Inhibition of TRPC6 channels ameliorates renal fibrosis and contributes to renal protection by soluble klotho

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Fibrosis is an exaggerated form of tissue repair that occurs with serious damage or repetitive injury and ultimately leads to organ failure due to the excessive scarring. Increased calcium ion entry through the TRPC6 channel has been associated with the pathogenesis of heart and glomerular diseases, but its role in renal interstitial fibrosis is unknown. We studied this by deletion of *Trpc6* in mice and found it decreased unilateral ureteral obstruction-induced interstitial fibrosis and blunted increased mRNA expression of fibrosis-related genes in the ureteral obstructed kidney relative to that in the kidney of wild-type mice. Administration of BTP2, a pyrazol derivative known to inhibit function of several TRPC channels, also ameliorated obstruction-induced renal fibrosis and gene expression in wild-type mice. BTP2 inhibited carbachol-activated TRPC3 and TRPC6 channel activities in HEK293 cells. Ureteral obstruction caused over a 10-fold increase in mRNA expression for TRPC3 as well as TRPC6 in the kidneys of obstructed relative to the sham-operated mice. The magnitude of protection against obstruction-induced fibrosis in *Trpc3* and *Trpc6* double knockout mice was not different from that in *Trpc6* knockout mice. Klotho, a membrane and soluble protein predominantly produced in the kidney, is known to confer protection against renal fibrosis. Administration of soluble klotho significantly reduced obstruction-induced renal fibrosis in wild-type mice, but not in *Trpc6* knockout mice, indicating that klotho and TRPC6 inhibition act in the same pathway to protect against obstruction-induced renal fibrosis. Thus klotho and TRPC6 may be pharmacologic targets for treating renal fibrosis.

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Chronic kidney disease (CKD) is an increasingly growing global health burden. In the United States and worldwide, CKD affects approximately 10% of the general population.[1] Fibrosis is a hallmark of progressive renal disease, regardless of the underlying cause.[2] After injury and nephron loss, the remaining nephrons hypertrophy to meet the increased demand. In severe cases, the process becomes maladaptive, causing injury to the remaining nephrons and further nephron loss, thus leading to a vicious cycle that eventually results in renal failure. Scar formation is an important part of tissue repair, yet excess leads to fibrosis and organ failure. Clinically, CKD progression correlates best with renal interstitial fibrosis rather than with glomerular pathology.[3] Therefore, a better understanding of the pathways that cause renal fibrosis and promote disease progression is important for developing effective therapeutic options.

Cells have adopted the calcium ion (Ca^{2+}) as an important signaling molecule.[4] Ca^{2+} signaling affects many aspects of cell functions, for example, cell proliferation and differentiation.[5] The canonical transient receptor potential (TRPC) family channels are nonselective, Ca^{2+}-permeable cation channels expressed in the plasma membrane of many tissues.[6] Within this channel family, TRPC6 has been increasingly recognized to be involved in the pathogenesis of many diseases, such as familial focal segmental glomerulosclerosis.[7]

Proliferation of fibroblasts and its activation into myofibroblasts is a key step in renal tissue repair and fibrosis.[2,8] Myofibroblasts are fibroblast-like mesenchymal cells with contractile capability. They often express α-smooth muscle actin (αSMA),[8] synthesize extracellular matrix (ECM) macromolecules, and remodel the extracellular matrix.[9] In the

kidney, following injury or in response to cytokines and other factors, myofibroblasts can be generated by different activation, differentiation, and transdifferentiation processes.[8] In skin and cardiac healing models, TRPC6 is necessary and sufficient for myofibroblast differentiation induced by angiotensin II (Ang-II) or transforming growth factor-β (TGF-β).[10] The self-sustaining abnormal Ca^{2+} entry and gene expression is responsible for disease pathogenesis of many gain-of-function TRPC6 channelopathies. *TRPC3* and *TRPC6* genes contain nuclear-factor-of-activated-T cells promoter response elements. Increased Ca^{2+} entry via TRPC3/6 may further stimulate expression of TRPC3/6 channels by activating calcineurin/nuclear-factor-of-activated-T cells signaling, causing positive feed-forward amplification and unremitting gene expression.[11] A recent study implicates TRPC3 in the pathogenesis of renal fibrosis.[12] The role of TRPC6 in renal fibrosis is yet unknown.

Klotho is a single-pass type-1 transmembrane protein predominantly produced in the kidney that exerts some antiaging function.[13] Membranous klotho interacts with fibroblast growth factor receptor and functions as a coreceptor for the ligand fibroblast growth factor 23, a bone-derived endocrine factor that negatively regulates body phosphate homeostasis.[14,15] Soluble klotho, cleaved from the ectodomain of membrane klotho, is a protein with pleiotropic functions including regulation of ion transport and growth factor signaling by acting as a paracrine or endocrine factor.[16] Previous studies have shown that loss of klotho aggravates renal fibrosis, whereas overexpression of klotho inhibits TGF-β1 signaling and suppresses renal fibrosis by antagonism of TGF-β1 and Wnt/β-catenin signaling.[17–19] Our recent data demonstrate heterozygous *klotho*-hypomorphic (*kl*/+) CKD mice have aggravated cardiac fibrosis relative to wild-type (WT) CKD mice, and that klotho protects against uremic cardiac pathology, at least partly, by down-regulation of TRPC6-mediated abnormal Ca^{2+} signaling in the heart.[20,21]

In the present study, we test the hypothesis that TRPC6 is critical for myofibroblast generation in kidney fibrosis. Given multiple potential pathways for soluble klotho to protect organs, we further investigated the relationship of TRPC6 with klotho's protective action on renal fibrosis. We report that inhibition of TRPC6 achieved by *Trpc6* gene deletion or pharmacological means protects against renal fibrosis and that TRPC6 inhibition acts in the same pathway of protection by soluble klotho. We discuss the implications of our results in light of current literature.

RESULTS

UUO induces renal fibrosis, and *Trpc6* KO in mice partially ameliorates UUO-induced fibrosis

Unilateral ureteral obstruction (UUO) is a common experimental model for the study of mechanisms of renal fibrosis. It has the advantage to induce accelerated renal fibrosis while recapitulating essential cellular processes in the progression of interstitial fibrosis, including cellular infiltration, myofibroblast generation and accumulation, increased extracellular matrix deposition, and tubular atrophy.[22] To elucidate the role of TRPC6 in the renal fibrosis, we asked if *Trpc6* knockout (KO) would alter fibrosis and expression of the genes involved in fibrogenic processes using a UUO experimental model. *Trpc6*$^{-/-}$ mice were previously described.[21] After 10 days, expression of multiple fibrosis-related gene markers was markedly increased in the obstructed kidney relative to contralateral sham kidney in WT and *Trpc6*$^{-/-}$ mice (Figure 1a–h). The increase in expression of mRNAs for collagen-1, connective tissue growth factor (CTGF), αSMA, matrix metalloproteinase (MMP)-2, vimentin, TGF-β1, and MMP-9 in UUO kidneys of *Trpc6* KO mice was blunted relative to WT mice. Expression of snail-1, a marker of mesenchymal-to-epithelial transitions in kidney fibrosis,[23] was not different in UUO kidneys between *Trpc6*$^{-/-}$ and WT mice (Figure 1h). Basal level expression of these genes in sham kidneys was not different between *Trpc6*$^{-/-}$ and WT mice. Histological evidence of fibrosis was evaluated by staining collagen using Masson trichrome staining. The increases in Masson trichrome-positive areas in WT UUO versus sham kidneys indicate marked interstitial fibrosis induced by UUO (Figure 1i and j; see Supplementary Figure S1 for low-magnification view). Consistent with the finding of UUO-increased *Trpc6* gene expression, *Trpc6* deletion decreased fibrosis as shown by decreased trichrome-positive areas. Basal Masson trichrome staining in sham kidneys of *Trpc6*$^{-/-}$ mice was comparable to staining in WT animals.

Pharmacological inhibition of TRPC ameliorates renal fibrosis

BTP2 (4-methyl-4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carbox anilide) is a TRPC channel blocker without subtype selectivity among members of the TRPC family.[24] It was shown that administration of BTP2 attenuated cardiac hypertrophy presumably by inhibiting cardiac TRPC3 and TRPC6 channels.[24] We first tested the effects of BTP2 on recombinant TRPC3/6/7 channel activities in HEK293 cells. As shown, BTP2 inhibited the activity of TRPC3 and TRPC6 with similar efficacy (Figure 2a and b) at concentrations in agreement with a previous report.[24] BTP2 also inhibited TRPC7 activity at similar concentrations (Figure 2c). At 10-fold higher concentration (100 μM), BTP2 had no effect on an inward-rectifying K^+ channel ROMK, renal outer medullary potassium channel, or TRPV5, a member of the V (vanilloid)-type TRP channel group (Figure 2d and e), demonstrating specificity of BTP2 for TRPC channels.

Having demonstrated the specificity and efficacy of BTP2 on diacylglycerol-sensitive TRPC3/6/7 channels, we next examined the effect of BTP2 on the development of renal fibrosis in UUO model. Daily intraperitoneal injection of BTP2 at 2 mg/kg for 7 days significantly ameliorated the