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FGF23 activates injury-primed renal fibroblasts via FGFR4-dependent signalling and enhancement of TGF-β autoinduction

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ABSTRACT

Bone-derived fibroblast growth factor 23 (FGF23) is an important endocrine regulator of mineral homeostasis with effects transduced by cognate FGF receptor (FGFR)1-α-Klotho complexes. Circulating FGF23 levels rise precipitously in patients with kidney disease and portend worse renal and cardiovascular outcomes. *De novo* expression of FGF23 has been found in the heart and kidney following injury but its significance remains unclear. Studies showing that exposure to chronically high FGF23 concentrations activates hypertrophic gene programs in the cardiomyocyte has spawned intense interest in other pathological off-target effects of FGF23 excess. In the kidney, observational evidence points to a concordance of ectopic renal FGF23 expression and the activation of local transforming growth factor (TGF)-β signalling. Although we have previously shown that FGF23 activates injury-primed renal fibroblasts *in vitro*, our understanding of the mechanism underpinning these effects was incomplete. Here we show that in the absence of α-Klotho, FGF23 augments pro-fibrotic signalling cascades in injury-primed renal fibroblasts via activation of FGFR4 and upregulation of the calcium transporter, transient receptor potential cation channel 6. The resultant rise in intracellular calcium and production of mitochondrial reactive oxygen species induced expression of NFAT responsive-genes and enhanced TGF-β1 autoinduction through non-canonical JNK-dependent pathways. Reconstitution with transmembrane α-Klotho, or its soluble ectodomain, restored classical Egr signalling and antagonised FGF23-driven myofibroblast differentiation. Thus, renal FGF23 may amplify local myofibroblast activation in injury and perpetuate pro-fibrotic signalling. These findings strengthen the rationale for exploring therapeutic inhibition of FGFR4 or restoration of α-Klotho as upstream regulators of off-target FGF23 effects.

1. Introduction

Fibroblast growth factor 23 (FGF23) is a central endocrine regulator of mineral homeostasis secreted by osteoblasts and osteocytes (Quarles, 2012). Circulating levels of FGF23 rise early in the course of disease in patients with renal impairment (Isakova et al., 2011a), initially as an adaptive response to maintain normal mineral handling in the face of a progressive loss in functioning renal mass and reduction in the ability to excrete phosphate.

High FGF23 concentrations are strongly predictive of cardiovascular dysfunction and mortality (Ford et al., 2012; Gutierrez et al., 2009; Isakova et al., 2011b; Kendrick et al., 2011), and the progression of Chronic Kidney Disease (CKD) in this setting (Fliser et al., 2007; Isakova et al., 2011b; Kendrick et al., 2011; Rebholz et al., 2015). Physiologically, FGF23 signals through binding to the C-isoform of one of several FGF receptors (FGFR), in complex with α-Klotho (αKL), an obligate co-receptor that confers tissue specificity, high-affinity binding and selective activation of downstream Egr-1/Erk signalling pathways principally in the kidney (Smith et al., 2014; Urakawa et al., 2006). As much as CKD can be considered a state of FGF23 excess, it is also state of αKL deficiency. The reduction in αKL appears to reflect suppressed expression rather than loss of functional tubules, with various mechanisms implicated: signalling from inflammatory cytokines, reactive oxygen species (ROS), activation of the renin-angiotensin system, uraemic toxins (e.g. indoxyl sulphate), dysregulated mineral metabolism itself and epigenetic changes such as increased histone acetylation and DNA hypermethylation of the αKL gene promoter [reviewed in Neyra and Hu, 2017].

Abbreviations: $[Ca^{2+}]_{ic}$, intracellular calcium concentration; CA-NFAT, constitutively-active NFAT; FGF, fibroblast growth factor; Fn1, fibronectin; Egr, early growth response protein; JNK, c-Jun N-terminal kinases; KL, α-klotho; NRKF, normal rat kidney fibroblast; PLC, phospholipase C; ROS, reactive oxygen species; SMA, smooth muscle actin; TGF, transforming growth factor; TRPC, transient receptor potential cation channel

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In contrast to FGFRs which are fairly ubiquitously expressed, αKL is restricted to very few tissues including the kidney, brain, parathyroid glands and testis (Kuro-o et al., 1997). In the kidney, αKL is highly expressed in the distal renal tubules, and, to a lesser extent, proximal renal tubules, as a ~130 kDa single-pass transmembrane protein (Tan et al., 2014). Cleavage of the extracellular domain by secretases yields a soluble form which is present in the circulation and exerts multiple paracrine and endocrine biological functions independently of FGF23 and discrete from those of the membrane receptor (Bloch et al., 2009; Neyra and Hu, 2016). Alternative splicing of the *Klotho* gene putatively generates a further soluble secreted form of the protein (Matsumura et al., 1998), although this product has not reproducibly detected since its original description and it is not considered to contribute appreciably to the circulating pool (Imura et al., 2004).

In the proximal renal tubule, αKL-dependent FGF23 signalling reduces phosphate reabsorption through the downregulation of sodium-dependent phosphate transporters (NaPi2a/2c). FGF23 also inhibits active $1,25(OH)_2$ vitamin D synthesis through inhibition of 1-α-hydroxylation of 25(OH)D by Cyp27b1 and upregulation of the 24-hydroxylase Cyp24a1 which converts $1,25(OH)_2D$ to the inactive $24,25(OH)_2D$ metabolite (Liu et al., 2006; Martin et al., 2012). However, recent landmark studies suggest that, in the absence of αKL, FGF23 excess can drive off-target hypertrophic gene programs in the cardiomyocyte via activation of FGFR4 and downstream Nuclear Factor of Activated T-cells (NFAT) signalling pathways. Pathologically, this manifests in left ventricular hypertrophy, cardiac fibrosis and dysfunction (Faul et al., 2011; Grabner et al., 2015). This has led to an interest in other potential off-target effects of exposure to chronically high levels of FGF23 seen in CKD.

Although FGF23 is principally made in bone (Ubaidus et al., 2009; Yoshiko et al., 2007), *de novo* expression of FGF23 has been observed in the cardiomyocyte (Andrukhova et al., 2015) and cardiac fibroblast (Hao et al., 2016) following ischaemic injury, and in the kidneys of animals with type 2 diabetic nephropathy (Zanchi et al., 2013), polycystic kidney disease (Spichtig et al., 2014) and following sub-total nephrectomy (Mace et al., 2017). Production at these extraosseous sites does not appear to be regulated by classical endocrine feedback loops ($1,25(OH)_2$ vitamin D or PTH) or by canonical FGFR signalling as occurs in bone (Martin et al., 2011; Wohrle et al., 2011) and may therefore be amenable to specific therapeutic targeting. Our own studies have also demonstrated tubular and interstitial expression of FGF23 in a well-established model of experimental renal fibrosis, unilateral ureteric obstruction (UUO) (Smith et al., 2017). However, the potential significance of such ectopic expression remains uncertain.

Comparative transcriptome analysis of kidney tissue from CKD and non-CKD models of FGF23 excess identified the activation of common pathways associated with fibrosis and inflammation, including transforming growth factor (TGF)-β1 related signalling (Dai et al., 2012), a master regulator of fibrosis (Meng et al., 2016a). This suggested that FGF23 may promote renal fibrosis, as appears to be the case in the heart (Hao et al., 2016), and is in line with other contemporary studies of uraemic animals, where renal FGF23 expression has been temporally correlated with local *Tgfβ1* expression (Mace et al., 2017). In addition to finding *de novo* expression of FGF23 in response to tubulointerstitial injury *in vivo*, we have also previously demonstrated that FGF23 could enhance fibrogenic signalling in fibroblasts derived from obstructed/injured kidneys - UUO fibroblasts (UUOF) (Smith et al., 2017). In these cells, FGF23 was found to enhance fibroblast differentiation (*de novo* αSMA expression) and upregulate the expression of extracellular matrix components (e.g. collagens, fibronectin). While mechanistically this appeared to be partly mediated by TGF-β1, inhibition of canonical TGF-β pathways did not suppress the pro-fibrotic effects of FGF23 completely (Smith et al., 2017). This pointed to the involvement of other signalling pathways which at the time awaited further elucidation. Intriguingly, in contrast to injury-primed cells, FGF23 failed to stimulate the same fibrogenic pathways in fibroblasts derived from normal rats kidneys (NRKF), and, if anything suppressed basal αSMA expression. Characterisation of these two cells types revealed marked differences in FGF and TGF-β surface receptor abundance, but critically, a total absence of αKL in UUOF (Smith et al., 2017).

The present study was performed to fully delineate the signalling mechanisms by which FGF23 promotes fibrogenesis in injury-primed renal fibroblasts, to explore the role of αKL in determining the response of fibroblasts to FGF23 and to provide potential novel targets for therapeutic manipulation.

2. Materials and methods

2.1. Growth factors, inhibitors and antibodies

Chemicals were analytical grade and purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Recombinant mouse FGF23 (NS0-derived 6-His-tagged Tyr25-Val251 [Arg179Gln]; #2629-FG) and recombinant human klotho (NS0-derived 6-His-tagged Glu34-Ser981 corresponding to the extracellular domain of αKL; #5334-KL) were obtained from R & D Systems (Minneapolis, MN, USA). We confirmed the biological activity of the recombinant mouse FGF23 by demonstrating a specific dose-dependent increase in Erg1 and SRE luciferase reporter activity in HEK293 cells transiently expressing V5-tagged membrane αKL (Smith et al., 2017). This is consistent with previous reports by other authors (de Jong et al., 2017; Faul et al., 2011), which have shown this source of recombinant protein to have biological activity *in vitro* (Erg1/Erk induction) and *in vivo* (i.e. phosphaturic). Antioxidant and cytoprotective effects of soluble klotho were confirmed as previously described (Hu et al., 2013; Wang et al., 2012). Details of the inhibitors used are listed in Table S1. None of the compounds were found to significantly reduce cell viability (MTT assay) over 24 h, when used at the stated concentrations. Primary and secondary antibodies used are listed in Table S2 and Table S3 respectively.

2.2. Fibroblast culture and treatments

Primary cultures of fibroblasts were propagated from normal kidneys (NRKF) and fibrotic kidneys (3 days after UUO; UUOF) from Sprague-Dawley rats, as described before (Grimwood and Masterson, 2009). Cultures were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2.2% HEPES, 1% L-glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL) in a humidified incubator at 37° C and 5% CO_2. Cells were characterised by immunocytochemical staining prior to use. They were positive for the mesenchymal marker vimentin, negative for the epithelial marker cytokeratin, and only occasionally positive for desmin (a marker of mesangial cells and some myofibroblasts). Based on αSMA staining, 100% of UUOF were myofibroblasts when cultured in this basal media. For experimental work, cells were seeded into 6-well plates (Costar, Corning, NY, USA) at 1×10^6 cells/well for qRT-PCR, Western blotting and flow cytometric analyses or in 25 cm^2 flasks (TPP, Trasadingen, Switzerland) at 5×10^6 cells/flask for mRNA profiling and immunoprecipitation studies. Transfections were performed in 6-well plates seeded at 1×10^6 cells/well or 24-well plates seeded at 5×10^4 cells/well. After attachment overnight and removal of floating cells, fibroblasts were typically cultured for a further 24–48 h in maintenance growth medium before switching to FBS-reduced media (1% FBS) for 24 h before all experimental interventions.

In general, our experimental approach was to look for effects of FGF23 (diluted in PBS) on fibroblast activation through changes in signal transduction (30 min), mRNA expression of fibrogenic mediators (24 h) and at the protein level (48 h) compared to vehicle treatment (PBS). mRNA and protein targets were selected to match those studied *in vivo* (Smith et al., 2017). In some experiments cells were pretreated with receptor inhibitors or cell-permeable inhibitors of signalling mediators (or vehicle) at the concentrations stated in the text for