FGF23 directly impairs endothelium-dependent vasorelaxation by increasing superoxide levels and reducing nitric oxide bioavailability

Neerupma Silswal,1 Chad D. Touchberry,1 Dorothy R. Daniel,1 Darla L. McCarthy,1 Shiqin Zhang,2 Jon Andresen,1 Jason R. Stubbs,2 and Michael J. Wacker1

1Muscle Biology Group, School of Medicine, University of Missouri-Kansas City, Kansas City, Missouri; and 2The Kidney Institute, University of Kansas Medical Center, Kansas City, Kansas

Submitted 6 June 2014; accepted in final form 15 July 2014


It is well known that patients with chronic kidney disease (CKD) have an increased risk of cardiovascular disease (CVD). Modification of the traditional risk factors for CVD (e.g., dyslipidemia, hypertension, anemia, and hyperhomocysteinemia) does not improve cardiovascular function in patients with CKD (32), suggesting that other factors may be responsible. Fibroblast growth factor 23 (FGF23) is a hormone secreted by osteocytes that serves as an important regulator of serum phosphate and vitamin D via direct actions on the kidney and parathyroid (6, 8). Recently, high circulating levels of FGF23 have been clinically associated with the development of CVD (3, 9, 33, 47, 55, 72) especially during CKD where serum FGF23 is substantially increased 10- to 1,000-fold (30, 37). Nevertheless, despite these clinical associations, there have been relatively few studies to determine whether FGF23 directly induces cardiovascular pathologies or if it is simply a pathological marker.

Recently, Faul et al. (16) and our research group (67) have shown that high concentrations of FGF23 have direct actions on the heart that alter contractile performance and induce cardiac hypertrophy. These data strongly suggest that FGF23 has the potential to directly initiate some of the pathological cardiovascular changes observed during CKD. In addition to direct actions on the heart, a growing body of clinical evidence suggests that circulating FGF23 levels are correlated with impaired vascular function. Due to its role in mineralization, FGF23 was clinically associated with vascular calcification (13, 14, 31, 48, 52, 62). However, most recently it has been shown that FGF23 does not promote (42, 54), but may actually prevent, vascular calcification (76). Nevertheless, FGF23 may have other effects on the vasculature. There are clinical associations between elevated FGF23 and atherosclerosis (44), impaired flow-mediated dilation (75), impaired vasoreactivity, and arterial stiffness (45), which provide a rationale to investigate the role of FGF23 in directly altering endothelial function.

Endothelial dysfunction is characterized by impaired endothelium-dependent relaxation and has been correlated with cardiovascular risk factors like hypertension, diabetes mellitus, and heart failure (53). Collectively, these findings have been important in identifying a possible role for FGF23 in vascular disease during CKD; however, the direct effects of FGF23 on endothelial function in models of vascular function, such as the aorta, remain unexplored.

In this study, we hypothesized that FGF23 would directly impair vasorelaxation by impairing endothelial production of nitric oxide (NO). To address this hypothesis, we explored the effects of FGF23 on the mouse aorta, where NO is the principle endothelium-derived relaxing factor (11). Using isometric tension myography, we examined the ability of FGF23 to alter contraction and relaxation of mouse aorta. Next, we explored the effects of FGF23 treatment on NO production in aortic segments. We repeated these experiments in a genetic animal model of CKD (Col4a3−/−) to elucidate the role of chronically elevated FGF23 in altering aortic function. Last, we explored the effects of FGF23 on steady-state levels of superoxide. Collectively, our results show that FGF23 directly impairs normal endothelial function of the aorta by reducing the bioavailability of NO and increasing superoxide.

Address for reprint requests and other correspondence: M. J. Wacker, School of Medicine, Univ. of Missouri-Kansas City, 2464 Charlotte St., Kansas City, MO 64108 (e-mail: wackermj@umkc.edu).

E426 0193-1849/14 Copyright © 2014 the American Physiological Society http://www.ajpendo.org
METHODS

Chemicals. Recombinant mouse FGF23 was purchased from R&D Systems (Minneapolis, MN). The FGFR inhibitor PD166866 was purchased from EMD Biosciences (San Diego, CA). Hanks' balanced salt solution (HBSS) and the membrane-permeable dye 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM) were obtained from Invitrogen (Carlsbad, CA). Total RNA isolation kits were purchased from IBI Scientific (Peosta, IA), and the real-time reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using a TaqMan RNA-to-CT one-step kit from ABI (Carlsbad, CA). Immunoblotting was carried out using primary antibodies for anti-eNOS (endothelial NOS) from BD Biosciences (San Jose, CA), anti-phospho-eNOS Thr495 from Cell Signaling (Beverly, MA) and anti-β-actin from Sigma (St. Louis, MO). All enzymes used for endothelial cell digestion were obtained from Worthington Biochemical (Lakewood, NJ). All remaining reagents were sourced from Sigma (St. Louis, MO).

Experimental animals. Twelve-week-old male C57BL/6j mice (Jackson Laboratory, Bar Harbor, ME) were used to study the acute effects of FGF23. We also used 9-wk-old male Col4a3<−/−> mice (background 129Sv/J) and litter-matched WT mice in this study. 1.18 KH2PO4, 1.19 MgSO4, 5.5 glucose, and 1.6 CaCl2) saturated at 37°C. Arterial rings were progressively stretched to 0.75 g/day and incubated with 10 μM PD166866 to determine whether the effects were receptor mediated, and tiron (1 mM) was used to elucidate the role of superoxide.

DAF-FM staining, visualization, and nitrate/nitrite assay. NO production in response to FGF23 in aortic rings was assessed and imaged using DAF-FM. Briefly, mouse aortic rings (~3 mm in length) were cleaned of fat and connective tissue and equilibrated for 30 min in HBSS at room temperature. DAF-FM (10 μM) was then added to the buffer for 30 min. The aortic rings were washed two times with fresh HBSS buffer and then incubated with vehicle, FGF23 (9,000 pg/ml), or l-NAME (NO synthase inhibitor; 100 μM) for 20 min. Finally, aortic rings were stimulated with ACh (10 μM) for 10 min and immediately snap-frozen with OCT embedding compound in isopentane prechilled with liquid nitrogen. Frozen rings were then cut into 10 μm sections and imaged by epifluorescence microscopy using an Olympus IX71 (Center Valley, PA) inverted microscope fitted with a Hamamatsu ORCA-R2 CCD camera (Bridgewater, NJ), a Sutter LB-1 epifluorescence source (Novato, CA), and Semrock filters (Rochester, NY) with optimized excitation and emission wavelengths (DAF-FM, 495/519 nm). All images were captured under constant exposure time and gain. The fluorescence intensity of the endothelial cell layer was quantified using Slidebook (Intelligent Imaging Innovations, Denver, CO). Three endothelial regions were randomly selected from each aortic section and quantified via mean fluorescence intensity.

For measurement of NO levels, aortic rings that underwent isometric tension myography experiments were preconstricted with PGF2α (10 μM) and then pretreated with vehicle, FGF23 (9,000 pg/ml), or l-NAME (100 μM) for 20 min and stimulated with ACh (1 mM-100 μM) were snap-frozen in liquid nitrogen. The following morning, aortic rings were homogenized in phosphate-buffered saline (PBS), and total nitrate/nitrite levels were measured using the Griess reaction method (Cayman Chemicals, Ann Arbor, MI). Briefly, aortic homogenates were incubated with nitrate reductase (10 μU) and NADPH (cofactor) for 3 h at 37°C. The total nitrate/nitrite in each sample was then determined by adding sulfanilamide (Griess reagent 1), followed by N-(1-naphthyl) ethylenediamine (Griess reagent 2). Absorbance was read at 540 nm after incubation of the mixture at 37°C for 10 min, and the concentrations (μM) of total nitrate/nitrite were calculated from a standard curve.

eNOS Western blots. The thoracic aorta was rapidly excised and placed in ice-cold HBSS, where blood, fat, and excess connective tissues were carefully removed. Segments 3–4 mm in length were mounted on pins in chambers of a DMT 610M wire myograph system (Danish Myo Technology, Aarhus N, Denmark) and then pretreated with vehicle, FGF23 (9,000 pg/ml) or l-NAME (100 μM) for 20 min preincubation with FGF23 (9,000 pg/ml). Aortic rings were incubated with the FGFR antagonist PD166866 (50 nM) prior to treatment with FGF23 to determine whether the effects were receptor mediated, and tiron (1 mM) was used to elucidate the role of superoxide.

For measurement of NO levels, aortic rings that underwent isometric tension myography experiments were preconstricted with PGF2α (10 μM) and then pretreated with vehicle, FGF23 (9,000 pg/ml), or l-NAME (100 μM) for 20 min and stimulated with ACh (1 mM-100 μM) were snap-frozen in liquid nitrogen. The following morning, aortic rings were homogenized in phosphate-buffered saline (PBS), and total nitrate/nitrite levels were measured using the Griess reaction method (Cayman Chemicals, Ann Arbor, MI). Briefly, aortic homogenates were incubated with nitrate reductase (10 μU) and NADPH (cofactor) for 3 h at 37°C. The total nitrate/nitrite in each sample was then determined by adding sulfanilamide (Griess reagent 1), followed by N-(1-naphthyl) ethylenediamine (Griess reagent 2). Absorbance was read at 540 nm after incubation of the mixture at 37°C for 10 min, and the concentrations (μM) of total nitrate/nitrite were calculated from a standard curve.

ACh. Aortic rings were rinsed once with fresh Krebs every 15 min and several times after KCl or ACh treatments.

The contractile response to FGF23 was determined by adding increasing concentrations of FGF23 (90, 900, and 9,000 pg/ml) to the bath and measuring the tension produced. FGF23-mediated relaxation of aortic segments was analyzed by adding increasing concentrations of FGF23 (90, 900, 9,000 pg/ml) to PGF2α-precontracted (10 μM) aortic rings and measuring the reduction in tension. To investigate the effect of FGF23 on endothelial function, aortic segments were preincubated for 20 min with different concentrations of FGF23 or vehicle (HBSS without Ca2+ and Mg2+, 0.001% BSA) prior to precontraction with PGF2α (10 μM) and then relaxation to ACh (1 mM-100 μM) was measured. For denudation experiments, forceps were inserted into the lumen, and the aortic ring was gently rolled over. This process was repeated five times. The effectiveness of endothelial removal was confirmed by the absence of relaxation by 1 μM ACh in aortic rings precontracted with 10 μM PGF2α.

To explore the involvement of smooth muscle in FGF23-mediated effects, aortic rings precontracted with PGF2α (10 μM) were tested for relaxation in response to increasing concentrations of sodium nitroprusside (SNP; 1 μM-100 μM) after 20 min preincubation with FGF23 (9,000 pg/ml). Aortic rings were incubated with the FGFR antagonist PD166866 (50 nM) prior to treatment with FGF23 to determine whether the effects were receptor mediated, and tiron (1 mM) was used to elucidate the role of superoxide.

E427
until immediately before grinding with a mortar and pestle in liquid nitrogen. The homogenized powder was then placed in cell extraction buffer from Invitrogen (Carlsbad, CA) supplemented with PMSF per the manufacturer’s instructions. Samples were kept on ice and vortexed, and cell debris was pelleted at 15,000 g for 10 min at 4°C. Protein (100 μg) was separated by SDS-PAGE and transferred to a 0.2-μm PVDF membrane using standard techniques. Membranes were blocked (LI-COR blocking buffer) for 1 h at room temperature, and incubated overnight with appropriate primary antibodies. After a washing, blots were exposed to fluorescent secondary antibodies, and bands were visualized using a LI-COR Biosciences Odyssey imager. Bands were quantified via densitometry.

Staining for superoxide using lucigenin-enhanced chemiluminescence and dihydroethidium. Primary mouse endothelial cells were isolated by enzymatic digestion. Briefly, sections of aortas 2–3 mm in length were placed in ice-cold (4°C) solution 1 [in mM: 140 NaCl, 5 KCl, 2 MgCl₂, 10 glucose, 10 HEPES (pH 7.4), and 0.1 CaCl₂]. Rings were first washed once in solution 1 and then placed into prewarmed new solution 1 containing dispase (19.3 U/ml) for 30 min at 37°C. After incubation, aortic rings were washed with solution 1 (2 × 3 min each) and then incubated at 37°C in solution 1 containing elastase (0.94 U/ml) for 10 min. Dissociated tissue was washed once with solution 1 and twice with solution 2 (solution 1 + 0.1% bovine serum albumin). Finally, aortic rings were cut open longitudinally into two pieces and triturated by passing the digest 10 times through the tip of a flame-polished Pasteur pipette. The aortic digest was dispersed in endothelial cell growth medium and allowed to grow for 5–6 days in a CO₂ incubator. Endothelial cells were easily identified by cobblestone morphology and confirmed by immunocytochemistry using an antibody specific for von Willebrand factor. Primary endothelial cells were treated for 20 min in HBSS followed by incubation with dihydroethidium (DHE; 5 mM) solution for 30 min at room temperature in the dark. Red fluorescence intensity was recorded using epifluorescence microscopy with optimized excitation and emission wavelengths (DHE, 518/603 nm) at ×20 magnification.

Aortic rings (3 mm in length) were preincubated for 30 min at 37°C in HBSS and then transferred into the wells of a 96-well plate containing vehicle, FGF23 (9,000 pg/ml), or FGF23 (9,000 pg/ml) and tiron (1 mM) in a total volume of 200 μl of HBSS-based assay solution containing 5 μM lucigenin. After 20 min of dark incubation, the luminescent reaction between superoxide and lucigenin was detected using a microplate luminometer (GloMax; Promega, Madison, WI). The tissue-dependent photon emission was monitored over a 20 min period.

Statistics. Data are plotted and expressed as means ± SE, and sample sizes are detailed in the figure legends. In myograph experiments, changes in isometric tension are expressed as %contraction or %relaxation. A two-factor ANOVA with a modified Bonferroni was used to determine the differences between concentration-response curves. A one-factor ANOVA with Tukey’s multiple comparison post hoc tests were used for analyzing gene expression (ΔCₚ and 2⁻∆ΔCₚ) DAF-FM, nitrate, and superoxide assays. Graphs and statistics were generated with GraphPad Prism (v. 5.01, San Diego, CA). Significance was set at P < 0.05.

RESULTS

**FGF23 and α-Klotho expression.** We quantified the expression levels of Fgfrs and α-Klotho in aortic tissue from C57BL/6J mice (Fig. 1A). The ΔCₚ values (Fig. 1B) were calculated using β-Actin as the reference gene, and 2⁻ΔΔCₚ analyses were performed and relative expression was presented (Fig. 1C). The order of expression from highest to lowest was Fgfr1, Fgfr2, Fgfr3, Fgfr4, and α-Klotho. Statistical analysis was conducted on the observed, nontransformed ΔCₚ values. Fgfr2, Fgfr3, Fgfr4, and α-Klotho had significantly higher ΔCₚ values than Fgfr1 (P < 0.05). Fgfr4 and α-Klotho had significantly higher ΔCₚ values than Fgfr2 and Fgfr3 (P < 0.05).

**FGF23: direct effects on vasocontraction and vasorelaxation.** Because FGF23 is correlated with endothelial dysfunction clinically, we began by determining whether FGF23 could directly induce vasocontraction or vasorelaxation. We examined the contractile responses of aortic rings to increasing concentrations of FGF23. In aortic rings with either intact endothelium or denuded endothelium, FGF23 did not cause vasocontraction or vasorelaxation. Because FGF23 is correlated with endothelial dysfunction clinically, we began by determining whether FGF23 could directly induce vasocontraction or vasorelaxation. We examined the contractile responses of aortic rings to increasing concentrations of FGF23. In aortic rings with either intact endothelium or denuded endothelium, FGF23 did not cause vasocontraction or vasorelaxation.

**FGF23-mediated effect on endothelial function.** To examine the role of FGF23 in NO-mediated vasorelaxation, aortic rings were pretreated with increasing concentrations of FGF23 (90, 900, 9,000 pg/ml) or vehicle. We then precontracted the aortic rings with PGF₂α (10 μM) aortic rings, increasing concentrations of FGF23 did not induce vascular relaxation compared with vehicle (P > 0.05; Fig. 2B). These data indicate that FGF23 does not directly induce contraction or relaxation independent of other vasoactive agents.

**FGF23-mediated effect on endothelial function.** To examine the role of FGF23 in NO-mediated vasorelaxation, aortic rings were pretreated with increasing concentrations of FGF23 (90, 900, 9,000 pg/ml) or vehicle. We then precontracted the aortic rings with PGF₂α (10 μM) and induced vasorelaxation with increasing concentrations of ACh (1 nM-100 M; Fig. 3, A–C). At 9,000 pg/ml, FGF23 significantly impaired (~36%) vasorelaxation (P < 0.05; Fig. 3, B–C), suggesting that path-

---

**Fig. 1.** Fibroblast growth factor (FGF) receptors (Fgfr1–4) and α-Klotho are expressed in mouse aortic tissue. A: real-time RT-PCR reaction (run in triplicate) showing average fluorescence values at each cycle number for Fgfr1–4, α-Klotho, and the reference gene β-actin from mouse aorta (n = 5). B: summary ΔCₚ (gene of interest minus β-Actin) data from aortic tissue for Fgfr1–4 and α-Klotho. C: summary data (2⁻ΔΔCₚ), which compares the relative expression of Fgfr1–4 and α-Klotho. Data are shown as means ± SE. *Statistically significant difference from Fgfr1 (P < 0.05); †statistically significant difference from Fgfr2 (P < 0.05); ‡statistically significant difference from Fgfr3 (P < 0.05).
ological concentrations of FGF23 induce endothelial dysfunction in mouse aorta. Importantly, preincubation with PD166866 (P < 0.05 vs. vehicle; Fig. 3D), an antagonist of FGFR (50), restored relaxation to ACh in FGF23-treated aortic rings. To rule out the role of vascular smooth muscle in endothelial impairment, we repeated the experiment and replaced ACh (endothelium dependent) with the NO donor SNP (endothelium independent). FGF23 (9,000 pg/ml) pretreatment did not impair relaxation (P > 0.05; Fig. 3E) to SNP in aortic rings precontracted with PGF2α, suggesting that smooth muscle responses to NO were unaffected by FGF23.

FGF23 and NO. To specifically determine whether FGF23 interferes with the bioavailability of NO, we imaged aortic sections loaded with DAF-FM (fluorescent indicator of NO). Aortic rings were preincubated with vehicle + ACh (10 μM), FGF23 (9,000 pg/ml) + ACh (10 μM), L-NAME (100 μM) + ACh (10 μM), or no treatment. The fluorescence intensity of the Veh + ACh-treated sections (Fig. 4A) was greater than the FGF23 + ACh (P < 0.05), l-NAME- (NOS inhibitor) + ACh (P < 0.05), or no-treatment aortic sections (P < 0.05; Fig. 4B). To further quantify the NO response to ACh, we utilized an NO metabolite detection kit to measure total nitrate concentration. Preincubation with FGF23 (9,000 pg/ml) significantly reduced (~52%; P < 0.05; Fig. 4C) the concentration of nitrate in ACh-stimulated aortic rings. In addition, as a negative control, aortic rings were treated with L-NAME (100 μM), which significantly reduced the concentration of nitrate (P < 0.05; Fig. 4C). Last, we investigated whether FGF23 was inhibiting eNOS in isolated aortas treated with either vehicle or FGF23 (9,000 pg/ml) in HBSS for 20 min at room temperature. Western blotting demonstrated that FGF23 did not alter phosphorylation of the inhibitory Thr495 site of eNOS (P > 0.05; Fig. 4D).

Col4a3−/− mice, receptor expression, endothelial function, and NO. We quantified the expression levels of Fgfrs and α-Klotho in aortic tissue from Col4a3−/− and WT mice.
The $\Delta C_T$ (Fig. 5A) values were calculated using $\beta$-Actin as the reference gene, and $2^{-\Delta C_T}$ and $2^{-\Delta \Delta C_T}$ analyses were performed. The order of expression from highest to lowest in the Col4a3$^{-/-}$ tissues was Fgfr1, Fgfr3, Fgfr4, and $\alpha$-Klotho. Statistical analysis was conducted on the observed, nontransformed $\Delta C_T$ values. Fgfr2, Fgfr4, and $\alpha$-Klotho had significantly higher $\Delta C_T$ values than Fgfr1 ($P < 0.05$). Fgfr3 and $\alpha$-Klotho had significantly higher $\Delta C_T$ values than Fgfr2 ($P < 0.05$). Last, $\alpha$-Klotho had significantly higher $\Delta C_T$ values than Fgfr3 and Fgfr4 ($P < 0.05$). The $2^{-\Delta \Delta C_T}$ analysis comparing WT and Col4a3$^{-/-}$ mice of the same genetic background revealed that Col4a3$^{-/-}$ mice have significant reductions in Fgfr1, Fgfr2, Fgfr3, Fgfr4, and $\alpha$-Klotho expression (Fig. 5C).

To explore the consequences of prolonged elevations in circulating FGF23, we studied aortas from Col4a3$^{-/-}$ mice. We did not observe any changes in isometric force development between Col4a3$^{-/-}$ and WT mice ($P > 0.05$; Fig. 6A).

Fig. 4. FGF23 impairs NO bioavailability in aortic rings. A: cross-sections of aortic rings incubated with DAF-FM and pretreated with: vehicle and ACh (10 $\mu$M), FGF23 (9,000 pg/ml) and ACh (10 $\mu$M), L-NAME (NOS inhibitor, 100 $\mu$M), and ACh (10 $\mu$M), or no treatment ($n = 4$). There was enhanced fluorescence (increased NO) in the endothelial layer (white arrow) in the Veh + ACh sections. Note reduction in fluorescence intensity in the endothelial layer of FGF23- and L-NAME-pretreated aortic rings. B: the quantified mean fluorescence intensity of DAF-FM-stained aortic sections ($n = 4$). C: nitrate levels after isometric tension experiments treated with vehicle and ACh (10 $\mu$M), FGF23 (9,000 pg/ml) and ACh (10 $\mu$M), or L-NAME (100 $\mu$M) and ACh (10 $\mu$M) ($n = 5$). D: Western immunoblots and densitometry quantification of phospho-endothelial NO synthase (p-eNOS; Thr495) and total eNOS, and actin from aortic rings treated with vehicle or FGF23 (9,000 pg/ml) for 20 min ($n = 4$). Total eNOS was normalized to actin and then normalized to p-eNOS to provide the p-eNOS/eNOS ratio.

Fig. 5. FGF receptors (Fgfr1–4) and $\alpha$-Klotho are expressed in aortic tissue of an animal model of chronic kidney disease (Col4a3$^{-/-}$ mice) that displays highly elevated serum FGF23 levels. A: summary $\Delta C_T$ (gene of interest minus $\beta$-Actin) data from Col4a3$^{-/-}$ aortic tissue for Fgfr1–4 and $\alpha$-Klotho. B: summary data ($2^{-\Delta C_T}$) for Col4a3$^{-/-}$ aortic tissue that compares the relative expression of Fgfr1–4 and $\alpha$-Klotho. C: $2^{-\Delta \Delta C_T}$ data comparing Col4a3$^{-/-}$ with age-matched WT mice from the same genetic background. Data are shown as means $\pm$ SE ($n = 6$). *Statistically significant difference from Fgfr1 or WT ($P < 0.05$); †statistically significant difference from Fgfr2 ($P < 0.05$); #statistically significant difference from Fgfr3 ($P < 0.05$); ^statistically significant difference from Fgfr4 ($P < 0.05$).
using aortic rings treated with increasing concentrations of PGF$_{2\alpha}$ (1 nM-100 μM). To examine the effects of a chronically elevated level of circulating FGF23 on endothelium-dependent relaxation, preconstricted (PGF$_{2\alpha}$; 10 μM) aortic rings of Col4a3$^{-/-}$ and WT mice were relaxed with increasing concentrations of ACh (1 nM-100 μM). We detected a significant reduction (~48%) in endothelium-dependent vasorelaxation in Col4a3$^{-/-}$ mice ($P < 0.05$; Fig. 6B). Similar to our WT ex vivo data, there was no impairment of relaxation with increasing concentrations of SNP (1 nM-100 μM) in preconstricted (PGF$_{2\alpha}$; 10 μM) aortic rings of Col4a3$^{-/-}$ mice compared with WT ($P > 0.05$; Fig. 6C), supporting the hypothesis that chronic elevations in FGF23 may reduce the bioavailability of NO generated upon endothelial stimulation by ACh. To further confirm the role of NO bioavailability in Col4a3$^{-/-}$ mice endothelial impairment, we measured the nitrate/nitrite levels in ACh (10 μM) stimulated aortic rings of Col4a3$^{-/-}$ and WT mice. Col4a3$^{-/-}$ mice aortic rings contained significantly reduced levels of nitrate compared with WT (~32%; $P < 0.05$; Fig. 6D). Taken together with our studies utilizing acute exogenous FGF23 exposure in WT mice, our data indicate that FGF23 may be mediating endothelium dysfunction and reducing NO bioavailability in an animal model of CKD.

FGF23 and superoxide. Because bioavailability of NO depends in part on the level of superoxide, we explored the effects of FGF23 on superoxide levels in both primary endothelial cells and aortic rings using DHE staining and the lucigenin assay, respectively (Fig. 7). Adult isolated endothelial cells treated with FGF23 (9,000 pg/ml) had increased superoxide levels (~62%; $P < 0.05$; Fig. 7, A and B) compared with vehicle, and the FGF23-mediated increase in superoxide levels was inhibited by pretreatment with tiron ($P < 0.05$; Fig. 7, A and B). NADPH was used as a positive control and significantly increased DHE fluorescence (~101%; $P < 0.05$; Fig. 7, A and B). In aortic rings, treatment with FGF23 (9,000 pg/ml) also significantly increased superoxide levels (~49%; $P < 0.05$; Fig. 7C), which was reduced to basal levels by preincubation with the superoxide scavenger tiron (1 mM) ($P < 0.05$; Fig. 7C). Repeating the aortic relaxation myography in the presence of tiron resulted in improved aortic relaxation ($P < 0.05$; Fig. 7D). These data suggest that FGF23 can interfere with the bioavailability of NO in aortic tissue potentially via increases in superoxide.

DISCUSSION

The epidemic of CVD in CKD has been an important area of research for decades. A report issued by The National Kidney Foundation Task Force of Cardiovascular Disease in Chronic Renal Disease data showed that the prevalence of CVD in dialysis patients was 10–30 times higher than in the general population (38). Today, CVD remains the major cause of mortality in CKD (22, 57, 66); however, it has been very difficult to discern the exact mechanisms responsible for this high cardiovascular morbidity and mortality. It has been suggested that FGF23 may mediate cardiovascular complications observed during CKD. In fact, previous data demonstrated FGF23 directly induces cardiac hypertrophy (16, 67), and our own findings have suggested that FGF23 alters cardiac contractility (67). Given the strong clinical associations between FGF23 and impaired vasoreactivity, we began to investigate the direct effects of exogenous FGF23 on the vasculature using mouse aorta. The major findings of this paper are as follows: 1) we detected the gene expression of all four subtypes of FGF receptors in mouse aorta and found that Fgfr1 was the most highly expressed Fgfr subtype; 2) we demonstrated that FGF23 inhibited endothelium-dependent relaxation in the aorta; 3) we found that FGF23-mediated impairment of endothelial function was due to a decrease in NO bioavailability; 4) we found that the high levels of FGF23 in the Col4a3$^{-/-}$ mice, which...
have CKD, were also associated with impaired endothelium-dependent relaxations, as well as low NO levels compared with age-matched WT mice; and 5) we determined that FGF23 increased endothelial superoxide and that superoxide scavenging prevented the endothelial dysfunction caused by FGF23. Taken together, our results are the first basic science investigation to indicate that high levels of FGF23 cause endothelial dysfunction by decreasing NO bioavailability and increasing superoxide.

Fgfr and α-Klotho expression. It is currently unknown which FGFRs are necessary for FGF23 to exert its effects on the aorta. Therefore, we first quantified the relative expression of all four subtypes of Fgfr in C57BL/6J mouse aorta by using real-time RT-PCR. Our normalized data show that Fgfr1–4 are expressed and that Fgfr1 is in the greatest abundance. Our findings of the level of relative expression for Fgfr1–4 are in agreement with a previous study using mouse aorta (18). Additionally, previous data in human umbilical vein have shown that Fgfrs are expressed in both the endothelial cell layer and the smooth muscle cell layer (2). At the protein level, FGFRs have been detected in the rat aorta (34), human aortic muscle cells (41), and human arteries (29). We were able to prevent impaired endothelial function of FGF23-treated aortic rings (C57BL/6J mice) by using an inhibitor of FGFR, PD166866, which has been shown to have a greater affinity for FGFR1 at our pretreated concentration of 50 nM (50). Although additional experiments are needed to elucidate the role of specific FGFRs, these data point to the possibility that FGF23 may be mediating its action in the aorta, in part, via FGFR1.

FGF23 is thought to exert its effects via high-affinity interactions with α-Klotho-FGFR1 complexes (36, 68), particularly in the parathyroid and kidney (20, 35). In contrast to some previous studies (18, 54), we were able to detect α-Klotho in C57BL/6J mouse aortic tissue. Our finding that α-Klotho is in low expression, however, is in agreement with two reports showing that α-Klotho protein is not detectable in human or mouse aorta (15, 42). Given that we found α-Klotho to be ~750-fold lower in expression than our highest-expressed gene, Fgfr1, we believe it is possible that FGF23 could exert effects independently of α-Klotho-FGFR complexes. It has been previously shown in the proximal tubules of the kidney that low concentrations of FGF23 are dependent on α-Klotho, whereas high concentrations of FGF23 are able to exert an effect independent of α-Klotho (1). Moreover, it has been suggested that in the absence of α-Klotho other FGFR subtypes can act in concert to mediate FGF23 effects in the kidney (39). Therefore, it seems likely that high concentrations of FGF23 can act through α-Klotho-independent pathways and may induce endothelial dysfunction (28). These conclusions are similar to those offered by Faul et al. (16) and our previous findings in adult mouse heart (67), suggesting that FGF23 acts in a Klotho-independent manner. There is a need for more research to explore the interaction among FGF23, FGFRs, and α-Klotho in the vasculature. Whereas our study compares the normalized gene expression of Fgfr1–Fgfr4 and α-Klotho, our conclusions are limited to gene expression. Future work will need to analyze protein expression/function as well as genetic inhibition or knockdown of FGFRs in the cardiovascular system to more fully elucidate the specific receptors responsible for our findings in the aorta.

Direct effect on the aorta. Elevated FGF23 levels are independently associated with endothelial dysfunction and arterial stiffness, which have been related to cardiovascular mortality (3, 45, 72, 75). Therefore, we determined whether FGF23 alone could alter vascular function. We observed that 9,000 pg/ml FGF23 alone did not cause any significant contraction of mouse aortic rings with or without endothelium. Similarly, we
were unable to detect relaxation of precontracted aortic rings upon FGF23 treatment. Our results are in agreement with a recent publication exploring the dilatory and contractile effects of 6,000 pg/ml FGF23 in mesenteric arteries (42). However, Six et al. (60) found that doses of FGF23 from 10,000–400,000 pg/ml directly cause contraction of mouse aortic vessels. These investigators used female mice at 8 wk, whereas our study utilized male mice at 12 wk. These findings suggest that the concentration of FGF23, sex, and type of vessel may complicate the interpretation of the direct vascular response to FGF23.

Although FGF23 did not independently alter vasoconstriction or relaxation, our data indicate that FGF23 induces endothelial dysfunction in response to ACh treatment. We report that the maximum relaxation of the aorta to ACh was diminished by 36% when FGF23-treated rings were compared with vehicle. In the endothelial layer of the aorta, ACh stimulates the production of NO, which penetrates the vascular smooth muscle layer and causes vasorelaxation (19). Therefore, we tested the NO donor SNP to determine whether FGF23-mediated endothelial dysfunction involved the vascular smooth muscle layer as well. We found no difference in the relaxation response to SNP in FGF23-incubated aortic rings, indicating that there is no impairment to the relaxation mechanisms of vascular smooth muscle when stimulated by NO. The impaired response to ACh in FGF23-preincubated aortic rings supports the hypothesis that there is reduced bioavailability of NO. Our data differ from the findings of Lindberg et al. (42), who reported that FGF23 pretreatment did not affect the relaxation of precontracted mesenteric arteries upon stimulation with ACh. The major difference between our two studies is the tissue selected for testing. The endothelium of the mesenteric artery modulates vascular tone using two mediators, NO and endothelium-derived hyperpolarizing factor (EDHF), whereas NO predominates in the aorta (64, 70). FGF23 may not cause endothelial dysfunction in the mesenteric artery because it is mediating its action by lowering NO bioavailability, which is not a major relaxing factor in mesenteric vascular bed. In contrast to our findings, Six et al. (60) did not observe impaired ACh relaxation in FGF23-pretreated aortic rings. It is possible that the concentration of FGF23 and/or sex differences between our two studies may explain the different observations. In summary, to our knowledge, we are the first to show that FGF23 can impair the relaxation of the aorta to ACh. Our findings in this regard offer direct support for the clinical associations observed between elevated circulating levels of FGF23 and vascular dysfunction (3, 45, 75).

**Effect on NO bioavailability.** Impairment of NO bioavailability is known to be a principal cause of endothelial dysfunction (5, 69). Thus, it is not surprising that we noted a reduction in NO after FGF23 treatment using two different methods. First, homogenates of FGF23-treated rings showed a decrease in total nitrate. Second, we noted a decrease in DAF-FM fluorescence in the endothelial layer of aortic cross-sections. To determine whether the reductions in NO were due to effects of FGF23 on eNOS, we measured the phosphorylation of eNOS on its inactivation site Thr495. There were no differences in the phosphorylation state or on total eNOS levels after FGF23 treatment, suggesting that other mechanisms are responsible for reduced NO bioavailability. These results correlate with a previous study showing that FGF23 did not alter the phosphorylation of the activation site Ser1177 (60). Future studies should explore other posttranslational regulatory sites for eNOS.

Our in vitro and ex vivo data are supported by clinical data showing that higher FGF23 levels were associated with an impaired vascular response in subjects with normal renal function (45). Furthermore, there is a significant link between elevated FGF23 levels and impaired flow-mediated dilation (brachial artery) in stage 3 and 4 CKD patients (75). Interestingly, FGF23 was not correlated with nitroglycerine-mediated dilation, which indicates that the effects are mediated through the endothelium and not smooth muscle, supporting our findings with exogenous FGF23 and the Col4a3⁻/⁻ model in this study.

**Endothelial dysfunction in Col4a3⁻/⁻ mice.** To translate our in vitro and ex vivo findings to an in vivo mouse model of CKD with high plasma FGF23 levels, we explored the vascular function of Col4a3⁻/⁻ mice. The Col4a3⁻/⁻ mouse is a model of human Alport syndrome in which there is a progression of CKD and a corresponding increase in serum FGF23 starting at 6 wk of age that peaks at >5,000 pg/ml by 12 wk (63). We quantified all four subtypes of Fgfrs in Col4a3⁻/⁻ mice. This strain of mice (129Sv/J) displays a different pattern of FGFR expression than C57BL/6J; however, both strains demonstrate Fgfr1 in highest expression and α-Klotho in lowest expression. In addition, we found that the Col4a3⁻/⁻ mice had significant reductions in Fgfr1⁻/⁻ and α-Klotho expression compared with WT mice of the same genetic background as the Col4a3⁻/⁻ mice. The reduction in these genes in a model of CKD is in agreement with recently published data from Lim et al. (41), who reported a decline in Fgfr1, Fgfr3, and Klotho protein expression in human arteries from patients with CKD. They hypothesized that metabolic stress factors found during CKD might promote changes in FGFR expression.

Consistent with the direct effects of exogenous FGF23 on aortic vascular function, we found an ~50% impairment in endothelial-dependent vasorelaxation in response to ACh, while endothelium-independent relaxations in response to SNP were preserved in Col4a3⁻/⁻ mice. We also found a 31% reduction in nitrate/nitrite in aortic homogenates of Col4a3⁻/⁻ mice compared with their WT littermate controls, which further supports the impaired vasorelaxation observed in Col4a3⁻/⁻ mice. It is important to point out that Col4a3⁻/⁻ mice present with significant elevations in serum phosphorus, parathyroid hormone, blood urea nitrogen, and creatinine, as well as significantly reduced serum calcium and 1,25-dihydroxyvitamin D (63). Nevertheless, our findings in the Col4a3⁻/⁻ mice strongly support our experiments with exogenously administered FGF23 and suggest that a mouse model of CKD that has elevated FGF23 is associated with defective NO signaling and impaired endothelial function. Further studies are needed to precisely elucidate the role of FGF23 in directly mediating endothelial dysfunction during CKD.

**Role of superoxide in endothelial dysfunction.** Because we did not observe a change in eNOS phosphorylation, it is possible that other mechanisms play a role in the endothelial dysfunction caused by FGF23. One such mechanism may be oxidative stress, as it is known to induce endothelial dysfunction (10). In CKD patients, increases in oxidative stress are correlated with the progression of CKD (25, 74), and endothelial dysfunction and oxidative stress are thought to increase...
arterial stiffness in renal patients (12, 74). One potential reason for decreased bioavailability of NO is an increased level of superoxide, which combines rapidly with NO and converts it to peroxynitrite (4). In our study, preincubation with FGF23 resulted in increased superoxide levels in primary endothelial cells as well as in aortic rings. The increases in superoxide were eliminated with the superoxide scavenger tiron. Importantly, pretreating aortic rings with tiron during myography studies prevented FGF23-induced endothelial dysfunction and restored Ach-mediated relaxation. Therefore, our data support the hypothesis that FGF23 increases superoxide levels, which results in reduced NO bioavailability, thus leading to endothelial impairment in the aorta. This same type of increase in reactive oxygen species, reduced NO levels, and impaired vascular function are observed in other diseases like diabetes (26). However, it is important for future studies to confirm these findings in vivo, as superoxide is an important signaling molecule for normal vascular function and additional mechanisms may also be involved.

It is becoming more widely accepted that oxidative stress may be an important link between CVD and CKD. Antioxidants have been used in previous clinical trials in end-stage CKD/hemodialysis patients and found to improve vascular function or cardiovascular outcomes (21, 65). The SPACE trial (secondary prevention with antioxidants of CVD in end-stage renal disease) found that 800 IU/day of vitamin E supplementation reduced the number of hemodialysis patients that reached a cardiovascular endpoint (7). Since FGF23 levels are significantly elevated during end stage kidney disease/hemodialysis and we have shown that FGF23 can directly impair endothelial function via increased superoxide levels, it is possible that antioxidant treatment during end stage CKD may be protective, in part, by reducing FGF23-mediated endothelial dysfunction. It will be important to continue testing antioxidant therapy in CKD patients to determine whether it improves cardiovascular outcomes.

Significance. Recently, clinical studies in CKD patients have identified an association between increased levels of FGF23 and vascular dysfunction (45), reduced ejection fraction (16, 55), and ultimately left ventricular hypertrophy (27, 46). While we (67) and others (16) have shown that FGF23 can directly alter cardiac function, the question arises: do FGF23’s effects on the endothelium contribute to cardiac pathology? Impaired endothelial function and reduced vasoreactivity in conduit arteries such as the aorta are an early marker of vascular disease and are associated with adverse outcomes like left ventricular dysfunction and heart failure (51). Factors that impair endothelial function are associated with increased arterial stiffness (49), which increases left ventricular afterload. Afterload is defined as the force against which the left ventricle must act in order to eject blood and is largely dependent on aortic tone. Increased afterload is well known to result in reduced fractional shortening (40, 61) and, hence, ejection fraction (73). Therefore, it may be possible that FGF23 decreases ejection fraction via its actions on the aortic endothelium. In support of this hypothesis, we (67) and others (16) have previously shown that mouse models of CKD present with contractile dysfunction (decrease in fractional shortening and ejection fraction). Interestingly, in our study we found that these changes in left ventricular dysfunction preceded gross cellular measures of hypertrophy (67), suggesting that the performance of the heart may also be influenced by other systemic factors (e.g., afterload). A similar trend has been noted in human patients with elevated FGF23 who display left ventricular dysfunction even after adjustment for glomerular filtration rate, left ventricular hypertrophy, and other confounding variables (55).

On the basis of these findings to date, we propose that elevated FGF23 has at least two distinct mechanisms of action on the cardiovascular system. One mechanism is a direct action on the heart itself to induce hypertrophy, alter cardiac contractility, and decrease ejection fraction (16, 67). The other mechanism of action is the induction of endothelial dysfunction in arteries such as the aorta that may also contribute to reductions in cardiac performance, the development of cardiac hypertrophy, and ultimately heart failure. Given the estimate that for every log10 increase in circulating FGF23 there is a decline in ejection fraction by 6.5% (56), that 70–80% of dialysis patients have established cardiac hypertrophy (17), and that 37% of patients will have heart failure (43), it is critical to understand the direct effects of FGF23 on cardiovascular tissues.

In summary, we have demonstrated that FGF23 can directly impair agonist-induced endothelium-dependent relaxation. Similar to the acute effects of exogenous FGF23, we also observed impaired endothelial-mediated relaxations in Col4a3−/− mice, which were attributed to low nitrate levels. Our data support the assertion that the endothelial impairment was due to a reduction in NO bioavailability due to an increase in steady-state levels of superoxide. Together, these results expand our understanding of FGF23’s role in vascular function by showing that high levels of a bone-secreted hormone can directly modulate the aorta, thereby causing endothelial dysfunction.

ACKNOWLEDGMENTS

We thank Dr. Lynda Bonewald for useful scientific discussions during data collection and manuscript preparation, Stephanie Bishop for assistance in editing of the manuscript, and Dr. Brett Mitchell for assistance and expertise in Western blotting eNOS from mouse aortic tissues.

GRANTS

This work was supported, in whole or in part, by the National Institutes of Health Grants IRC2 AR-058962-0110 (M. J. Wacker and J. Andresen) and NIH K08 DK-087949 (J. Stubbs), Missouri Life Sciences Research Board Grant 09-1101 (M. J. Wacker and J. Andresen), American Heart Association Grants 11SDG5330016 (M. J. Wacker), SDG 0735053N (J. Andresen), and 11POST77650044 (N. Silswal).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

1. Andrukhova O, Zeitz U, Goetz R, Mohammadi M, Lanske B, Erben RG. FGF23 acts directly on renal proximal tubules to induce phosphaturia...


FGF23 IMPAIRS ENDOTHELIAL FUNCTION


