FGF23 is a novel regulator of intracellular calcium and cardiac contractility in addition to cardiac hypertrophy

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FGF23 is a novel regulator of intracellular calcium and cardiac contractility in addition to cardiac hypertrophy. Am J Physiol Endocrinol Metab 304: E863–E873, 2013. First published February 26, 2013; doi:10.1152/ajpendo.00596.2012.—Fibroblast growth factor 23 (FGF23) is a hormone released primarily by osteocytes that regulates phosphate and vitamin D metabolism. Recent observational studies in humans suggest that circulating FGF23 is independently associated with cardiac hypertrophy and increased mortality, but it is unknown whether FGF23 can directly alter cardiac function. We found that FGF23 significantly increased cardiomyocyte cell size in vitro, the expression of gene markers of cardiac hypertrophy, and total protein content of cardiac muscle. In addition, FGF1R and FGF3 mRNA were the most abundantly expressed FGF receptors in cardiomyocytes, and the coreceptor α-klotho was expressed at very low levels. We tested an animal model of chronic kidney disease (Col4a3−/− mice) that has elevated serum FGF23. We found elevations in common hypertrophy gene markers in Col4a3−/− hearts compared with wild type but did not observe changes in wall thickness or cell size by week 10. However, the Col4a3−/− hearts did show reduced fractional shortening (~17%) and ejection fraction (~11%). Acute exposure of primary cardiomyocytes to FGF23 resulted in elevated intracellular Ca2+ ([Ca2+]i; F/F0 + 86%) which was blocked by verapamil pretreatment. FGF23 also increased ventricular muscle strip contractility (67%), which was inhibited by FGF receptor antagonism. We hypothesize that although FGF23 can acutely increase [Ca2+]i, chronically this may lead to decreases in contractile function or stimulate cardiac hypertrophy, as observed with other stress hormones. In conclusion, FGF23 is a novel bone/heart endocrine factor and may be an important mediator of cardiac Ca2+ regulation and contractile function during chronic kidney disease.

Fibroblast growth factor 23; pathological cardiac hypertrophy; chronic kidney disease; Col4a3; cardiac function; α-klotho

FGF23 are markedly elevated 100- to 1,000-fold in patients with chronic kidney disease (CKD) (24, 31) and are independently associated with cardiovascular morbidity and mortality (8, 22, 26, 34, 35, 48, 52). Specifically, an association between left ventricular (LV) hypertrophy and serum FGF23 levels has been established in CKD patients (20, 36, 52).

Nevertheless, despite strong associations between FGF23 and adverse outcomes, it remains relatively unknown whether FGF23 is simply a marker of cardiac disease risk or a direct mediator of cardiac pathophysiology and cardiac performance. Only one study to date has analyzed the direct effects of FGF23 on the heart both in vitro and in vivo (13). This important work by Fau1 et al. (13) shows that FGF23 can directly induce hypertrophy in isolated neonatal cardiomyocytes as well as with intramyocardial FGF23 injections. These authors also demonstrated that a FGF receptor (FGF1R) antagonist reduced the LV hypertrophy in a 5/6 nephrectomy rat model of CKD. These findings are significant in that FGF23 may be an important player in directly inducing cardiac hypertrophy during CKD.

Moving forward, our laboratory has explored and addressed crucial questions that require answers, such as whether the hypertrophic effects of FGF23 can also be replicated in adult cardiomyocytes in vitro and observed in another animal model of CKD (Col4a3−/−) that has elevated serum FGF23. Also, can FGF23 directly alter intracellular Ca2+ ([Ca2+]i) and cardiac contractility? Previous studies have found a clinical association between FGF23 and LV mass (22, 35, 52) as well as declines in cardiac performance as measured by reduction in ejection fraction (19). However, to date no investigation has determined whether FGF23 can alter cardiac function independent of changes in cardiac hypertrophy. Determining what direct effects FGF23 may have on the heart will not only reveal how FGF23 may alter cardiac function on a beat-to-beat basis but also yield insights into how this hormone may induce chronic pathologies such as hypertrophy or heart failure.

MATERIALS AND METHODS

Materials. Recombinant mouse FGF23 was purchased from R & D Systems (Minneapolis, MN). The FGF1R inhibitor PD-166866 was purchased from EMD biosciences (San Diego, CA). Organ baths, stimulating electrodes, and LabChart 6 software were obtained from AD Instruments (Colorado Springs, CO). The stimulation unit (SD9) was purchased from Grass Technologies (Quincy, MA). Hanks’ balanced salt solution (HBSS) and Flou-4 AM were obtained from Invitrogen (Carlsbad, CA). Enzymes for cardiomyocyte isolation were obtained from Worthington (Lakewood, NJ). Total RNA isolation kits are available from Qiagen (Valencia, CA). First published February 26, 2013; doi:10.1152/ajpendo.00596.2012.

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 1 step kit from ABI (Carlsbad, CA). β-Tubulin and phospho-ERK1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). NCX1 primary antibodies were purchased from Swant (Marly, Switzerland). Claycomb’s medium and fetal bovine serum were purchased from Sigma-Aldrich (St. Louis, MO). All remaining reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Experimental animals. Twelve-week-old wild-type (WT) male CD1 mice (Harlan Laboratories, Madison, WI) were used in experiments using exogenous FGF23. In addition, male and female 10-week-old Col4a3−/− mice (background SV129) and age/litter-matched WT mice were also used in this study. The Col4a3−/− mice are a model of human autosomal-recessive Alport syndrome and can have serum levels of FGF23 >5,000 pg/ml by 12 wk (46). The Col4a3−/− mice develop nonhypertensive progressive renal fibrosis (17, 18). By 12 wk, Col4a3−/− mice present with significant elevations in serum phosphorus, parathyroid hormone, blood urea nitrogen, and creatine compared with WT mice (46). In addition, at week 12, Col4a3−/− mice have significantly reduced serum calcium and 1,25-dihydroxyvitamin D (46). All mice were housed in a temperature-controlled (22 ± 2°C) room with a 12:12-h light-dark cycle. Animals were fed ad libitum. All protocols were approved by the Animal Care and Use Committee of the University Missouri-Kansas City School of Medicine and the University of Kansas Medical Center.

HL-1 cell culture. HL-1 cardiomyocytes were plated (5,000/cm²) in flasks precoated with 0.00125% fibronectin and 0.02% gelatin. Cells were cultured for 24 h in Claycomb’s medium (supplemented with 10% FBS, 2 mM l-glutamine, 0.1 mM norepinephrine, 0.3 mM ascorbic acid, 100 U/ml penicillin, and 100 mg/ml streptomycin), as described previously (50, 51). Prior to experiments, cells were rendered quiescent in a minimal media (0.5% FBS, 2 mM l-glutamine, and penicillin-streptomycin, without norepinephrine) for 48 h prior to treatment. Cells were treated with vehicle, FGF23, and FGF23 + PD-166866 (50 nM) for 48 h prior to analysis. Cells were collected and analyzed for changes in cell size by flow cytometry using FACSCalibur (FSC).

Isolation of primary cardiac myocytes. Following cervical dislocation the heart was rapidly excised, extraneous tissue was removed, and the aorta was cannulated under a dissecting microscope. Cardiomyocytes were isolated in a standard manner, utilizing retrograde perfusion via the aorta using a Langendorff perfusion apparatus with phosphate and the University of Kansas Medical Center.

libitum. All protocols were approved by the Animal Care and Use Committee and the University of Kansas Medical Center.

apoAlp (10 μM FGF23) were tested five times, which

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were quickly excised and placed into an ice-cold cardioprotective medium that included the addition of 2,3-butanediol monoxyde (30 mM), as described previously (50). Tissue cultures were treated with vehicle, FGF23, or FGF23 + PD-166866 (50 nM).

Total protein and Western blot. Clamp-frozen ventricular tissues were weighed and homogenized in a 1:2.1 (vol/wt) ratio of ice-cold cell extraction buffer (Invitrogen), as described previously (56). Total protein concentration of the samples was determined by use of the microbicinchoninic acid protein assay (Pierce Chemical) and then normalized to tissue weight (μg/mg). Protein samples (20–50 μg) were run on 4–20% SDS-PAGE gels, and Western blots were performed using standard techniques. Because downstream changes in the signaling pathway are reliant on a net increase in ERK phosphorylation and total ERK protein expression is unlikely to change over 15 min, p-ERK blots were normalized to β-tubulin as a loading control. Whole heart lysates from WT and Col4a3−/− mice were used for analysis of NCX1 via Western blotting and normalized to β-tubulin.

Echocardiography on Col4a3−/− mice. Mice were weighed and anesthetized with isoflurane inhalation (3% for induction, 1% for maintenance). The anterior chest was shaved, and the mice were placed on a heating pad in the left lateral decubitus position. A rectal temperature probe was placed to ensure that the body temperature remained at 37.0°C during the study. Left ventricle structure and function were assessed by previously validated two-dimensional M-mode and Doppler echocardiographic techniques (9, 61). Echocardiographic images were obtained using a Philips HDI 5000 SonosCT ultrasound system equipped with a 12–5 MHz phased-array probe fitted with a 0.3-cm standoff and a 15–7 MHz broadband linear probe. Digital images were analyzed offline according to modified American Society for Echocardiography standards (42) using the ProSolv image analysis software (version 3.5; Problem Solving Concepts). LV end-diastolic and end-systolic diameters and anterior and posterior wall thickness in diastole were measured from M-mode tracings obtained at the midpapillary level (41). LV ejection fraction was derived from M-mode parameters. LV mass was estimated from the M-mode data, and LV end-diastolic and end-systolic volumes were calculated using the formula of Teichholz et al. (49). Analysis of data was performed by an investigator blinded to the treatment assigned.

Histology. Hearts were removed and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned (5 μm). Sections were deparaffinized and stained with wheat germ agglutinin or picrosirius red. Measurements of the cross-sectional area (n > 250 cells from both male and female animals) were obtained from images using Slidebook software (Intelligent Imaging Innovations, Denver, CO).

Ca²⁺ imaging. Imaging during FGF23 perfusion was conducted as reported previously (50, 51, 56). Briefly, cells were loaded at room temperature with the fluorescent Ca²⁺ indicator Fluo-4-AM (2 μM) for 20 min. Cells were washed three times in HBSS and allowed to deestryer for 10 min at room temperature. Intracellular Ca²⁺ levels were measured with an inverted microscope with fluorescent imaging capabilities (Olympus IX51 (Olympus, Melville, NY) and Hamamatsu Orca-ERGA charge-coupled device cameras (Hamamatsu, Bridgewater, NJ)). Semrock Bright Line filter set (Semrock, Rochester, NY), EXFO X-cite metal halide light source (EXFO, Mississauga, ON, Canada), and Slidebook ratiometric software (Intelligent Imaging Innovations). Diluted FGF23 (18,000 pg/ml) was carefully perfused to the plates at a rate of 0.3 ml/min. In these experiments, the five treatment conditions [vehicle, FGF23 alone, PD-166866 (50 nM) + FGF23, FGF23 in the presence of 0 μM extracellular Ca²⁺, and verapamil (10 μM + FGF23)] were tested five times, which

were repeated in three to five different animals. The fluorescent changes from each cell were averaged, and the data were grouped by animal and then used for data analysis. All data are presented as the peak increase in fluorescence (F) after FGF23 application divided by the initial fluorescence before FGF23 application (F₀). A F/F₀ of 1 indicates no change in fluorescence from the baseline. Cells were tested for viability with KCl (80 mM) at the end of each experiment and included in the data set only
if the response to KCI was greater than a 50% increase from baseline fluorescence.

Cardiac contractility measurements. CD1 mice were euthanized by cervical dislocation. The mouse hearts used for the muscle strip experiments were quickly excised and placed into an ice-cold cardioprotective Ringer’s solution (with Ca²⁺) that included the addition of 2,3-butanedione monoxime (30 mM) for 30 min, as described previously (50). Briefly, LV muscle strips were prepared (1–2 mm wide by 6–8 mm long) in the cardioprotective solution. The strips were tied on the proximal and distal ends with a silk thread. The muscle strips were then rinsed three times (5 min each) in Ringer’s solution (with Ca²⁺, pH 7.4) to remove the 2,3-butanedione monoxime. The muscle strips were hung vertically and attached to a force transducer between bipolar platinum-stimulating electrodes suspended in 25-ml glass tissue chambers and bubbled under 100% O₂. Heart muscles were stretched to the length of maximum force development in Ringer’s solution (pH 7.4, without 2,3-butanedione monoxime) and stimulated with pulses of 1 Hz for 5 ms. The stimulation voltage was set 20% of the muscle strips were then rinsed three times (5 min each) in Ringer’s solution (with Ca²⁺, pH 7.4) to remove the 2,3-butanedione monoxime. The muscle strips were hung vertically and attached to a force transducer between bipolar platinum-stimulating electrodes suspended in 25-ml glass tissue chambers and bubbled under 100% O₂. Heart muscles were stretched to the length of maximum force development in Ringer’s solution (pH 7.4, without 2,3-butanedione monoxime) and stimulated with pulses of 1 Hz for 5 ms. The stimulation voltage was set 20% above threshold, and the muscles in the chamber were superfused with Ringer’s solution (with Ca²⁺, pH 7.4). Muscles were allowed to stabilize for 90 min prior to experimentation and provided with fresh media changes every 30 min. Muscles were paced at 1 Hz to obtain a stable baseline and treated with either vehicle or FGF23. The contractile data were recorded and analyzed on the LabChart 6 software. Waveform changes were analyzed in the segments corresponding to peak isometric tension (mN). Slope (mN/s) was analyzed by taking the average slope from 10 to 90% of the peak. τ (s) was fitted at the baseline using data from 95 to 0% of peak. Strip experiments were normalized within each condition to baseline levels of contractility and presented as a relative change from baseline contractile data.

Statistical analysis. All graphs were made and statistical procedures performed using GraphPad Prism 5.0. Data are presented as means ± SE. Data were compared using either a paired t-test or a one-way analysis of variance, and significance was set at the P ≤ 0.05 level. When necessary, the one-way analysis of variance was followed with appropriate post hoc tests. A Bonferroni post hoc adjustment was used to correct for two to three comparisons to avoid type I error. In cases where we made at least three comparisons, we utilized a Tukey Post hoc adjustment to avoid type II error. FSC data was analyzed using FlowJo Version 8.8.6 probability binning population comparison software (Tree Star) using a modified Cox Chi Squared Test [T(X)]. A value of T(X) > 4 implies that the two distributions are different with a P value of <0.01 (99% confidence).

RESULTS

Markers of cardiac hypertrophy with exogenous FGF23. We began this series of studies by testing the hypothesis that FGF23 directly induces hypertrophy in cardiomyocytes. Flow cytometry revealed a concentration-dependent increase in HL-1 cell surface area of cardiomyocytes exposed to FGF23 [T(X) = 63, P < 0.05; Fig. 1A and B]. Twenty-four-hour exposure of ventricular muscle strips to FGF23 (900 pg/ml) resulted in increased expression of early growth response 1 (EGR1), atrial natriuretic peptide (ANP), and brain natriuretic peptide (BNP) (P < 0.05; Fig. 2A). In addition, 48-h exposure to FGF23 increased gene expression of β-myosin heavy chain (β-MHC) and skeletal muscle α-actin (SkAct) (P < 0.05; Fig. 2A). No statistically significant changes were noted for c-Myc, c-Fos, or c-Jun following FGF23 treatment. It is well known that FGFR signaling in the kidney involves the activation of the MAPK cascade, particularly ERK (58). In cardiomyocytes, ERK phosphorylation is known to induce the EGR1 transcription factor as well as increase fetal gene expression associated with pathological hypertrophy (33). ERK phosphorylation in isolated cardiac muscle strips was increased significantly 15 min after treatment with FGF23 when compared with vehicle-treated strips (p < 0.05; Fig. 2B). In addition, FGF23 increased protein synthesis 8% (P < 0.05; Fig. 2C), and this increase was inhibited by the preaddition of PD-166866. These data suggest that changes in cell size are FGFR mediated and are not due simply to cell swelling.

FGFR and α-klotho gene expression. We quantified the expression levels of FGFRs and α-klotho in isolated cardiomyocytes (Fig. 3A). The Δ-cycle threshold (ΔCT) values were calculated using β-actin as the reference gene, and 2−ΔCT calculations were performed. The order of expression from highest to lowest was FGFR3, FGFR1, FGFR4, FGFR2, and α-klotho. For ease and clarity of data presentation, we calculated the relative expression of each FGFR by comparing it with α-klotho, as shown in Fig. 3B. Statistical analysis was not conducted on these data since it was transformed and normalized to the lowest-expressed gene. Statistical analysis was conducted on the raw ΔCT values. FGFR3, FGFR1, and FGFR4 were significantly higher compared with α-klotho (p < 0.05). FGFR3 and FGFR1 did not differ statistically from one another (P > 0.05), but both were statistically higher than FGFR4 and FGFR2 (P < 0.05). FGFR4 and FGFR2 did not differ from one another (P > 0.05). Similar expression results were confirmed using GAPDH as the housekeeping gene.

Col4a3−/− mice. Since we observed hypertrophic signaling occurring with exposure to FGF23, we analyzed ventricular heart tissue from 10-wk-old Col4a3−/− mice for markers of pathological hypertrophy. The Col4a3−/− mouse is a model of human cardiomyopathy and is well characterized. 

Fig. 1. Fibroblast growth factor 23 (FGF23) increases cell size in a dose-dependent manner. A: representative forward-scatter histograms (FSC-H) of HL-1 cardiomyocytes treated with vehicle or FGF23 (900 pg/ml) for 48 h; T(X) = 63. B: summary of forward-scatter (FSC-H) data on cardiomyocytes treated with increasing doses of FGF23 (9–900 pg/ml) using flow cytometry [T(X) = 63, P < 0.01]. FSC-H analysis of >10,000 live gated cells/sample (n = 5 experiments). Results from independent experiments were normalized to vehicle controls and averaged. *Statistical difference from vehicle; †statistical difference from FGF23 treatment (9 pg/ml).
Alport syndrome, in which there is progression of CKD, and therefore, it has has elevated levels of FGF23 starting at week 6 (46). We observed increases in ANP, β-MHC, and SkAct in Col4a3<sup>-/-</sup> hearts compared with hearts of their WT littermates (P < 0.05; Fig. 4A). Interestingly, we did not see gross morphological evidence of hypertrophy in the Col4a3<sup>-/-</sup> mice. There were no changes in anterior or posterior wall thickness compared with WT controls as measured by echocardiography (P > 0.05; Fig. 4B). We did not detect an increase in heart size by estimates in LV mass from the echocardiogram or by comparing heart weights with tibia length (P > 0.05; Fig. 4C). In addition, the average cardiomyocyte cross-sectional area based on the histological analysis from both male and female mice were similar in Col4a3<sup>-/-</sup> and WT hearts (Fig. 4, D and E). A randomly selected subsample of cross-sections was stained with picrosirius red, and we did not observe any differences in fibrosis between WT and Col4a3<sup>-/-</sup> mice (mean% fibrosis of heart sections = 0.46 vs. 0.49%, respectively; n = 2). Representative M-mode tracings from echocardiography are shown in Fig. 5A. Col4a3<sup>-/-</sup> mice did exhibit declines in LV function, as determined by reductions in both fractional shortening and ejection fraction (−17 and −11%, respectively, P < 0.05; Fig. 5B). Because there appeared to be a decrease in contractile function without hypertrophy, we hypothesized that there may be Ca<sup>2+</sup>-handling issues, and therefore, we tested three major Ca<sup>2+</sup>-handling genes. Interestingly, Col4a3<sup>-/-</sup> mouse hearts showed a significant upregulation of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger 1 (NCX1) mRNA (1.76 ± 0.33-fold, P < 0.05; n = 5), however, there was not a significant increase in NCX1 protein expression (1.15 ± 0.04-fold, P > 0.05; n = 5). In addition, there were no significant increases in calsequestrin (Cai) (1.39 ± 0.28-fold, P > 0.05; n = 5) or sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>/ATPase (SERCA) (1.02 ± 0.04-fold, P > 0.05; n = 5) mRNA expression in the Col4a3<sup>-/-</sup> mouse hearts.

Ca<sup>2+</sup><sup>+</sup> imaging of primary cardiomyocytes. To determine whether FGF23 can modulate the levels of [Ca<sup>2+</sup>]<sup>+</sup>, we re-
corded the Ca\textsuperscript{2+} responses in primary cardiomyocytes with the fluorescent Ca\textsuperscript{2+} indicator Fluo-4 AM. Figure 6, A and B, displays a representative response of a cardiomyocyte to FGF23 (18,000 pg/ml). The myocyte displayed a spontaneous Ca\textsuperscript{2+} oscillation prior to treatment, which after FGF23 had a large and transient increase in [Ca\textsuperscript{2+}], FGF23 increased F/F\textsubscript{o} by 86% on average compared with vehicle (P < 0.05; Fig. 6C). We were able to prevent these increases in [Ca\textsuperscript{2+}]i by pretreating cells with PD-166866 (P < 0.05; Fig. 6C). In addition, we were able to eliminate the increases in [Ca\textsuperscript{2+}]i by eliminating extracellular Ca\textsuperscript{2+} upon pretreatment of the cells with the L-type voltage-gated Ca\textsuperscript{2+} channel antagonist verapamil (10 \mu M, P < 0.05; Fig. 6D). The average time for a peak response to perfused FGF23 was 186.6 ± 6.6 s. The average time for a peak response to perfused KCl was 68.8 ± 5.6 s, and the average F/F\textsubscript{o} response to KCl was 3.17 ± 0.41. The dead-space time of our perfusion system in these experiments was ~45 s. To test whether prolonged exposure to FGF23 induced an increase in resting levels of Ca\textsuperscript{2+}, we loaded primary cardiomyocytes with the ratiometric fluorescent Ca\textsuperscript{2+} indicator Fura-2-AM. Cardiomyocytes treated with FGF23 for 2 h increased [Ca\textsuperscript{2+}]i by 25% (P < 0.05; data not shown).

**Contractility.** Since FGF23 acutely increased [Ca\textsuperscript{2+}]i, we explored the effect of exogenous FGF23 in cardiac muscle contractility. We compared the contractile responses elicited by increasing concentrations of FGF23 compared with vehicle. Peak changes in contractility were noted between 15 and 20 min following FGF23 treatment. Figure 7A displays raw tracings of paced ventricular muscle strips following treatment with vehicle and FGF23 (9,000 pg/ml). Increasing concentrations of FGF23 (900 and 9,000 pg/ml) increased isometric force when compared with vehicle (P < 0.05; Fig. 7B). We analyzed the effects of FGF23 on specific characteristics of each contractile waveform. FGF23 (900 and 9,000 pg/ml) increased the slope of contraction (r < 0.05; Fig. 7C) and the overall area (P < 0.05; Fig. 7C) of the contractile waveform (i.e., the integral/impulse) when compared with vehicle. However, FGF23 had no effect on \( \tau \), the time constant of decay (rate of relaxation), compared with vehicle (Fig. 7C). To test whether the changes in contractility were receptor mediated, we repeated a series of experiments with PD-166866 (50 nM) that eliminated the increases in isometric tension, slope, and overall area induced by FGF23 (P < 0.05; Fig. 7D). In addition, FGF23 + PD-166866 had no effect on \( \tau \).

**DISCUSSION**

There have been several recent clinical reports suggesting that FGF23 may alter heart function, particularly during CKD (15, 21, 22, 35). However, there have been very few studies...
Fig. 6. FGF23 treatment increases intracellular Ca$^{2+}$ ([Ca$^{2+}$]) in primary cardiomyocytes. A: acute fluo-4 changes in [Ca$^{2+}$]$_i$ in primary cardiomyocytes immediately following treatment with FGF23 in the presence of extracellular Ca$^{2+}$. For the fluorescent images, warmer colors (yellow, red) indicated increased fluorescence (increase in [Ca$^{2+}$]). B: fluo-4 image of a primary cardiomyocyte at baseline and at the peak fluorometric response following acute FGF23 treatment. C: summary data showing the average acute changes in fluorescence mediated by FGF23 and during receptor antagonism (PD-166866; n = 10–30 cells, 3–5 animals). Measurements are indicated as a change in fluorescence after treatment divided by the initial fluorescence (F/F$_{0}$). D: summary data showing the average changes in fluorescence mediated by FGF23 treatment in the absence of extracellular Ca$^{2+}$ (0 mM, +0 Ca$^{2+}$) and following pretreatment with verapamil (Verap; n = 10–30 cells, 3–5 animals). *Statistical difference from vehicle; †statistical difference from FGF23 treatment.

that have attempted to address the direct effects of FGF23 on the myocardium. Therefore, we sought to determine what alterations in cardiac function would occur during exposure to FGF23. The major findings of this study are as follows. 1) Exposure to FGF23 causes a dose-dependent increase in cell size as well as increased protein synthesis and expression of common hypertrophy markers; 2) the Col4a3$^{-/-}$ mouse model of CKD that is known to have elevated FGF23 demonstrates increased gene expression of markers of pathological hypertrophy but does not show increases in cardiomyocyte size or ventricular wall thickness; 3) Col4a3$^{-/-}$ mice have alterations in contractile function that appear to precede the potential development of pathological hypertrophy; 4) acute exposure to FGF23 increases [Ca$^{2+}$]$_i$ in adult ventricular myocytes; and 5) acute FGF23 exposure alters cardiac contractility by increasing force, rate of force development, and the area under the curve (integral).

Hypertrophy. The normal level of FGF23 in the plasma in healthy patients is 13.3 ± 19.0 pg/ml; however, in CKD plasma, FGF23 levels can rise 100- to 1,000-fold higher than patients with normal renal function (25). In patients with CKD, these elevated serum levels of FGF23 have been clinically associated with increased LV mass and increased risk of LV hypertrophy (20, 35, 52). Moreover, FGF23 levels have been shown to predict outcomes in patients with systolic heart failure (39). However, despite these strong clinical associations, there has been only a single basic science study to date to show that FGF23 directly induces a hypertrophic phenotype in the heart (13). Therefore, we determined whether the hypertrophic effects of FGF23 could also be replicated in adult cardiomyocytes in vitro and whether hypertrophy is present in another animal model of chronic kidney disease (Col4a3$^{-/-}$) that has elevated serum FGF23.

To investigate the effects of FGF23 on hypertrophy, we first performed concentration response experiments analyzing changes in cell size with HL-1 cardiomyocytes. We utilized concentrations of 90, 900, and 9,000 pg/ml, as that represents an approximate baseline level (in WT mice), and then 10- and 100-fold higher concentrations, which would be expected during CKD. HL-1 cardiomyocytes were utilized since they maintain phenotypic characteristics of adult myocytes (7), have been used previously in models of cardiac hypertrophy (5, 6, 30), do not contain fibroblasts, and can be used in large population numbers over extended periods of time to more accurately detect changes in cell size. Similarly to Faul et al. (13), who used neonatal cardiomyocytes, our data show that FGF23 induced HL-1 cell growth up to 24% in a concentration-dependent manner. To validate that FGF23 could have a direct effect on cardiac tissue, we analyzed early growth response genes and fetal genes associated with pathological cardiac hypertrophy in ventricular muscle strips. Similarly to previous reports (13, 53), we were unable to induce EGR1 expression 1 h following FGF23 exposure (data not shown); however, we report that FGF23 can induce EGR1 expression in cardiac tissue 24 h following administration. The expression of c-Myc (a mediator of growth signaling in cardiomyocytes) did not reach statistical significance despite increasing more than twofold following treatment with FGF23. In addition, FGF23 treatment also resulted in the elevated expression of BNP, ANP, β-MHC, and SkAct, which are well-known markers of cardiac hypertrophy. Specifically, there have been significant correlations between elevated FGF23 and elevated BNP plasma levels in patients with left ventricular hypertrophy (19, 43). BNP in particular is used as a diagnostic indicator for heart failure (12), suggesting that FGF23 may directly promote the progression of heart failure.

Previous research in noncardiac tissue has shown that FGF23 is a potent inducer of ERK phosphorylation and subsequent EGR1 expression (1, 53, 58). In cardiac muscle strips treated with FGF23, we noted a p-ERK response within 15 min.
of treatment. Interestingly, it has been reported previously that FGF23 did not increase cardiac expression of p-ERK at 30 and 60 min following exposure in neonatal cardiomyocytes (13). However, these authors did show that the ERK inhibitor U-0126 was able to attenuate increases in FGF23-induced cell size (13). These differences with our findings for p-ERK may be due to differences in age (neonatal vs. adult), the tissue type (isolated myocytes vs. whole tissue), or the timing of the measurements in these studies.

Finally, we applied exogenous FGF23 to cultured adult ventricular muscle strips and measured changes in total protein content. FGF23 increased protein synthesis following 48 h of treatment, and this effect was also dependent on FGFR activation, as it was eliminated by pretreatment with PD-166866. Taken together, our data lend strength to the hypothesis that FGF23 can directly induce cardiac hypertrophy.

FGFRs and α-klotho. It is currently unknown which receptors are necessary for FGF23 to exert its effects on the heart. FGF23 is known to bind FGFR1–4 with varying degrees of affinity (59, 60), and previous reports using end point RT-PCR and immunohistochemistry in neonatal cardiomyocytes and adult hearts have shown that FGFR1–4 are present (13, 23). Our data now extend these findings by using real-time RT-PCR to quantitate relative expression levels in isolated primary cardiomyocytes, which showed that FGFR3 and FGFR1 are the most abundantly expressed. Since we were able to block the acute and chronic effects FGF23 with PD-166866, a selective inhibitor of FGFR1 at 50 nM (37), and FGFR1 is abundantly expressed, it seems likely that FGFR1 is an important mediator of FGF23 cell signaling in the myocycardium.

FGF23 is thought to have a high binding affinity for FGFR1-α-klotho complexes in other tissues, like the kidney and parathyroid. In contrast to previous studies (13, 29, 47, 53), we have detected α-klotho expression in the heart. Our ability to detect α-klotho may be attributed to the increased sensitivity of the one-step real-time RT-PCR procedure (54). However, given that we found α-klotho to be ~3,750-fold lower in expression than our highest-expressed gene, FGFR3, we support the hypothesis that α-klotho likely plays a limited role in FGF23 signaling in cardiomyocytes (13). Moreover, it is possible that other FGFRs in addition to FGFR1 may be involved in FGF23-mediated actions on cardiomyocytes. Previous studies have suggested that FGFR1, FGFR3, and FGFR4 can act in concert to mediate FGF23 effects in the kidney (32). It has also been suggested that, in the absence of α-klotho, FGF23 has a high affinity for FGFR4 (23, 60) and may mediate effects on the heart (13). However, we would hypothesize that FGFR4 as well as FGFR2 may play limited roles due to their lower expression levels. Taken together, our data lend support to the hypothesis that FGF23 signaling in the heart may be independent of α-klotho expression and suggest that FGFR1 and FGFR3 may be critical to signaling in the heart. Nevertheless, a more thorough inquiry into exact mechanisms responsible for FGFR cardiac signaling awaits further investigation.

Evidence of cardiac dysfunction in Col4a3−/− mice. It has been shown recently that the 5/6-nephrectomized rat model of
CKD develops left ventricular hypertrophy that was significantly attenuated with the FGFR antagonist PD-173074 (13). Interestingly, another study found that a FGF23-neutralizing antibody did not reduce the hypertrophy in this animal model (44). Therefore, we were interested in exploring whether a different model of CKD with elevated FGF23 could develop cardiac hypertrophy. The Col4a3−/− mouse is a model of autosomal-recessive Alport syndrome. This mouse model is nonhypertensive and has a progressive increase in the serum levels of FGF23 that precedes elevations of traditional markers of renal dysfunction (17, 18). From weeks 4 to 6, FGF23 levels increase from ~130 to ~260 pg/ml, respectively. However, serum FGF23 levels increase exponentially from weeks 8 to 12 (440 to 5,400 pg/ml, respectively) (46). Therefore, we used Col4a3−/− mice to explore the connection between elevated serum FGF23 and cardiac hypertrophy. Similarly to the 5/6-nephrectomized mice (13, 44), Col4a3−/− mice presented with increased gene expression markers of hypertrophy (ANP, SkAct, and βMHC); however, there were no significant increases in anterior/posterior wall thickness, nor was there increased cardiac mass by echocardiography or necropsy. Furthermore, there was no increase in average cardiomyocyte size based on histological analysis. The Col4a3−/− mouse model has significantly increased rates of mortality beginning at 10 wk, which prevented further characterization of these animals. We postulate that, given additional time, these mice may also have demonstrated significant increases in cell size given that the hypertrophic gene markers were increased at 10 wk.

Although we did not observe hypertrophy, there were significant decreases in the contractile parameters (fractional shortening and ejection fraction). In the 5/6-nephrectomized animals, Fauel et al. (13) reported a decline in ejection fraction (although it did not reach statistical significance), which was observed concurrent with increases in hypertrophy. This decrease in ejection fraction was eliminated by a FGFR antagonist (13). Interestingly, the changes in contractility in the Col4a3−/− mice in our study occurred without significant hypertrophy or fibrosis and in a CKD animal model that does not demonstrate chronic hypertension. Thus the Col4a3−/− mouse model may provide an interesting tool for studying cardiac effects of FGF23. Differences in cardiac function between animal models with high FGF23 need to continue to be explored to fully elucidate cardiac effects of FGF23 in vivo during CKD.

The contractile deficits we observed in the animal model led us to explore whether FGF23 is altering Ca2+ handling genes or directly altering Ca2+ levels. We first explored Ca2+-handling genes that are known to increase during heart failure. The Col4a3−/− mouse had a significant upregulation of NCX1 mRNA but not SERCA or Cal. Interestingly, we did not observe a significant increase in NCX1 protein expression in Col4a3−/− mice compared with WT. Because the NCX1 is an important regulator of [Ca2+], in cardiomyocytes and is increased during cardiac hypertrophy (28, 40), future studies concerning the effects of FGF23 on the myocardium may be warranted.

[Ca2+], Next, we wanted to explore whether FGF23 could be directly altering [Ca2+]. Acute exposure of primary cardiomyocytes to FGF23 increased [Ca2+], significantly, and we were able to eliminate this increase via pretreatment with PD-166866. Removing extracellular Ca2+ also abolished the FGF23-evoked increase in [Ca2+], suggesting that FGF23 likely opens a Ca2+ channel on the cellular membrane of cardiomyocytes to augment contraction rather than altering release of Ca2+ from internal stores. To test this hypothesis, we pretreated cardiomyocytes with the L-type Ca2+ channel blocker verapamil. Pretreatment with verapamil also completely inhibited Ca2+ entry, suggesting that FGF23 can affect L-type gating. Furthermore, prolonged exposure to FGF23 resulted in a 25% increase in basal levels of Ca2+, suggesting that over time FGF23 may lead to Ca2+ overload. Increased basal levels of Ca2+ have been linked to remodeling and hypertrophy of the heart (3, 11, 16). Thus [Ca2+]i may be a critical link between elevated FGF23, acute alterations in cardiac function, long-term remodeling, hypertrophy, and ultimately heart failure.

**Cardiac contractility.** Finally, to determine the acute effects of this elevated [Ca2+], we explored the effects of exogenous FGF23 on isolated ventricular muscle contractility. We hypothesized that the increases in [Ca2+]i were large enough to acutely improve cardiac contractility. We have shown for the first time that FGF23 significantly increases isometric tension, slope, and the area of contraction in isolated cardiac muscle. During cardiac excitation contraction coupling, force is generated on a beat-to-beat basis by a 10-fold increase in cytosolic Ca2+ by a process known as Ca2+-induced Ca2+ release (CICR). As a cardiac myocyte depolarizes, [Ca2+]i, begins to accumulate principally from the opening of L-type voltage-gated Ca2+ channels, and this triggers CICR from RyR2 to drive muscle contraction. Therefore, [Ca2+]i increases are tightly coupled to increased cardiac contractility. Our data showing an increase not only in the magnitude but also in the slope and area suggest that FGF23 may alter the CICR mechanism, allowing for greater [Ca2+]i, thus promoting faster and more powerful contractions. Our changes in tension, slope, and area lend strength to our calcium imaging data showing that FGF23 promotes Ca2+ entry via L-type Ca2+ channels. During relaxation, a return to Ca2+ homeostasis is controlled principally by SERCA and the NCX, with minor contribution from the plasma membrane Ca2+-ATPase. If FGF23 is increasing [Ca2+]i and the contraction by slowing Ca2+ removal, then there should be a corresponding increase in τ (rate of relaxation following contraction). Our data show that FGF23 does not affect τ, demonstrating that it is unlikely to have a major acute effect on SERCA, NCX, or Ca2+-ATPase transporter function.

Receptor antagonism with PD-166866 was able to eliminate the changes in the contractile waveforms, demonstrating that FGFRs mediate the effects of FGF23 in the myocardium.

**Significance.** Although an axis of signaling has been reported between bone and brain, gut, kidney, parathyroid, and adipose, potential endocrine cross-talk between bone and heart has not been well explored. A major question that arises from the current investigations on FGF23 and the heart is whether FGF23 is having both physiological and pathological effects on the myocardium. One possible physiological benefit of FGF23 altering cardiac contractility may be increasing renal phosphate clearance. Acutely increasing cardiac contractility may be a mechanism to increase cardiac output and renal blood flow. In addition, FGF23 may act on the heart to promote the expression of ANP/BNP, which would increase vasodilation, natriuresis, and diuresis to clear excess phosphate. However, in CKD the increases in ANP/BNP are unable to improve renal
clearance of phosphate. This accumulation of serum phosphate then elevates serum FGF23, which at high concentrations may promote cardiac dysfunction. This cycle appears to continue in CKD since increased ANP/BNP and FGF23 are predictive of CKD progression (10, 45) and cardiac pathologies (15, 21, 22, 26, 35, 43, 52).

From our findings, we propose that FGF23 is behaving like other well-characterized stress hormones (i.e., norepinephrine, epinephrine, and angiotensin II). Acutely, these hormones are inotropic and act to restore homeostasis; however, over the long term, chronic elevations in these hormones and \([Ca^{2+}]_i\), can cause contractile dysfunction, remodeling of the heart, and progression to cardiac hypertrophy. Similarly, our findings have led us to propose that endogenous FGF23 increases \([Ca^{2+}]_i\), in cardiomyocytes. This finding is important because increases in \([Ca^{2+}]_i\), can initially be diverted to the excitation-contraction coupling process and increased stimulation of CICR to improve cardiac contractility. However, long-term exposure to FGF23 may create disruptions in \([Ca^{2+}]_i\) homeostasis that then activate transcriptional remodeling mechanisms that contribute to long-term impairments in contractile function and ultimately cardiac hypertrophy. For example, it has been shown that FGF23 can activate the Ca\(^{2+}\)-sensitive calcineurin-NFAT signaling pathway (13), suggesting that \([Ca^{2+}]_i\), may be an important trigger for hypertrophic signaling in cardiomyocytes. Thus, FGF23’s induction of \([Ca^{2+}]_i\) signals appears to be important for controlling both transcriptional regulation and contractility. Importantly, our data in the Col4a1-/- mice suggest that left ventricular dysfunction may precede the development of cardiac hypertrophy. This may have important implications on a patient’s quality of life and may also serve as an important clinical diagnostic marker for severity of disease. In summary, our data show that FGF23 may have additional effects on the heart, in addition to hypertrophy, specifically related to calcium handling and cardiac contractility. Therefore, our studies provide an important rationale to further investigate the mechanisms for direct effects of this important bone endocrine factor on the heart.

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DISCLOSURES

All authors state that they have no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS


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