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Authors

Lau, Wei Ling
Liu, Shuman
Vaziri, Nosratola D

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Chronic Kidney Disease Results in Deficiency of ABCC6, the Novel Inhibitor of Vascular Calcification

Wei Ling Lau Shuman Liu Nosratola D. Vaziri

Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, Calif., USA

Key Words

ABCC6 · Chronic kidney disease · Cardiovascular disease

Abstract

Background: Chronic kidney disease (CKD) is associated with arterial medial calcification which plays a major role in the pathogenesis of cardiovascular disease in this population. Several factors are known to promote soft tissue and accelerated arterial calcification in CKD including systemic inflammation, altered calcium and phosphate homeostasis, hypertension, and deficiency of endogenous calcification inhibitors. The ABCC6 transporter (ATP-binding cassette sub-family C number 6), also known as multidrug resistance-associated protein 6 (MRP6), is highly expressed in the liver and kidney. Mutation of *ABCC6* results in pseudoxanthoma elasticum, an inherited disorder characterized by arterial and soft tissue calcification. Given the prevalence of arterial medial calcification in CKD, the present study was undertaken to test the hypothesis that CKD may lead to acquired ABCC6 deficiency. **Methods:** CKD was induced via 5/6 nephrectomy in male Sprague-Dawley rats and by adenine-containing diet to cause chronic interstitial nephropathy in female DBA/2J mice. Sham-operated rats and mice fed regular diet served as controls. Liver and kidney tissues were harvested and processed for ABCC6 protein and mRNA analysis. **Results:** ABCC6 protein levels were significantly reduced in the liver and kidney tissues from CKD rats and mice. However, ABCC6 mRNA

levels were unchanged, pointing to post-transcriptional or post-translational mechanisms for the observed ABCC6 deficiency. Additionally, plasma levels of the calcification inhibitor fetuin-A were significantly decreased in CKD animals compared to controls. **Conclusions:** CKD results in acquired ABCC6 transporter deficiency. To our knowledge this abnormality has not been previously reported and may contribute to CKD-associated vascular and soft tissue calcification.

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Introduction

Arterial medial calcification develops as part of normal aging but is accelerated in acquired metabolic diseases such as chronic kidney disease (CKD) and diabetes mellitus. Vascular calcification contributes to the high prevalence of cardiovascular (CV) morbidity and death in CKD. Individuals with CKD are more likely to die of CV causes than to develop end-stage kidney failure [1]. In the dialysis population, this disparate mortality risk is most marked in the younger age group (25–34 years old), where the CV death rate is 500-fold greater than in age-matched healthy controls [1]. Risk factors in the uremic milieu include abnormal phosphorus and calcium me-

W.L.L. and S.L. contributed equally to this work.

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N.D. Vaziri, MD, MACP
Division of Nephrology and Hypertension, University of California Irvine Medical Center
Suite 400, City Tower, 333 City Blvd West
Orange, CA 92868 (USA)
E-Mail ndvaziri@uci.edu

tabolism, transformation of vascular smooth muscle cells, bone disorders, existence of co-morbid conditions such as hypertension and diabetes mellitus, and deficiency of endogenous calcification inhibitors such as Klotho, matrix glutamate protein (MGP), pyrophosphate and fetuin-A [2–5].

Mutations in the *ABCC6* (ATP-binding cassette sub-family C number 6) gene result in the heritable disorder, pseudoxanthoma elasticum (PXE, OMIM 264800) [6]. *ABCC6* is primarily expressed in the liver and kidney [7, 8], and encodes the transmembrane *ABCC6* protein (a.k.a. MRP6, multidrug resistance-associated protein 6). PXE has a prevalence of 1 in 25,000 [9] and is characterized by ectopic calcification and fragmentation of elastic fibers of connective tissues, including skin, vascular walls and the eyes [10]. *ABCC6* has also been identified as the causal gene within the *Dyscalc1* locus that predisposes certain mouse strains (such as C3H/HeJ and DBA/2J) to dystrophic cardiac calcinosis and other ectopic soft tissue calcification [11, 12].

Given the prevalence of arterial medial calcification in CKD and the strong association of hereditary *ABCC6* deficiency with arterial/ectopic calcification, the present study was undertaken to test the hypothesis that CKD may lead to acquired *ABCC6* deficiency. To this end, *ABCC6* protein and mRNA levels were determined in the kidney and liver tissues of rats with CKD induced by 5/6 nephrectomy, in mice with adenine-induced chronic interstitial nephropathy, and in their normal control counterparts. The study revealed significant reduction of *ABCC6* protein abundance despite its normal mRNA levels in liver and kidney of the CKD animals.

Methods

Experimental Animals

Male Sprague-Dawley rats were randomized to the CKD and control groups. The CKD group underwent partial resection of the left kidney followed by right total nephrectomy 5 days later. Sham-operated rats served as controls. To exclude possible effect of differences in species, gender, or the underlying cause of CKD, we studied female DBA/2J mice fed a diet containing 0.2% adenine for 18 days to induce chronic interstitial nephropathy, and these mice were observed for 6 weeks. The CKD mice ($n = 5$) were re-exposed to 0.2% adenine-containing diet on week 3 during the observation period. The control mice (CTL, $n = 5$) were maintained on regular chow.

All experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee. Further details are available in online supplementary methods (for all online suppl. material, see www.karger.com/doi/10.1159/000365014).

Blood and Urine Chemistries

Blood chemistries were measured using commercially available kits: QuantiChrom™ Urea and Creatinine Assay Kits (BioAssay Systems, Hayward, Calif., USA) for blood urea nitrogen and creatinine; the *o*-cresolphthalein complexone kit from Teco Diagnostics (Anaheim, Calif., USA) for calcium; EnzChek Phosphate Assay Kit (Life Technologies, Grand Island, N.Y., USA). Plasma fetuin-A was measured using the rat ELISA kit (MyBioSource, Inc., San Diego, Calif., USA, catalog # MBS297234). Urinary protein excretion was calculated from 24-hour urine collections in CTL and CKD rats.

Western Blot Analysis

Tissue lysates were prepared from liver and kidney samples and subjected to Western blot using *ABCC6* primary antibody (sc-5787 at 2 μ g/ml; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and data were normalized to GAPDH. Further details are available in online supplementary methods.

TaqMan Real-Time PCR

Total mRNA was isolated from liver and kidney samples and first-strand cDNA was synthesized using standard methods. Relative RNA expression was quantified using TaqMan® gene expression assays with pre-made exon-spanning primers and TaqMan probe mixes (Applied Biosystems, Carlsbad, Calif., USA) targeting *ABCC6* and *18s*. For details please refer to Supplementary Methods.

Statistical Analysis

Student's *t* test was used in statistical evaluation of the data, which are shown as mean \pm SEM. *p* values < 0.05 were considered significant. Figures were generated using GraphPad Prism 4 software (GraphPad Software, San Diego, Calif., USA).

Results

General Data

CKD animals exhibited significant decrease in body weight: average weight in CTL rats was 417 ± 4.2 versus 391 ± 14 g in CKD rats; average body weights in the CTL and CKD mice were 24.4 ± 0.9 and 19.6 ± 0.5 g, respectively. Plasma creatinine was 0.16 ± 0.05 mg/dl in CTL rats versus 0.59 ± 0.08 mg/dl in the CKD rats, and CKD rats had significant proteinuria 75.1 ± 13.4 versus 10.2 ± 4.5 mg/day in CTL rats. Serum urea nitrogen concentration was 17 ± 0.7 mg/dl in CTL and 60.3 ± 3.7 mg/dl in CKD mice. Serum calcium and phosphorus were not significantly different between the study groups (data not shown). Plasma fetuin-A levels in CKD rats (20.1 ± 1.6 mg/l) was significantly lower than that found in CTL rats (24.8 ± 1.1 mg/l, $p = 0.03$).

ABCC6 Protein and mRNA Data

ABCC6 protein abundance in the liver and kidney tissues was significantly ($p < 0.05$) lower in the CKD rats and mice than in their control counterparts (fig. 1). In

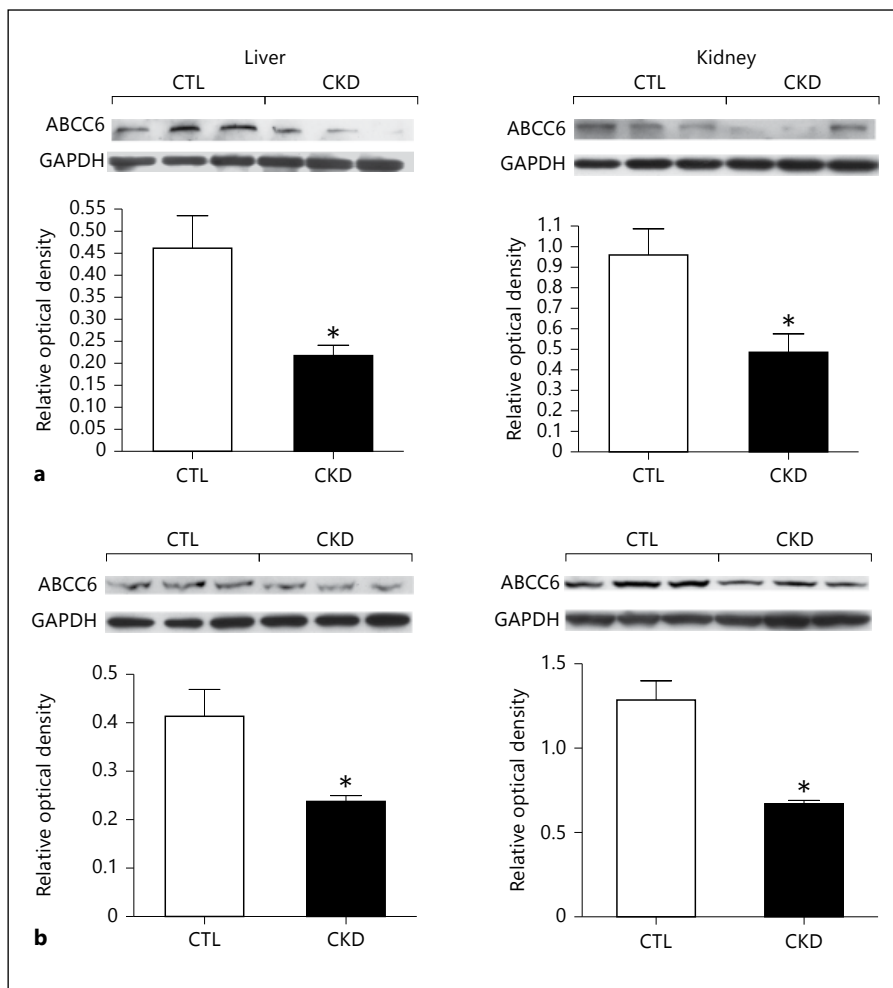


Fig. 1. Representative Western blots and group data depicting the abundance of ABCC6 protein in the liver and kidney tissues of CTL and CKD animals. **a** CKD induced in rats via 5/6 nephrectomy. **b** CKD induced in mice via adenine diet. * $p < 0.05$ compared to CTL.

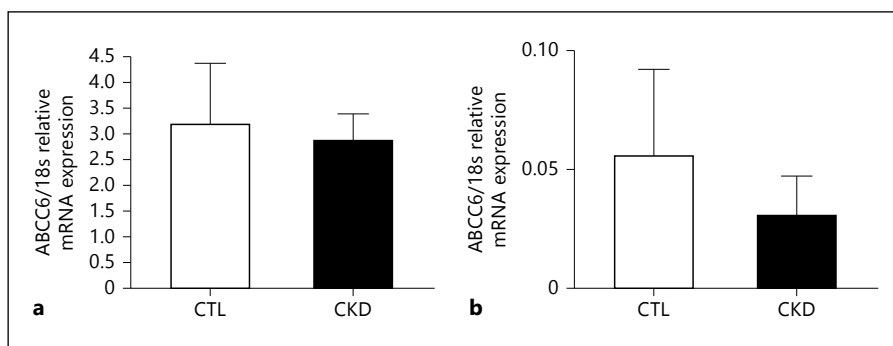


Fig. 2. Relative mRNA levels of ABCC6 were unchanged in the **(a)** liver and **(b)** kidney tissues from CTL and CKD rats, $n = 4$ per group.

contrast, ABCC6 mRNA expression in liver and kidney tissue was not significantly different between the control and CKD rats (fig. 2). These observations point to the post-transcriptional or post-translational mechanisms of the observed ABCC6 protein deficiency in the CKD animals.

Discussion

The present study revealed that CKD in experimental animals results in significant deficiency of the ABCC6 transporter which is a novel inhibitor of vascular calcification. Acquired ABCC6 deficiency was present in male

rats with CKD induced by 5/6 nephrectomy and in female mice with adenine-induced chronic interstitial nephropathy, thus excluding a possible effect of differences in species, gender, or the underlying cause of CKD. To our knowledge, this phenomenon has not previously been demonstrated in an in vivo CKD model.

ABCC6 mutations are known to cause the rare monogenic condition PXE, that is characterized by elastin degradation and ectopic calcification in the medial vascular wall, skin and eyes [6]. The vascular calcification localizes to the tunica media (with resultant stiffening/loss of vessel distensibility) rather than intimal disease. In fact, in mice on an *apoE*^{-/-} background deletion of ABCC6 results in significant medial calcification but not atherosclerotic plaque calcification or change in atherosclerotic lesion size [13]. Medial calcification is highly prevalent in CKD and is evident in pediatric dialysis patients who lack traditional atherosclerosis risk factors (smoking, diabetes mellitus, older age) and do not have intimal disease [14]. By promoting vascular calcification, acquired ABCC6 deficiency shown for the first time here may play a role in the pathogenesis of CKD-associated accelerated CV disease.

As mentioned in the Introduction, ABCC6 was previously identified via DNA fine mapping as the major causal gene underlying dystrophic cardiac calcification in mice [12]. Meng et al. [15] found a close correlation between ABCC6 expression levels and regulators in the BMP2-Wnt signaling pathway. Towler and colleagues [16] showed a major role for BMP2-Msx2-Wnt/ β -catenin signaling in the calcification of aortic valves and the medial arterial wall, in a transgenic mouse model. Msx2 and Wnt/ β -catenin signaling has also been implicated in calcific aortic valvular disease in humans [17].

Several pathways can be proposed by which ABCC6 deficiency can increase the risk of CV disease in CKD. ABCC6 deficiency in PXE results in the deficiency of circulating factors (such as fetuin-A) which leads to loss of physiological suppression of artery calcification despite normal calcium and phosphate homeostasis [18, 19]. In this context, overexpression of fetuin-A in *Abcc6*^{-/-} mice has been shown to attenuate soft tissue mineralization in these animals [20]. ABCC6 deficiency in our CKD animals was accompanied by significant reduction in circulating fetuin-A level – these events can work in concert to promote vascular/soft tissue calcification. Another point of interest is that mutations in the γ -glutamyl carboxylase GGCX (the enzyme responsible for γ -carboxylation of MGP to its functional form) have been described in patients with a PXE-like phenotype [21]. Abnormalities in

circulating levels of fetuin-A and MGP are present in CKD and correlate with CV disease [22–27], however the influence of ABCC6 deficiency has yet to be fully explored.

Jansen et al. [28] recently reported that medium from HEK293 cells overexpressing ABCC6 inhibited mineralization of cultured chondrocytic cells via excretion of large amounts of nucleoside triphosphates, which are hydrolyzed to produce the calcification inhibitor, pyrophosphate. There is also an ‘elastosis hypothesis’ in PXE that implicates abnormal elastin turnover as the primary defect, and calcification of the medial wall smooth muscle cells as a secondary consequence [29–31]. Of note, breakage of elastic fibers is present in the aortic wall of CKD mice [32, 33], and fragmentation of medial elastic fibers has been described in CKD patients [34, 35].

The ABCC6-transported substrate or substrates, which modulate ectopic calcification and other pathologic changes in PXE, are not yet known. Rutsch et al. [36] proposed a schematic within vascular smooth muscle cells whereby ABCC6 may participate in inhibition of calcification via modulating adenosine signaling. Organic anions have been postulated to be primary substrates based on transporter activity when ABCC6 was expressed in Sf9 insect cells [37]. However, confirmatory evidence of clinically relevant ABCC6 substrates has been elusive.

In summary, this is the first report of liver and kidney ABCC6 deficiency in CKD. If true in humans, acquired deficiency of this transporter may contribute to accelerated arterial calcification in patients with CKD. Further research to elucidate causative pathways and clinical relevance of this abnormality is needed.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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