Mammalian target of rapamycin signaling inhibition ameliorates vascular calcification via Klotho upregulation

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Vascular calcification (VC) is a major risk factor for cardiovascular mortality in chronic renal failure (CRF) patients, but the pathogenesis remains partially unknown and effective therapeutic targets should be urgently explored. Here we pursued the therapeutic role of rapamycin in CRF-related VC. Mammalian target of rapamycin (mTOR) signal was activated in the aortic wall of CRF rats. As expected, oral rapamycin administration significantly reduced VC by inhibiting mTOR in rats with CRF. Further *in vitro* results showed that activation of mTOR by both pharmacological agent and genetic method promoted, while inhibition of mTOR reduced, inorganic phosphate-induced vascular smooth muscle cell (VSMC) calcification and chondrogenic/osteogenic gene expression, which were independent of autophagy and apoptosis. Interestingly, the expression of *Klotho*, an antiaging gene that suppresses VC, was reduced in calcified vasculature, whereas rapamycin reversed membrane and secreted Klotho decline through mTOR inhibition. When mTOR signaling was enhanced by either mTOR overexpression or deletion of tuberous sclerosis 1, Klotho mRNA was further decreased in phosphate-treated VSMCs, suggesting a vital association between mTOR signaling and *Klotho* expression. More importantly, rapamycin failed to reduce VC in the absence of Klotho by using either siRNA knockdown of *Klotho* or *Klotho* knockout mice. Thus, Klotho has a critical role in mediating the observed decrease in calcification by rapamycin *in vitro* and *in vivo*.

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Vascular calcification (VC) is a major risk factor for cardiovascular mortality in patients with chronic renal failure (CRF).[1–3] It may occur in the tunica intima and tunica media. The former is usually found in advanced atherosclerotic lesions, whereas the latter is independent of atherosclerosis but has a strong relationship with aging, metabolic syndrome, and CRF. Accumulated evidence has suggested that vascular smooth muscle cells (VSMCs) dedifferentiating from the contractile phenotype to the osteogenic phenotype, in which mineralized matrix and osteogenic proteins are produced, promote the VC process.[4,5]

Serum inorganic phosphate (Pi) level is closely correlated with the severity of VC and contributes to high mortality and morbidity in vascular abnormality.[6] Dysregulation of phosphate metabolism is common in CRF patients and drives VC.[6] *In vivo* and *in vitro* studies have shown that Pi is a crucial regulator in VC, and the underlying mechanisms include NaPi-III cotransporter–mediated Pi uptake, increased Cbfα-1 expression and subsequent VSMC osteogenic phenotype transition, and so on.[6–8] We have recently demonstrated that mitochondrial reactive oxygen species–activated nuclear factor-κB promotes Pi-induced calcification, whereas reactive oxygen species-induced autophagy counteracts Pi-induced calcium deposition.[9,10] However, the pathogenesis of CRF-related VC remains largely unknown, and effective therapeutic targets should be urgently explored.

Mammalian target of rapamycin (mTOR) is a highly conserved 289-kDa protein kinase that is broadly expressed in multiple organs and cells, including VSMCs. mTOR signaling is vital in body energy metabolism, longevity, and regulates cell proliferation.[11] Rapamycin inhibits mTOR activity. Studies have shown that rapamycin supplementation reduces high-fat diet–induced atherosclerosis in apolipoprotein E–deficient ($apoE^{-/-}$) mice,[12] suppresses vascular inflammation, and enhances the stability of atherosclerotic plaques.[13] In addition, rapamycin might be involved in regulating phosphate homeostasis[14] and participate in osteoblastic differentiation.[15–18] Rapamycin is used as an immunosuppressive agent after kidney transplantation in specific

cases, and mTOR signaling inhibition might be associated with renal function reserve in chronic kidney disease (CKD).[19,20]

Therefore, the therapeutic role of rapamycin in CRF-related VC was pursued in the present study. The results demonstrated that rapamycin blocked mTOR signaling, increased *Klotho* expression, and then reduced Pi-induced VC *in vitro* and *in vivo*.

RESULTS

mTOR signaling inhibition by rapamycin suppresses CRF-related VC

To explore the role of mTOR signaling inhibition in CRF-related VC, rapamycin (0.4 mg/kg per day) was administered to the CRF rats by gavage. Rapamycin treatment significantly reduced CRF-activated mTOR signaling, which was evidenced by increased phospho-p70 S6 kinase (P-S6K; downstream of mTOR and reflects its activation) protein level in vehicle-administered CRF rats and decreased in rapamycin-treated CRF rats (Figure 1a). Importantly, calcium deposition in the abdominal aorta, as assessed by von Kossa staining and calcium content assay, was increased in CRF rats, whereas rapamycin administration significantly ameliorated the calcium deposition (Figure 1b and c), which was accompanied with reduced osteogenic/chondrogenic differentiation of contractile VSMCs in the aortas of CRF rats, because SM-α-actin decreased and Cbfα-1 increased in the aortic wall of CRF rats, and these were reversed by rapamycin treatment (Figure 1d). Furthermore, rapamycin administration significantly decreased the expression levels of CRF-induced osteogenic genes (*msh homeobox 2 (Msx2), core-binding factor α1 (Cbfα-1),* and *osterix*) and chondrogenic genes (*aggrecan* and *Sox9*), whereas it rescued the expression of smooth muscle lineage markers (*SM-α-actin* and *SM22α*) compared with that in vehicle-administered CRF rats (Figure 1e). Meanwhile, calcification inhibitors (*matrix gla protein (Mgp)* and *osteopontin (Opn);* serum pyrophosphate (PPi)) were decreased in CRF rats, and *Mgp* and *Opn* were rescued by rapamycin, but not PPi (Figure 1e and f). Furthermore, *ex vivo* treatment of rapamycin (30 nmol/l) obviously reduced calcium deposition in Pi-induced human aortic tissue calcification (Figure 1g). These results indicate that mTOR signaling inhibition by rapamycin protects against hyperphosphatemia-induced VC.

mTOR activation mediates Pi-induced VSMC calcification in both bovine and human aortic smooth muscle cells

To test whether Pi could activate mTOR signaling *in vitro*, bovine aortic smooth muscle cells (BASMCs) were treated with Pi (3.0 nmol/l) for 4–24 h and 7 days. Western blot analyses showed that Pi increased P-mTOR and its downstream target protein P-S6K levels from 8 to 24 h, and even at day 7 (Figure 2a and Supplementary Figures 1a and b online). In addition, Pi-induced P-S6K elevation in BASMCs was augmented by leucine (4 mmol/l), but it was blocked by rapamycin (30 nmol/l) (Figure 2b). More importantly, Pi-induced calcium deposition, as assessed by alizarin red staining, was also augmented by leucine and reduced by rapamycin (Supplementary Figure 2 online). These results were further confirmed by calcium content measurement in BASMCs. The results showed that rapamycin (15 and 30 nmol/l) suppressed, whereas leucine (2 and 4 mmol/l) promoted, Pi-induced calcification (Figure 2c and d). To confirm the pharmacological results from leucine and rapamycin, wild-type (WT) mTOR and kinase-dead mTOR-expression plasmids were transfected to BASMCs, and then the mTOR signaling activity was verified with the P-S6K level (Figure 2e and f, upper panel). Calcium content assay showed that mTOR overexpression significantly promoted Pi-induced calcification, whereas kinase-dead mTOR significantly suppressed Pi-induced calcification (Figure 2e and f, lower panel). Furthermore, aortic smooth muscle cells of TSC1 flox-flox mice were cultured and treated with adenovirus-expressing Cre recombinase to knock out TSC1, leading to the activation of mTOR signaling (Supplementary Figure 3 online). Compared with cells infected with adenovirus-expressing GFP, the cells infected with Cre adenovirus had significantly increased calcium content (Figure 2g).

In addition, similar results were observed in human aortic smooth muscle cells (T/G HASMCs): i.e., Pi increased P-mTOR and P-S6K levels with time (Figure 3a and Supplementary Figure 4a and b online), and leucine (4 mmol/l) augmented and rapamycin (30 nmol/l) blocked Pi-induced P-S6K elevation (Figure 3b) and calcium deposition (Figure 3c and d). These results suggest that mTOR activation mediates Pi-induced calcification *in vitro*. To further test whether rapamycin could suppress VC after osteoblastic differentiation, HASMCs were treated with Pi for 3 days, and then treated with rapamycin plus Pi for an additional 7 days. Calcium content results showed that rapamycin could still reduce VC, although the inhibitive effect was less than that of rapamycin added simultaneously with Pi (Figure 3e).

mTOR activation mediates Pi-induced phenotypic transition in both BASMCs and HASMCs

To determine whether mTOR signaling modulates BASMC and HASMC phenotypic transition, the mRNA levels of osteogenic genes and smooth muscle lineage markers were detected by real-time PCR. Pi significantly upregulated *Msx2* and *Cbfα-1* levels, which were augmented by leucine, but reduced by rapamycin (Figure 4a and b), whereas the mRNA levels of *SM-α-actin* and *SM22α* were significantly reduced in the Pi-treated group, which were intensified by leucine and rescued by rapamycin (Figure 4c and d).

The role of autophagy in rapamycin-inhibited VC

Our previous data have demonstrated that Pi-induced autophagy counteracts VC by reducing matrix vesicle release.[9] In addition, rapamycin is a well-known autophagy inducer. Indeed, rapamycin treatment did enhance autophagy in the aortic wall of CRF rats (data are not shown) and in cultured BASMCs (Figure 5a). However, when autophagy was inhibited by 3-MA (Figure 5a), rapamycin still ameliorated