Chapter 5

Fibroblast Growth Factor 23 Modifies the Pharmacological Effects of Angiotensin Receptor Blockade in Experimental Renal Fibrosis

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ABSTRACT

Background. Blockade of the renin-angiotensin-aldosterone system (RAAS) retards progression of chronic kidney disease. Yet, in many patients, the renoprotective effect is incomplete. A high circulating level of the phosphaturic hormone fibroblast growth factor 23 is associated with an impaired response to RAAS blockade-based therapy in clinical studies. Therefore, we addressed whether administration of recombinant fibroblast growth factor 23 (FGF23) interferes with the efficacy of angiotensin receptor blocker (ARB) treatment in a mouse model of renal fibrosis [unilateral ureteral obstruction (UOO)].

Methods. UOO mice were treated with losartan (100 mg/L in drinking water), recombinant FGF23 (160 ng/kg i.p. twice daily), their combination or vehicle (n = 10 per group). Seven days after the UOO procedure, kidney tissue was analyzed for markers of RAAS activity, inflammation and fibrosis using real-time PCR and immunohistochemistry.

Results. In the contralateral (non-affected) kidneys of ARB-treated UOO mice, administration of FGF23 reversed the induction of renin, ACE, ACE2 and AT1 receptor mRNA expression, suggesting interference with the physiological response to RAAS blockade by FGF23. Furthermore, recombinant FGF23 infusion prevented ARB-induced klotho up-regulation in contralateral kidneys. In the UOO kidneys, klotho was majorly reduced in all groups. Pro-inflammatory gene expression (MCP-1, TNF-α) induced in UOO kidneys was reduced by ARB treatment; this anti-inflammatory effect was reversed by FGF23. In contrast, ARB-induced reduction of (pre-)fibrotic gene expression was not reversed by FGF23.

Conclusion. Our findings show pharmacological interaction between exogenous FGF23 and losartan, thus serving as a proof of principle for crosstalk between the FGF23-klotho axis and RAAS.
5.1 Introduction

Blockade of the renin–angiotensin–aldosterone system (RAAS) retards the progression of chronic kidney disease (CKD) by reducing blood pressure and proteinuria and by exerting anti-inflammatory and antifibrotic effects\(^1\). However, the renoprotective effect of RAAS blockade is incomplete in many patients and renal function may deteriorate despite optimally dosed RAAS blockade. Therefore, it is conceivable that additional pathological mechanisms may hamper or bypass renoprotection by RAAS blockade.

Recent data suggest interaction between RAAS blockade and the vitamin D–fibroblast growth factor 23 (FGF23)–klotho axis\(^2\). More specifically, a higher plasma level of the phosphaturic hormone FGF23 has been associated with an impaired response to intensified RAAS blockade–based antiproteinuric therapy in CKD patients\(^3\). This is consistent with the prior observation that a higher serum phosphate level is associated with a reduced renoprotective efficacy of angiotensin-converting enzyme (ACE) inhibition in the REIN trial\(^4\). Serum FGF23 levels rise early during the course of CKD, and elevated FGF23 levels are strongly associated with progressive renal function loss\(^5,6\) and mortality\(^7\) in CKD. Together, these findings suggest that FGF23 may impair the renoprotective effects of RAAS blockade, thus contributing to negative outcomes. Alternatively, it is plausible that FGF23 in itself may contribute to the development of renal damage. Yet, the renal effects of exogenous FGF23 infusion are unknown.

In light of these observations, first, we studied whether exogenous FGF23 administration modulates renal RAAS genes and aggravates renal inflammation and fibrosis. This was investigated in the mouse unilateral ureteral obstruction (UUO) model, characterized by severe renal fibrosis in the UUO kidney and an unaffected contralateral kidney, allowing simultaneous evaluation of pharmacological and physiological effects on functional kidney tissue. Second, we investigated whether FGF23 administration modulates the pharmacological effects of RAAS blockade in fibrotic and unaffected kidney and, subsequently, whether FGF23 impairs the capacity of RAAS blockade to reduce renal inflammation and fibrosis in the UUO model.

5.2 Materials and Method

5.2.1 Animal model

Male C57BL/6 mice 8–10 weeks old were obtained from Harlan (Zeist, The Netherlands) and were housed under standard conditions. All experimental procedures were approved by the Animal Ethics Committee of the University of Groningen (DEC6475A).

The UUO model was used as a model of renal inflammation and fibrosis. The UUO procedure was performed under isoflurane anaesthesia. Buprenorphine 0.05–0.1 mg/kg, administered subcutaneously every 8 h, was used as postoperative analgesic. The left kidney was exposed via midline abdominal incision and the ureter was ligated at two sites. Sham-operated mice underwent the same procedure, with the difference that the left ureter was only exposed and manipulated, but not ligated.

UUO mice were divided into four treatment groups (all \(n = 10\)). Group I received vehicle treatment (MOPS/Na\(_2\)SO\(_4\)/EDTA/DTT buffer in PBS, i.p. twice daily), while group II received mouse recombinant FGF23 [Tyr25-Val251 (Arg179Gln), 6-His tagged]
via i.p. injections (160 µg/kg, twice daily). Both the buffer and the recombinant FGF23 were obtained from R&D Systems (Minneapolis, MN, USA). Group III was treated with the angiotensin II receptor blocker (ARB) losartan (100 mg/L in drinking water; Santa Cruz Biotechnology, Heidelberg, Germany) combined with i.p. vehicle treatment. Group IV received the combination of losartan and recombinant FGF23. Sham control mice were not treated with either losartan or recombinant FGF23 and served as time controls.

Treatment with FGF23 started 3 days before UUO. The dose of FGF23 was chosen based on a pilot experiment in which this dose demonstrated optimal efficacy to lower serum phosphate levels (Supplementary material, Figure 5.7).

Measurement of serum phosphate, calcium, creatinine and urea took place at 3 and 7 days after UUO surgery, using a Roche Modular (Roche Diagnostics, Lewes, UK). Plasma levels of C-terminal FGF23 were measured for both time points in the sham, vehicle-treated and FGF23-treated groups using a C-terminal mouse FGF23 ELISA (Immutopics, San Clemente, CA, USA). Animals were sacrificed seven days after UUO surgery. After sacrifice, kidneys were flushed and harvested. One half of each kidney was snap frozen in liquid nitrogen and subsequently stored at −80°C, the other half was fixed in 4% formalin solution and further processed for paraffin embedding.

5.2.2 Quantitative PCR

Total RNA was isolated from whole kidney lysates using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. Subsequently, cDNA was synthesized from 1 μg of RNA using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR was performed using the SensiMix SYBR No-ROX Kit (Bioline Reagents, London, UK) and QuantiTect primer assays (Qiagen) or custom-designed primers (Biolegio, Nijmegen, The Netherlands) in a CFX384 thermal cycler (Bio-Rad Laboratories, Berkeley, CA, USA). Primer assay product codes and sequences are provided in Supplementary material, Table 5.2. Transcript expression levels were normalized to the ribosomal housekeeping gene 36b4 (RPLP0) and are presented as 2−ΔCt.

5.2.3 Immunohistochemistry and quantification

Paraffin sections (3 µm) were stained for fibronectin (rabbit-anti-fibronectin; ab6584, Abcam, Cambridge, UK), α-smooth muscle actin (α-SMA) (mouse-anti-α-SMA clone ASM-1, mIgG2a; Progen Biotechnik, Heidelberg, Germany) and CD3+ mature T-lymphocytes (rabbit-anti-CD3; A0452, DAKO, Glostrup, Denmark). Sections were deparaffinized, rehydrated and subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris-HCl buffer (pH 9.0) at 80°C. Endogenous peroxidase activity was blocked with 0.3% H2O2/PBS for 30 min followed by incubation with primary antibodies for 1 h at room temperature. The primary antibody was followed by sequential incubations with HRP-labelled secondary antibodies (DAKO) for 30 min at room temperature. Peroxidase activity was developed through incubation for 10 min with 3,3’-diaminobenzidine tetrachloride (DAB; fibronectin and α-SMA) or for 15 min with 3-amino-9-ethylcarbazole (AEC; CD3).

The extent of interstitial target expression was measured using computer-assisted morphometry in 10 randomly selected tubulo-interstitial cortical fields per section. The area
percentage that was positive for staining was determined using ImageJ software (version 1.44p, National Institutes of Health, Bethesda, MD, USA). Blood vessels and glomeruli were excluded from the analysis. The ultimate score was calculated by averaging all fields per section.

5.2.4 Statistical analysis

Statistical analysis was performed using SPSS Statistics 20.0.0.2 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Mann–Whitney U tests were used to test group pairs based on an a priori hypothesis. The Kruskal–Wallis one-way analysis of variance was used for comparison of multiple groups. Results were considered significant at the $P < 0.05$ level. All data are displayed as mean ± SD or median [interquartile range (IQR)].

5.3 Results

5.3.1 Biochemical parameters

Plasma C-terminal FGF23 levels were slightly but significantly higher in vehicle-treated UUO animals than those in sham animals; FGF23 infusion led to significantly higher plasma FGF23 levels both at 3 and 7 days (Figure 5.1). Plasma FGF23 did not change significantly over time in the VEH ($P = 0.14$) or FGF23 infusion ($P = 0.15$) groups. Serum creatinine and BUN levels were slightly higher in UUO animals both 3 and 7 days after induction of the model; no significant differences were observed between the UUO treatment groups (Table 5.1). Three days after UUO induction, recombinant FGF23 reduced serum phosphate compared with the UUO + vehicle group, irrespective of co-treatment with losartan. This effect was no longer observed after 7 days. Body weight was similar in all groups.

5.3.2 Renal EGR1 expression

To demonstrate the biological activity of FGF23 treatment, we quantified early growth response 1 (EGR1) gene expression in both UUO and contralateral (unaffected) kidneys. In UUO kidneys, no differences in EGR1 expression were observed between groups (Figure 5.2). In contrast, in the contralateral kidneys, FGF23 administration induced a sig-

\[\text{Table 5.1: Biochemical parameters during the study.} \quad * P < 0.05 \text{ versus sham, } ** P < 0.05 \text{ versus vehicle (VEH). Data displayed as mean ±SD.} \]

<table>
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<td>UO FGF23</td>
<td>ARB FGF23 + ARB</td>
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<td>24.3 ± 1.0</td>
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<td>BUN (mmol/L)</td>
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<td>12.6 ± 1.1*</td>
</tr>
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<td>Phosphate (mmol/L)</td>
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<td>2.2 ± 0.2</td>
<td>1.8 ± 0.1***</td>
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</table>
Figure 5.1: Plasma C-terminal FGF23 levels in sham, vehicle-treated and FGF23-treated mice, 3 days and 7 days after UUO surgery. **P \leq 0.01 versus sham. ##P < 0.05 versus vehicle. ###P < 0.001 versus vehicle. Data shown as median with IQR.

Figure 5.2: EGR1 gene expression (normalized to 36b4) in UUO (red bars) and contralateral kidneys (blue bars) 7 days after UUO surgery. ###P < 0.001 versus contralateral vehicle. Data shown as median with IQR.

significant increase in EGR1 expression over vehicle controls; a similar trend was observed in animals treated with FGF23/ARB (P = 0.07).

5.3.3 Renal α-klotho gene expression

FGF23 signalling relies on the presence of the membrane-bound co-factor α-klotho for its renal effects. Therefore, we quantified α-klotho mRNA expression in both UUO and contralateral kidneys. UUO kidneys from all treatment groups showed significantly lower α-klotho expression levels than the sham control group (Figure 5.3). Conversely, in the
contralateral kidneys, α-klotho expression was similar in vehicle-treated UUO mice and sham controls. FGF23 treatment alone did not significantly influence klotho expression. However, ARB treatment increased α-klotho mRNA expression compared with vehicle controls, while this ARB-induced increase in klotho expression was absent in the group co-treated with FGF23.

5.3.4 Renal RAAS genes

We further explored the effects of intervention with FGF23, ARB or their combination on the expression of renin, ACE, ACE2 and angiotensin type 1 receptor (AT1R) genes in the contralateral kidneys of UUO animals. Losartan induced a major increase in renal renin mRNA expression, which was blunted in animals co-treated with FGF23 (Figure 5.4A). Similarly, losartan treatment increased renal expression of ACE and AT1R in the contralateral kidney, which was prevented by co-treatment with FGF23 (Figure 5.4B and D). Lastly, ACE2 mRNA expression was significantly decreased upon FGF23/ARB co-treatment compared with ARB monotherapy (Figure 5.4C). None of the RAAS genes was affected by FGF23 monotherapy compared with vehicle controls.

5.3.5 Renal inflammation

The effects of FGF23 infusion on renal inflammation were assessed by measuring monocyte chemoattractant protein-1 (MCP-1) and TNF-α gene expression and T-cell accumulation in UUO kidneys. Pro-inflammatory gene expression and T-cell accumulation were strongly increased compared with sham animals (Figure 5.5A–D). In losartan-treated animals, the expression of both renal MCP-1 and TNF-α was decreased compared with the vehicle controls (Figure 5.5A and B). Co-treatment with FGF23 partially but significantly reversed the effects of losartan treatment on MCP-1 (Figure 5.5A) and tended to reverse TNF-α
expression (Figure 5.5B). Neither ARB nor ARB + FGF23 treatment changed the renal accumulation of T cells as reflected by CD3⁺ cells, with notable interindividual differences within the groups (Figure 5.5C). FGF23 in itself did not affect any of the inflammatory markers. No inflammation was present in the contralateral kidneys (data not shown).

5.3.6 Tubular damage and (pre-)fibrotic changes

The tubular damage marker Lcn2 [neutrophil gelatinase-associated lipocalin (NGAL)], the pre-fibrotic marker α-SMA and the fibrosis markers collagen-1 and fibronectin were all strongly upregulated in UUO animals compared with the sham animals (Figure 5.6A–E). ARB treatment significantly reduced renal Lcn2, α-SMA and collagen-1 gene expression (Figure 5.6A–C), although these effects were only minimal and non-significant at the protein level for α-SMA and fibronectin (Figure 5.6D–E). The antifibrotic effect of ARB was not influenced by co-treatment with FGF23. FGF23 monotherapy also did not significantly influence any of the renal (pre-)fibrotic markers.
Figure 5.5: Renal inflammation quantified by (A) MCP-1 and (B) TNF-α mRNA expression and (C) renal T-cell accumulation in UUO kidneys. Renal pro-inflammatory genes were reduced by ARB treatment; this effect was (partly) reversed by FGF23 co-treatment. (D) Representative images of immunostaining for the T-cell marker CD3 in all groups. **P < 0.001 versus sham, # P < 0.05 versus vehicle, ## P < 0.01 versus vehicle, $ P < 0.05 versus ARB. Data shown as median with IQR.

5.4 Discussion

Despite the proven efficacy of ACE inhibitor and ARB therapy in retarding CKD progression, RAAS blockade is unable to prevent renal function loss in the majority of patients. A suboptimal response to RAAS blockade–based therapy is associated with higher levels of serum phosphate\(^4\) and its regulating hormone FGF23\(^3\). These associations prompted us to prospectively study whether FGF23 interferes with the pharmacological and renoprotective efficacy of ARB treatment in a mouse model of renal fibrosis. We also studied potential direct renal adverse effects of FGF23 in a model of renal fibrosis (UUO).

Our main finding is that FGF23 interferes with the pharmacological effects of ARB treatment, as reflected by an interaction with the effects on renal function loss in the majority of patients. A suboptimal response to RAAS blockade–based therapy is associated with higher levels of serum phosphate\(^4\) and its regulating hormone FGF23\(^3\). These associations prompted us to prospectively study whether FGF23 interferes with the pharmacological and renoprotective efficacy of ARB treatment in a mouse model of renal fibrosis. We also studied potential direct renal adverse effects of FGF23 in a model of renal fibrosis (UUO).

Our results suggest that FGF23 influences the pharmacological response to RAAS blockade. ARB treatment induced upregulation of renin, ACE and AR1R gene expression in the contralateral (unaffected) kidney. In addition, FGF23 co-treatment appeared to attenuate the anti-inflammatory effects of ARB therapy at the gene expression level. In contrast, our data do not support the assumption of a direct nephrotoxic or pro-inflammatory effect of FGF23.

Our results suggest that FGF23 influences the pharmacological response to RAAS blockade. ARB treatment induced upregulation of renin, ACE and AR1R gene expression in the contralateral kidney, consistent with previously published data\(^8,9\). Together, we interpret these findings as a physiological response to ARB treatment. Of note, the effects of ARB treatment on renal RAAS genes were reversed by FGF23 co-treatment, suggesting interference with the pharmacological effects of ARB. Furthermore, our results are in keeping with previously published work that revealed downregulation of ACE2 in
Chapter 5

Figure 5.6: Renal mRNA expression of the tubular damage marker (A) Lcn2, (B) pre-fibrotic marker α-SMA, (C) fibrosis marker procollagen-1 and interstitial protein expression of (D) α-SMA and (E) fibronectin in UUO kidneys. Renal (pre-)fibrotic genes were reduced by ARB treatment (A-C); this effect was non-significant at the protein level (D-E) and FGF23 co-treatment did not affect ARB efficacy at the mRNA level (A-C). Representative images for (F) α-SMA and (G) fibronectin staining in all groups. ***P < 0.001 versus sham, # P < 0.05 versus vehicle, ### P < 0.001 versus vehicle. Data shown as median with IQR.

transgenic mouse models of elevated FGF2310.

Although FGF23 in itself did not modify ACE2 expression in our study, the induction of ACE2 expression by losartan treatment was notably absent in the FGF23/losartan co-
Fibroblast Growth Factor 23 treatment group. ACE2 promotes the degradation of angiotensin I and angiotensin II and thereby has important renoprotective as well as cardioprotective properties\textsuperscript{11,12}. Impaired upregulation of ACE2 in response to ARB treatment may therefore contribute to therapy resistance and to an adverse cardio-renal outcome during high FGF23 conditions. The implications of the prevention of ARB-induced ACE and AT1R upregulation by FGF23 infusion are less clear, although these observations are in keeping with the hypothesis that FGF23 interferes with the pharmacological effects of RAAS blockade.

In addition to these effects on RAAS gene expression, klotho expression was significantly increased in the contralateral kidneys of ARB-treated mice. This finding is concordant with previous publications\textsuperscript{13}. In keeping with the observed pattern of RAAS gene expression, FGF23 administration alone did not alter klotho expression, while FGF23 co-treatment induced a significant interaction with the effects of losartan. Klotho is known to have important anti-inflammatory properties, but its expression is also negatively affected by existing inflammation\textsuperscript{14}. Thus, although we hypothesize that the observed effect of FGF23 on klotho indicates attenuated RAAS blockade efficacy, we cannot discriminate whether klotho is a mediator or a target of the interaction between FGF23 and losartan.

Losartan treatment significantly reduced expression of the pro-inflammatory genes MCP-1 and TNF-\(\alpha\) in the UUO kidneys, in line with previous studies\textsuperscript{15,16}. In addition, losartan exerted a minor antifibrotic effect, which was observed at the mRNA level, but not at the protein level. FGF23 infusion in itself did not significantly influence any marker of renal inflammation or fibrosis. While FGF23 injection did not directly affect inflammation or fibrosis, our results suggest that FGF23 treatment did interfere to some extent with the renoprotective effect of losartan. Primarily, renal MCP-1 expression was significantly higher in the FGF23/losartan combination group than in the losartan monotherapy group, while TNF-\(\alpha\) exhibited a similar pattern.

Together, these findings suggest that the interaction between FGF23 and RAAS blockade, at the level of RAAS genes, is at least partly paralleled by impaired renoprotection of ARB treatment during high FGF23 conditions, independent of other factors such as renal function. Yet, this interaction between FGF23 and ARB treatment efficacy was less clear in the UUO kidneys (for parameters of inflammation and fibrosis) than in the contralateral kidneys (for RAAS gene expression). Furthermore, despite the observed changes in gene expression, the interventions in our study did not result in significant differences at the protein level. Therefore, we cannot conclude from our data that FGF23 directly attenuates the renoprotective efficacy of RAAS blockade in this model.

The lack of effect at the protein level is unlikely to be explained by insufficient dosing or technical failure of FGF23 administration in our model. First, we measured a significant and sustained elevation of plasma C-terminal FGF23 levels in FGF23-treated mice compared with vehicle controls. Second, FGF23 induced EGR1 expression, a commonly used marker for \(\alpha\)-klotho-dependent FGF23 signalling activity, in the contralateral kidney\textsuperscript{17}, as well as (transient) hypophosphatemia 3 days after UUO. The reversal of hypophosphatemia after 7 days may be mediated by changes at the receptor level, as suggested by the trend in reduced renal klotho expression in the contralateral kidneys of FGF23-treated animals (Figure 5.3) or by FGF23-mediated inhibition of PTH or active vitamin D. Alternatively, previously published work suggests that the mitigation of hypophosphatemia could be explained through the upregulation of NaPi-2b in jejunal enterocytes.

We hypothesize that the limited effect of FGF23 on the therapeutic response to ARB
treatment, which is notably discrepant with the observations in the contralateral kidneys, can be explained by the severity of renal damage due to ureteral obstruction, and the absence of proteinuria as a driving force in the UUO model. This idea is supported by the high renal expression of fibrosis markers in all UUO groups. Also, klotho expression was uniformly downregulated in the UUO kidney. The pattern of EGR1 expression, which was similar but less pronounced in the UUO kidneys than in the contralateral kidneys, suggests that the very severe renal damage may have limited FGF23 signalling. Thus, any klotho-mediated effects of FGF23 may be understated in this experiment. Lastly, use of the UUO model precludes any conclusions on the interaction between FGF23 and RAAS blockade efficacy in the context of proteinuria-driven, chronic, bilateral renal disease, which should be explored in future studies.

These limitations notwithstanding, the UUO model allowed us to study the pharmacological effects of FGF23 infusion, as a single intervention and combined with ARB treatment, in both injured and healthy kidneys. The UUO model does not rely on a nephrotoxic agent, which could introduce confounding pharmacological interactions. Other advantages of the model include the well-defined moment of onset in all animals and uniformity of renal injury. To our knowledge, our study is the first to study the renal effects of recombinant FGF23 infusion.

In conclusion, our data suggest that FGF23 interferes with the pharmacological effects of RAAS blockade and may also attenuate the effect of RAAS blockade on pro-inflammatory gene expression. Further studies are needed to determine the magnitude and clinical relevance of this crosstalk between FGF23 and the RAAS.
References


Supplementary material

Table

Table 5.2: Sequences / Product codes of qPCR primers.

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Figure

Figure 5.7: Results of a pilot study to identify the optimal recombinant FGF23 dose for the induction of hypophosphatemia. Mice (n = 5 per group) were treated with either a 40 µg/kg or a 160µg/kg dose of FGF23, administered twice with a 12-hour interval, using peritoneal injections. Blood samples were taken 12 hours after the second injection. We observed a significant decrease in serum phosphate (p<0.05) in mice treated with the high dose, but not in mice treated with the low dose, as compared to vehicle control animals.