protein diets was 2.5%, in accordance with previous reports [17,18].

At week 6, rats were housed in metabolic cages for 24 h and food and water intake and urine volume were recorded. Collected urine was centrifuged at 3000 $\times g$ for 15 min, and the supernatant was stored at −30 °C until analyzed. At sacrifice, blood, aorta, kidney, and liver were collected. Serum was separated by centrifugation at 5000 $\times g$ and stored at −80 °C until analysis. Aortas were weighed using a microbalance. Aorta, kidney, and liver were dissected into several samples; one was immersed in formalin for histological analysis, and the others were stored at −80 °C until later analyses.

2.3. Determination of biochemical parameters

Serum and urine levels of calcium, phosphate, albumin, urea nitrogen, and creatinine were measured with an automated analyzer (Hitachi Co., Ltd., Tokyo, Japan). Creatinine clearance was calculated using the standard formula: urinary creatinine level × urine volume / serum creatinine level (dL/day). Fractional excretion of phosphate was calculated using the standard formula: (urinary phosphate concentration × serum creatinine concentration) / (urinary creatinine concentration × serum phosphate concentration). Serum calcitriol was measured by radioimmunoassay (SRL, Inc., Tokyo, Japan). Serum levels of fibroblast growth factor 23 (Kainos Laboratories Inc., Tokyo, Japan), fetuin-A (Aviscera Bioscience Inc., CA, USA), and tumor necrosis factor-alpha (TNF-α) (R&D Systems, MN, USA), and urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) (JaICA, Shizuoka, Japan), were determined using commercially available rat ELISA kits, according to the manufacturers' instructions.

2.4. Examination of aortic medial calcification and serum calcium-phosphate precipitates

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 6 N hydrochloride for 24 h. The calcium and phosphate contents 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).

Rat serum (500 μL) was centrifuged at 16,000 $\times g$ for 2 h. The pellets after ultracentrifugation were calcium-phosphate precipitates composed of calcium, phosphate, fetuin-A, and other proteins, and assumed to contain calciprotein particles (CPP) [17]. Next, we treated these pellets with 100 μL of 6 N hydrochloride and measured the total calcium content of the hydrochloride-treated solution [17,18]. We defined these calcium-phosphate precipitates as CPP in the present study.

2.5. Real-time PCR

Real-time PCR was performed as previously described [16]. Total RNA was extracted from frozen rat tissue stored in liquid nitrogen using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. Complementary DNA was synthesized by reverse transcription using a PrimeScript™ RT reagent kit (Perfect Real Time; Takara Bio Inc., Otsu, Japan). Real-time quantitative PCR was performed using SYBR Premix Ex Taq™ (Takara Bio Inc.), Applied Biosystems 7500 Real-time PCR systems (Applied Biosystems, CA, USA), and primers for rat glyceraldehyde-3-phosphate dehydrogenase (RA015380), runt-related transcription factor 2 (RA045439), rat TNF-α (RA043092), albumin (RA065413), and fetuin-A (RA065433), all from Takara Bio Inc. 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 $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde-3-phosphate dehydrogenase as an internal reference.