PiT-2, a type III sodium-dependent phosphate transporter, protects against vascular calcification in mice with chronic kidney disease fed a high-phosphate diet

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PiT-2, a type III sodium-dependent phosphate transporter, is a causative gene for the brain arteriolar calcification in people with familial basal ganglion calcification. Here we examined the effect of PiT-2 haploinsufficiency on vascular calcification in uremic mice using wild-type and global PiT-2 heterozygous knockout mice. PiT-2 haploinsufficiency enhanced the development of vascular calcification in mice with chronic kidney disease fed a high-phosphate diet. No differences were observed in the serum mineral biomarkers and kidney function between the wild-type and PiT-2 heterozygous knockout groups. Micro computed tomography analyses of femurs showed that haploinsufficiency of PiT-2 decreased trabecular bone mineral density in uremia. *In vitro*, sodium-dependent phosphate uptake was decreased in cultured vascular smooth muscle cells isolated from PiT-2 heterozygous knockout mice compared with those from wild-type mice. PiT-2 haploinsufficiency increased phosphate-induced calcification of cultured vascular smooth muscle cells compared to the wild-type. Furthermore, compared to wild-type vascular smooth muscle cells, PiT-2 deficient vascular smooth muscle cells had lower osteoprotegerin levels and increased matrix calcification, which was attenuated by osteoprotegerin supplementation. Thus, PiT-2 in vascular smooth muscle cells protects against phosphate-induced vascular calcification and may be a therapeutic target in the chronic kidney disease population.

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Vascular calcification (VC), a component of chronic kidney disease (CKD)–mineral and bone disorder, increases the risk of cardiovascular disease and death in the CKD population.[1,2] Among various risk factors for VC, phosphate (P) is the most powerful inducer.[3–5] One of the mechanisms by which P induces VC is through direct effects on vascular smooth muscle cells (VSMCs) via PiT-1, a type III sodium (Na)-dependent P transporter.[5,6] Increased extracellular P interacts with PiT-1, thereby inducing osteochondrogenic transition and apoptosis of VSMCs, degradation of extracellular matrix, and release of unstable matrix vesicles/exosomes.[7–11] Currently, PiT-1 in VSMCs is considered as a therapeutic target for VC.

PiT-2 is the second member of the type III Na-dependent P transporter that is expressed in various tissues, including VSMCs, renal proximal tubules, bones, and brain.[12–14] Compared with PiT-1, the function of PiT-2 in VC has been much less investigated. However, recent gene linkage analyses disclosed that mutations in PiT-2 underlie a majority of the cases of familial basal ganglion calcification. Familial basal ganglion calcification is a rare hereditary disorder characterized by arteriolar calcifications in the hypothalamic and basal ganglion regions of the brain that leads to a variety of adverse neurologic symptoms.[15–18] PiT-2 deficiency in mice recapitulates the brain calcification observed in people and is associated with elevated P levels in cerebrospinal fluid.[19,20] We have very recently shown that PiT-2 deficiency in mice decreases bone mineral density (BMD) in the craniofacial and long bones.[21] To date, however, it is still not known how PiT-2 exerts its protective function against calcification in brain vessels, or whether it also protects against VC in the peripheral circulation, either normally or under disease conditions.[22,23] Thus, our aim was to elucidate the role of PiT-2 on VC using global PiT-2 heterozygous (HET) knockout mice and primary cultured VSMCs.[20]

RESULTS

PiT-2 targeting, genotype and phenotype of wild-type and HET mice

Figure 1a shows the gene targeting scheme for generating mice with a knockout-first allele of PiT-2. In the present study

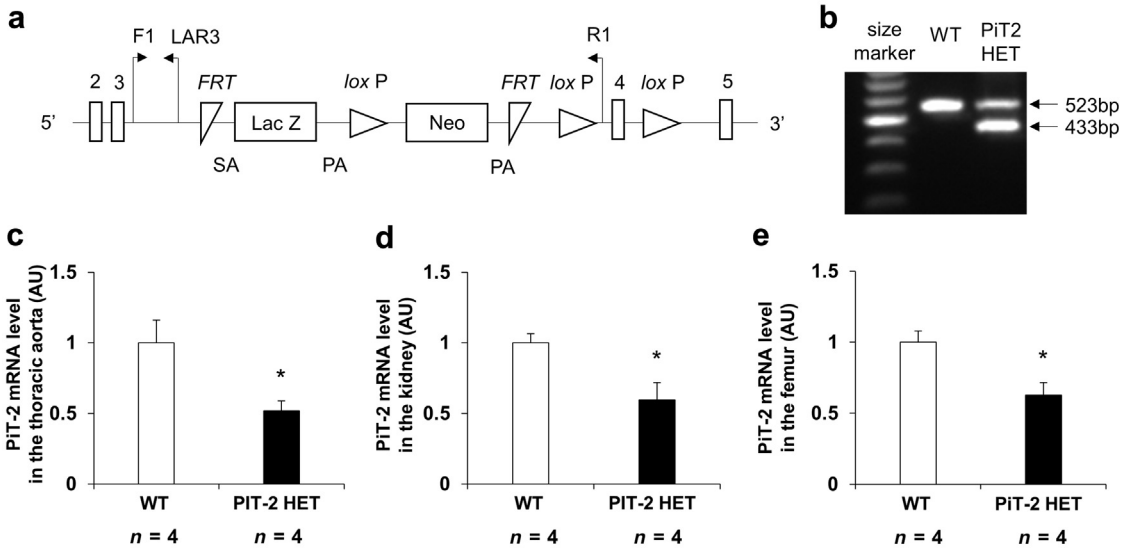

Figure 1 | Genomic design of a knock-out first allele and characterization of PiT-2 HET mice. (**a**) Presentation of the knockout-first allele. (**b**) Expression of genomic DNA determined by polymerase chain reaction in wild-type (WT) and PiT-2 heterozygous (HET) mice. (**c–e**) PiT-2 mRNA level in the (**c**) aorta, (**d**) kidney, and (**e**) femur. Three samples from each genotype were used for quantitative real-time–polymerase chain reaction and 6 samples for biochemical determination. Data are expressed as mean ± SEM and compared by unpaired *t* test. A 2-tailed *P* value of <0.05 was considered statistically significant. $^*P < 0.05$ versus WT. AU, arbitrary unit; bp, base pair; F1, forward primer recognition site; FRT, flippase recognition target; LAR3, ligation amplification reaction; Neo, neomycin phosphotransferase; PA, polyadenylation; PiT-2 HET, PiT-2 heterozygous knockout; R1, reverse primer recognition site; SA, splice acceptor. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

PiT-2 HET mice were used instead of PiT-2 knockout mice because PiT-2 knockout mice showed early mortality and were susceptible to physical stress.[20] DNA extracted from tail biopsy specimens showed that PiT-2 HET mice carried both wild-type (WT) and knockout-first alleles (Figure 1b). As shown in Figure 1c–e, mRNA expression of PiT-2 in the aorta, kidney, and femur of PiT-2 HET C57BL/6 mice was decreased by approximately 40% compared with WT C57BL/6 mice, as expected. Alizarin Red S staining of the abdominal aorta and brain revealed no calcification in 10-week-old female WT and PiT-2 HET C57BL/6 mice fed a standard diet (Supplemental Figure 1).

PiT-2 haploinsufficiency did not alter renal P handling in mice with intact kidney function

To determine the impact of PiT-2 haploinsufficiency on renal P handling in mice with intact kidneys, metabolic cage studies were performed. Twenty-week-old female WT and PiT-2 HET mice backcrossed onto a DBA/2 strain for at least 5 generations were fed a normal (0.5%) P (NP) diet for 2 weeks and then switched to a high (1.5%) P (HP) diet for another 11 days (Supplementary Figure S1A). As shown in Figure 2a–c, when mice were fed an NP diet, no significant differences were observed in serum levels of P, calcium (Ca), and creatinine (Cr) between WT and PiT-2 HET groups. Likewise, after the diets were changed from 0.5% P to 1.5% P, no significant differences in serum P, Ca, and Cr levels and fractional excretion of Ca were observed between genotypes (Figure 2a–d). As for fractional excretion of P, HP diet feeding significantly increased FEP in both genotypes when compared with NP diet feeding (Figure 2e). However, there was no significant difference in fractional excretion of P between WT and PiT-2 HET mice fed either an NP or HP diet.

PiT-2 protected against VC in CKD mice fed a high P diet

Next we determined the role of PiT-2 haploinsufficiency in VC induced by CKD after NP or HP diet feeding (Supplementary Figure S1B). For these studies, female PiT-2 HET and WT mice were bred onto a DBA/2 background for at least 5 generations to enhance their susceptibility to VC.[24–26] CKD was induced surgically using a 2-step 5/6 nephrectomy procedure as previously described.[27,28]

None of the mice in any of the groups died during the study period. As shown in Table 1, there were no significant differences among the 4 groups regarding the baseline serum levels of urea nitrogen, Cr, Ca, and P. By contrast, as shown in Table 2, moderate renal insufficiency was induced in CKD mice according to serum urea nitrogen and Cr levels, and these were not significantly different between genotypes or diets. In addition, PiT-2 haploinsufficiency did not significantly alter serum levels of Ca, P, or alkaline phosphatase in response to NP or HP diet. Consistent with previous findings,[25] serum fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) levels in the CKD/HP groups were significantly higher than those in the CKD/NP groups, regardless of genotype. Interestingly, the serum osteoprotegerin (OPG) level in PiT-2 HET-CKD/HP mice was slightly, but significantly lower than in WT-CKD/HP mice.

Uremic mice fed an NP diet did not develop calcification in the aorta, as determined by Alizarin Red S staining (Figure 3a).