The Role of FGF23 on Cardiac and Vascular Function in Experimental Chronic Kidney Disease

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CHAPTER 5

The calcimimetic R568 lowers FGF23 but does not improve vascular function in renal failure

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Abstract

Cardiovascular causes account for approximately 50% of mortality in patients with chronic kidney disease (CKD). Calcimimetics are used in CKD patients to treat hyperparathyroidism, but are also associated with a decrease in vascular calcification. We hypothesized that the calcimimetic R568 improves microvascular function in experimental CKD. Eight week-old male C57Bl/6 mice were subjected to partial nephrectomy (5/6Nx) and injected with vehicle or R568 (30 mg/kg/day). After 6 weeks of renal failure, *ex vivo* vascular function was assessed in muscle resistance arteries using pressure myography. Myocardial blood flow was assessed *in vivo* using myocardial contrast echocardiography (MCE). Plasma c-term FGF23 was increased six weeks after surgery in 5/6Nx mice + vehicle (from 187.4 ± 6.4 at baseline vs. 350.7 ± 15.3 pg/ml after 6 weeks, p=0.043). R568 treatment statistically significantly lowered plasma FGF23 concentration in 5/6Nx mice compared to vehicle treatment (286.5 ± 12. vs. 350.7 ± 15.3 pg/ml, p=0.003). In mice with renal failure, R568 treatment did not restore *ex vivo* sensitivity to the endothelium-dependent vasodilator acetylcholine (vehicle vs. R568: log EC50 -7.0±0.2 vs. -6.7±0.1, p=0.23) or the maximal vasodilatation in response to acetylcholine (vehicle vs. R568: 80.4±3.8 vs. 86.6±4.2 a.u., p=0.29). In addition, R568 treatment did not attenuate ex vivo sensitivity to the endothelium-independent vasodilator sodium nitroprusside (SNP) (vehicle vs. R568: log EC50 -7.0±0.2 vs. -6.7±0.1 p=0.19) or the maximal vasodilatation in response to SNP (vehicle vs. R568: 60.4±5.0 vs. 61.0±4.7 a.u., p=0.94). Responses to the vasoconstrictor endothelin were also comparable between groups. *In vivo* microvascular tissue blood volume during acetylcholine infusion into the heart was not increased compared to baseline in both vehicle and R568 treated mice (117±12 and 119±11%, p=0.20 and p=0.16, respectively) and not different between groups (p=0.81). Microvascular blood flow during acetylcholine infusion was only significantly increased in vehicle treated mice and not in R568 treated mice (183±14 and 145±17%, p=0.008 and p=0.19 respectively), but not different between groups (p=0.19). We conclude that the calcimimetic R568 does not improve endothelial function in this mouse model of renal failure. Therefore, our data do not support a role of calcimetics to improve vascular dysfunction in CKD.

Introduction

Chronic kidney disease (CKD) is a major public health problem worldwide with a prevalence of approximately 10% in the United Kingdom (UK) population and 14% in the US population. [1,2] Of note, patients with CKD frequently die from cardiovascular complications before reaching end stage renal disease (ESRD). [3] In those progressing to ESRD an additional 50% die from cardiovascular disease.

Mineral metabolism is altered early in CKD patients [4], characterized by elevations in serum fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH), followed by phosphate, and a decrease in serum 1,25 dihydroxyvitamin D. FGF23 is amongst the first factors that changes in CKD patients [5, 6] and is a strong predictor of cardiovascular events in both the general population and CKD patients. [7-9] Moreover, serum FGF23 concentrations are associated with increased arterial stiffness and impaired vasoreactivity in CKD patients. [10-12] Lowering FGF23 in CKD patients would thus qualify as a new target to combat cardiovascular mortality. Indeed, we previously showed that in an early model of experimental CKD, blocking FGF23 prevented endothelial dysfunction.[13] Unfortunately, FGF23 antibodies cannot be used as a drug in CKD patients, since the consequent increase in plasma phosphate concentrations may increase mortality, as was shown in animal models. [14]

Calcimimetics have originally been developed to treat secondary hyperparathyroidism, but were also shown to decrease FGF23 concentrations in patients on dialysis [15] and improved cardiovascular outcome proportionally to the decrease of FGF23 concentrations. [16] In a small study in patients receiving hemodialysis, treatment with a calcimimetic improved endothelial function by increasing serum nitric oxide (NO) production, but FGF23 serum levels were not measured in that study. [17]

All clinical studies mentioned above have been conducted in patients on dialysis, but whether calcimimetics also improve cardiovascular outcomes in CKD patients prior to dialysis is not known. Here we tested the hypothesis that treatment with the calcimimetic R568 in experimental moderate CKD improves vascular function by lowering FGF23.

Methods

Animals

All male C57BL/6 mice (Charles River Laboratories, Leiden, The Netherlands) were housed under standardized conditions and received water and food *ad libitum*. All experiments were approved and conducted following the guidelines of the local animal ethical committee at the VU University Medical Center, Amsterdam, The Netherlands and complied with Dutch government guidelines.

Induction of CKD

Eight-week old male C57BL/6 mice were subjected to either 5/6 nephrectomy (5/6Nx), as described before,[18] or sham surgery under isoflurane anesthesia and preoperative analgesia (Buprenorphine; Temgesic (Schering-Plough), 0.05 mg/kg intramuscular). Briefly, an abdominal dorsal midline incision was made and the left kidney was decapsulated, after which the upper and lower poles were removed by a bipolar electrocoagulator. In the same procedure the complete decapsulated right kidney was removed after ligation of the renal blood vessels and the ureter. After surgery all mice received subcutaneous injections of postoperative analgesia for two consecutive days (Ketoprofen; Ketofen (Merial S.A.S.), 5 mg/kg). In control mice, sham surgery was performed, which included decapsulation of both kidneys, but no removal of kidney tissue. The rest of the protocol was followed as mentioned above. Six weeks after surgery mice were placed into individual metabolic cages (Tecniplast, Milan, Italy) for collection of 24-hours urine samples. Evaporation of urine was minimized by the addition of paraffin oil to the collection tube.

Intraperitoneal injections with calcimimetic R568

To reduce FGF23 levels in CKD, mice were injected intraperitoneally (i.p.) with the calcimimetic R568 in a dose of 30 mg/kg/day (kindly provided by Amgen Inc. Thousand Oaks, CA). The control group received vehicle treatment. A first R568 suspension was given on the day of nephrectomy surgery and repeated with intraperitoneal injections (10 ml/kg) every 48 hours for the remainder of the study. Six weeks after surgery mice were placed into individual metabolic cages (Tecniplast, Milan, Italy) for collection of 24-hour urine samples, as described above.

CKD-related parameters, electrolytes and FGF23 in plasma

Blood was collected by either tail vein incision or heart-puncture at sacrifice at the end of all experiments, divided into EDTA- and heparin-coagulated microtainers (BD Microtainer tubes, Plymouth, UK) and centrifuged for 10 minutes at 3000rpm at 4°C. Plasma samples were stored at -80°C. Urea, creatinine and phosphate concentrations from EDTA-anticoagulated plasma were determined by in-hospital services using automatic biochemical analyzers. Ca^{2+} concentrations from heparin- anticoagulated plasma or urine samples were determined using a commercial serum standard (Precinorm U, Roche, Switzerland) and measured as described previously.[19] Circulating C-terminal FGF23 concentrations from EDTA- anticoagulated plasma were measured in duplicate using a rodent specific ELISA assay (Immutopics International, San Clemente, CA, USA), according to the manufacturers protocol.-

Ex vivo measurement of resistance artery function

Vasoreactivity of resistance arteries, isolated from the gracilis muscle of mice, was characterized *ex vivo* by pressure myography as described previously.[20] After isolation, resistance arteries were mounted in a pressure myograph after which the diameter was continuously monitored. Resistance arteries were incubated for 45 minutes with KCl (25 mmol/L) to induce vasoconstriction. Acute endothelium-dependent vasodilation of resistance arteries was determined by evaluating diameter responses to acetylcholine (Ach) at 5 different concentrations ranging from 10^{-9} - 10^{-5} mol/L. To assess effects of the calcimimetic on endothelium-independent vasodilation, acute effects of sodium nitroprusside (SNP) were studied at 6 different concentrations ranging from 10^{-9} - 10^{-4} mol/L. Finally, acute endothelium-independent vasoconstriction was studied using endothelin (ET-1) (Sigma-Aldrich, St. Louis, MO, USA) studied at 5 different concentrations (10^{-11} - 10^{-7}, mol/L). To determine acute effects of FGF23 on endothelial function 10 ng/ml of FGF23 (R&D Systems, Minneapolis, MN, USA, catalog # 2629-FG-025/CF) was added to the pressure myograph, one hour prior to the first acetylcholine application.

Myocardial blood flow measurements

To evaluate effects of CKD on myocardial blood flow, mice were anesthetized with intraperitoneal injections of fentanyl, midazolam and acepromazine. A venous line was placed in the jugular vein for infusion of both microbubbles (for ultrasound contrast) and vasodilators. Myocardial contrast echocardiography (MCE) was performed with a Siemens-Acuson Sequoia C512 with a 17L5 transducer (Siemens-Acuson, Mountain View, CA, USA), as described previously.[21] A combination of 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC; Avanti Polar Lipids, Alabama, USA) and polyoxyethylene stearate (PEG40; Sigma, St. Louis, MO, USA), in a molecular ratio of 3:1, was solved in a 0.9% saline, glycerol (Life Sciences) mixture (volume 3:2) in a 2 ml tube with perfluorobutane gas (C4F10(g), F2 Chemicals, Lancashire, UK) in the cap space. Microbubbles were produced by means of mechanical agitation using a VialmixTM (Lantheus Medical Imaging, North Billerica, MA, USA) high-speed shaker. Subsequently, microbubble size distribution and concentration were determined using a Multisizer 3 (Beckham Coulter Nederland, Woerden, the Netherlands), after which microbubbles were diluted in saline to a final concentration of $1*10^9$ microbubbles/ml. Microbubbles were infused at a rate of 7.5 µl/min for two minutes to reach a systemic steady state. Four real-time inflow curves of >10 seconds each, in a long-axis view of the heart in end-systolic phase of the cardiac cycle, were recorded after destruction of the microbubbles by a sequence of 8 high-energy pulses (mechanical index of 1.7). Thirty minutes after baseline measurements were acquired, a vasodilator (acetylcholine (5 µg/kg/min, 15 mg/L) or SNP (3 µg/kg/min, 9 mg/L)) was infused intravenously to assess myocardial

blood flow reserve. After 5 minutes of continuous infusion of the vasodilator and 2 minutes infusion of microbubbles, four real-time inflow curves were obtained as described above.

Microbubble inflow curves were analyzed off-line using the Image Processing toolbox in MATLAB (Mathworks, Natick, MA, USA). Average intensity was measured in a region of interest (ROI), which was manually drawn on the myocardial wall of the left ventricle. No corrections had to be made since microbubble concentrations were equal across all mice and measurements. Video-intensities within the ROI were then fitted to the exponential function $y=A(1-e^{-\beta t})$, whereby y is signal video-intensity at any given time, β is the initial slope of the curve, representing microvascular filling velocity (MFV), and A is the plateau video-intensity, representing the microvascular blood volume (MBV) and t is the time after the start of the inflow curve.

Statistics

Data are presented as mean ± SEM. The number of mice in individual experiments is shown in figure legends. Differences between groups were assessed by Mann-Whitney and within groups by Wilcoxon. For *ex vivo* vascular function analysis linear mixed models were used to test whether the relation between relative increase (outcome/dependent variable) and the vasoactive substance concentration (acetylcholine, SNP or endothelin) differed between groups. The mixed models included a random intercept for mice and fixed effects for groups, vasoactive substance concentration level indicator and their interaction. Main interest was in testing the interaction between groups and vasoactive substance concentration. In case the interaction was not significant, the interaction was removed from the model and the main effect of groups was considered in order to see if groups differed in the relative increase (averaged over the concentrations).

2-tailed *P* values of less than 0.05 were considered statistically significant. Outliers were removed from data sets when samples were more than three times the interquartile range. Analyses were performed using SPSS version 22.

Results

R568 treatment in 5/6Nx mice decreases plasma FGF23 concentrations.

Water intake, urine production, food intake and feces production were not different six weeks after surgery between mice after 5/6 nephrectomy and treated with either vehicle (5/6Nx + vehicle) or R568 (5/6Nx + R568) (Table 1). Plasma samples were pooled to determine phosphate, creatinine and urea concentrations. Six weeks after surgery, plasma phosphate was decreased by 22% in 5/6Nx mice + vehicle, and by 17% in 5/6Nx mice + R568 (Table 2). Plasma creatinine was increased 1.9-fold in 5/6Nx mice + vehicle and 1.8-fold in 5/6Nx mice

+ R568 six weeks after surgery. Plasma urea was increased 3.5-fold in 5/6Nx mice + vehicle and 3.6-fold in 5/6Nx mice + R568 six weeks after surgery.

Plasma c-term FGF23 was increased six weeks after surgery in 5/6Nx mice + vehicle (from 187.4 ± 6.4 at baseline vs. 350.7 ± 15.3 pg/ml after 6 weeks, p=0.043, Figure 1). After six weeks R568 treatment in 5/6Nx mice lowered plasma FGF23 concentrations as compared to vehicle treatment (286.5 ± 12.1 vs. 350.7 ± 15.3 pg/ml, p=0.003, respectively, Figure 1), although plasma FGF23 levels still remained significantly increased in 5/6Nx mice + R568 after six weeks as compared to baseline (286.5 ± 12.1 vs. 187.0 ± 7.1 pg/ml, p=0.043, respectively, Figure 1).

Table 1. Effects of vehicle of R568 treatment in 5/6Nx mice after six weeks on general physiological parameters.

	5/6Nx + vehicle	5/6Nx + R568
Water intake (ml/24h)	5.8 ± 0.2	4.4 ± 0.9
Urine production (ml/24h)	2.6 ± 0.2	2.2 ± 0.5
Food intake (gr/24h)	3.0 ± 0.3	2.6 ± 0.4
Feces production (gr/24h)	0.80 ± 0.07	0.73 ± 0.14

Water intake, urine production, food intake and feces production of mice subjected 5/6Nx surgery and treated with vehicle of R568 for six weeks. Data are mean ± SEM. N=7-8 for both groups.

Table 2. Pooled plasma samples of 5/6Nx mice treated with vehicle or R568.

	5/6Nx + vehicle t=0	5/6Nx + vehicle t=6	5/6Nx + R568 t=0	5/6Nx + R568 t=6
Phosphate (mmol/L)	2.04	1.59	1.98	1.65
Creatinine (µmol/L)	12	23	13	24
Urea (mmol/L)	7.7	27.0	7.7	27.9

Plasma phosphate concentrations in 5/6Nx treated with vehicle were decreased with 22% and in 5/6Nx treated with R568 with 17% after 6 weeks. Plasma creatinine was increased 1.9-fold and 1.8-fold in 5/6Nx mice treated with vehicle or R568 respectively. Plasma urea was increased 3.5-fold and 3.6-fold in 5/6Nx mice treated with vehicle or R568 respectively.
N=16-17 for t=0 and n=13-15 for t=6.

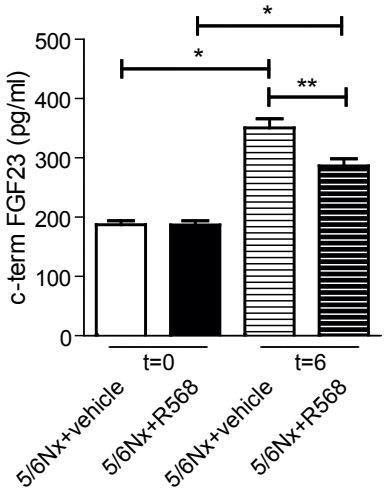

Figure 1. R568 treatment in 5/6Nx mice decreases plasma FGF23 levels after 6 weeks.

5/6Nx surgery increased plasma FGF23 levels 6 weeks after surgery (t=6) by 1.9 fold. R568 treatment decreased FGF23 levels 6 weeks after 5/6Nx surgery as compared to vehicle treatment.
Data are mean ± SEM, * $P \leq 0.05$ and ** $P \leq 0.01$. N=5 for both groups at t=0, n=15 for 5/6Nx+vehicle and n=13 for 5/6Nx+R568 at t=6.

R568 treatment in 5/6Nx mice does not alter vasomotor function ex vivo.

In 5/6Nx mice R568 treatment did not improve *ex vivo* sensitivity to the endothelium-dependent vasodilator acetylcholine (Ach) compared to 5/6Nx mice with vehicle treatment (vehicle vs. R568: log EC50 3.0±0.1 vs. 2.9±0.1 a.u., p=0.25, Figure 2A) or the maximal vasodilatation upon Ach (vehicle vs. R568: 80.4±3.8 vs. 86.6±4.2 % diameter change, p=0.17, Figure 2D). In addition, R568 treatment did not improve *ex vivo* sensitivity to the endothelium-independent vasodilator sodium nitroprusside (SNP) (vehicle vs. R568: log EC50 -7.0±0.2 vs. -6.7±0.09 a.u., p=0.11, Figure 2B) or the maximal vasodilatation upon SNP (vehicle vs. R568: 60.4±5.0 vs. 61.0±4.7 % diameter change, p=0.56, Figure 2E), compared to vehicle treatment in 5/6Nx mice. Vascular responses to the vasoconstrictor endothelin were comparable between groups (Figure 2C) and the maximum vasoconstriction upon endothelin was also comparable (vehicle vs. R568: -69.9±5.3 vs. -66.3±4.6 % diameter change, p=0.46, Figure 2F).

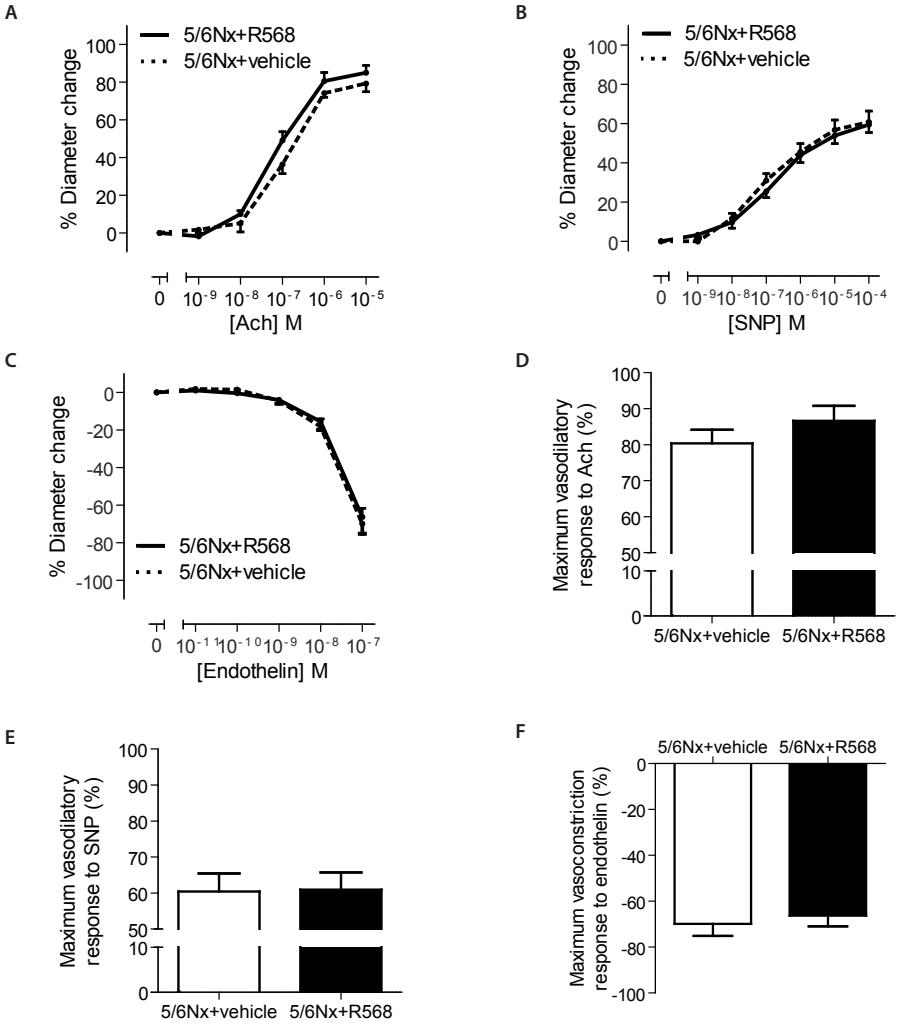

Figure 2. R568 treatment in 5/6Nx mice does not ameliorate endothelium-dependent and endothelium-independent vascular function.

(A) Six weeks of R568 treatment in 5/6Nx mice does not improve endothelium-dependent vasodilator responses of the gracilis artery as compared to vehicle treatment. Solid line: 5/6Nx+R568; dotted line: 5/6Nx+vehicle. **(B)** R568 treatment in 5/6Nx mice does not improve vasodilator responses of gracilis resistance arteries to the endothelium-independent vasodilator sodium nitroprusside (SNP). Solid line: 5/6Nx+R568; dotted line: 5/6Nx+vehicle. **(C)** Six weeks of R568 treatment in 5/6Nx mice does not improve does not change vasoconstrictor responses to endothelin. Solid line: 5/6Nx+R568; dotted line: 5/6Nx+vehicle. **(D)** The maximum vasodilatory response upon acetylcholine (Ach) treatment is not changes in 5/6Nx mice treated with R568 as compared to 5/6Nx mice treated with vehicle. **(E)** R568 treatment in 5/6Nx mice does not alter the maximum vasodilatory upon the endothelium-independent vasodilator SNP as compared to vehicle treatment. **(F)** 6 weeks of R568 treatment in 5/6Nx does not change maximum vasoconstriction response to endothelin as compared to vehicle treatment.
Data are mean ± SEM. N=7 for 5/6Nx+vehicle and n=8 for 5/6Nx+R568.

R568 treatment in 5/6Nx mice does not alter myocardial blood flow.

Acetylcholine did not increase microvascular blood volume in the heart *in vivo* in either vehicle- or R568-treated 5/6Nx mice (117±12, p=0.16 and 119±11%, p=0.14, respectively, Figure 3A) and there was no difference between groups (p=0.82). Acetylcholine infusion increased microvascular filling velocity in the heart in both vehicle and R568 treated 5/6Nx mice (177±32, p=0.012 and 128±9%, p=0.012, respectively, Figure 3B), but was not different between groups (p=0.67). Acetylcholine significantly increased myocardial blood flow in both vehicle treated and in R568 treated 5/6Nx mice (183±14, p=0.012 and 146±17%, p=0.025 respectively, Figure 3C), but these responses were not different between groups (p=0.17).

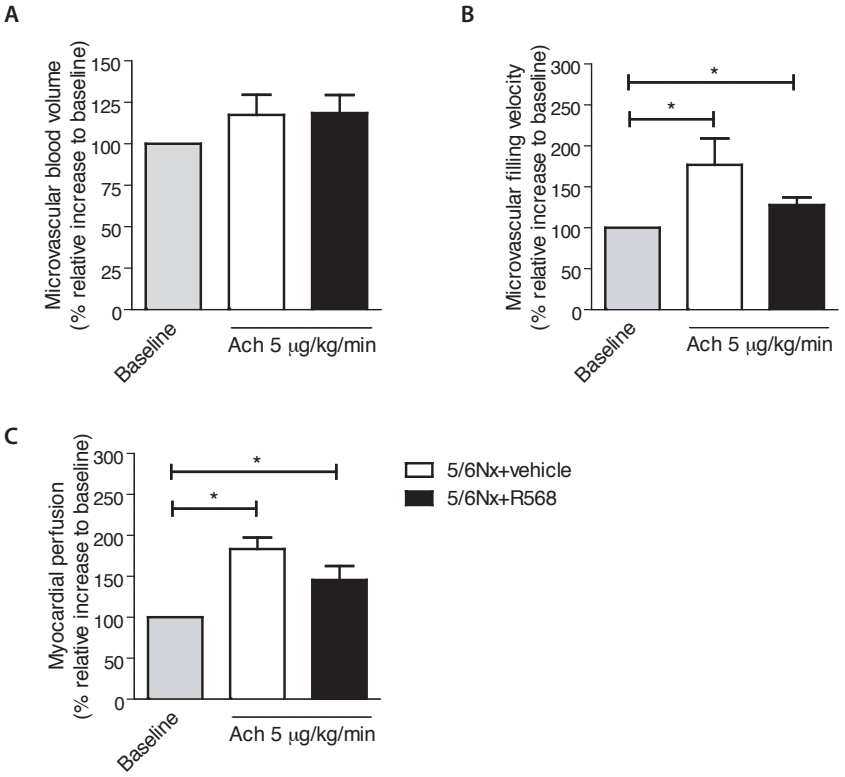

Figure 3. Microvascular blood volume, filling velocity and perfusion in the myocardium are not changed upon R568 treatment in 5/6Nx mice.

(A) Microvascular blood volume is comparable in both 5/6Nx mice treated with R568 or vehicle after acetylcholine (Ach) administration. **(B)** Microvascular filling velocity after Ach administration was not different between 5/6Nx mice treated with R568 or vehicle. **(C)** R568 treatment in 5/6Nx mice did not change myocardial perfusion upon Ach administration as compared to vehicle treatment.
Data are mean ± SEM, * $P \leq 0.05$. N=8-9 for both groups.

Discussion

The main findings of our study are that the calcimimetic R568 attenuates the renal failure-induced increase of plasma FGF23, but does not improve peripheral or myocardial vascular function in experimental CKD. R568 lowering of circulating FGF23 levels did not change endothelium-dependent or -independent vasodilation, nor smooth muscle contractility.

In the present study the gracilis artery was used, a resistance artery in muscle important for regulation of arterial blood pressure and muscle tissue perfusion, and as such part of the microcirculation. We have previously shown endothelial dysfunction in mice with mild CKD, while vascular smooth muscle cell function was intact.[13] In the present study endothelial function was not different between renal failure mice receiving R568 or vehicle, indicating that lowering FGF23 by a calcimimetic in this model of CKD does not prevent endothelial dysfunction. As expected, vasodilator and contractile vascular smooth muscle cell function was not altered, since CKD in this model induces endothelial but not smooth muscle dysfunction.[13]

A prospective clinical trial using the calcimimetic cinacalcet was negative on its primary endpoint,[22] but a posthoc analysis suggested a benefit for cardiovascular events other than of assumed atherosclerotic origin like stroke and acute myocardial infarction. This suggests that especially myocardial function may benefit from calcimimetic treatments. Moreover in another posthoc analysis of the EVOLVE trial it was shown that improved outcome was associated with a decline of FGF23.[16]

A previous study indicated that peripheral endothelial dysfunction predicts the occurrence of major adverse cardiac events [23], which might in part be explained by myocardial perfusion defects. In the present study we observed no changes in myocardial endothelial function upon calcimimeticum treatment of mice with CKD, despite substantial decline of FGF23 by the calcimimentic R568, although the decline in FGF23 here was less pronounced than was seen at week 20 in the EVOLVE trial, which found a decline of over 50%.[16] Perfusion of the endothelium-dependent vasodilator acetylcholine increased both microvascular filling velocity and myocardial blood flow in mice with CKD, but this was not further improved in CKD mice receiving the calcimimetic. This may indicate that also in CKD patients, endothelial dysfunction in the myocardial vasculature may not be attributed to increased FGF23 concentrations, but rather by other uremic toxins. In addition, the suggested improvement in heart failure seen in the EVOLVE trial may not be the consequence of improved myocardial microcirculation.

The lack of improved endothelial function after R568 treatment in this study might be explained by other vascular effects of R568. Moe et al. showed that rats with renal failure treated with R568 reduced vascular calcification,[24] which could explain improved cardiovascular outcome in CKD patients treated with calcimimetics. This was in line with the ADVANCE trial showing attenuated progression of coronary calcification in a secondary

outcome.[25] We did not measure serum calcium, so we are not able to take into account calcimimetic-induced declines of serum calcium levels. Serum phosphate on the other hand was not different between groups.

Another possible explanation why we failed to observe an effect of R568 on peripheral endothelial function is a lack of severe endothelial dysfunction in CKD mice. We previously observed a maximal response of 55% to acetylcholine in CKD mice, while in this study the maximum response to acetylcholine in CKD mice was 80%. Therefore, it is possible that endothelial dysfunction was only mildly present in CKD mice, which makes it difficult to observe R568-induced improved endothelial function.

Our study has several limitations. We did not include a parallel control group without renal failure. Moreover we did not measure plasma PTH concentrations, due to the small blood sample size obtained from mice. Although this would have been an important biomarker of R568 bioactivity, in our experiment we did notice a statistically significant decline of FGF23 which cannot be explained by other ways than by the calcimimetic. We can however not rule out that the final dose we selected was too low. Although in the EVOLVE trial the percentage of plasma FGF23 decline was higher than in our experiment, we studied early CKD and a lower decline in FGF23 was expected.

In summary, R568 attenuates the renal failure-induced of FGF23 but does not prevent peripheral or myocardial endothelial dysfunction. Further research is needed to determine if more aggressive lowering of FGF23 in early CKD patients may improve cardiovascular outcome.

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