FGF23 induces cardiac hypertrophy independent of Angiotensin II

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Abstract

Chronic kidney disease (CKD) affects 10% of Americans. Patients live an average of only 4.25 years after diagnosis with the last stage of CKD (kidney failure). A leading cause of death in these patients is not kidney failure, but rather cardiovascular complications. One of the leading cardiovascular diseases in these patients is left ventricular hypertrophy (LVH), which affects 90% of stage 5 CKD patients, and 50-75% of stage 2-4 CKD patients. My study examined neonatal rat cardiomyocytes by adding blockers and hormones to show that fibroblast growth factor 23 (FGF23) induces LVH independently from an angiotensin II (ANGII). Additionally, my study showed that while FGF23 independently triggers LVH, the presence of FGF23 leads to increased amounts of ANGII. These findings have the potential to improve treatment for CKD patients suffering from LVH, by showing that in order to decrease FGF23, one must administer an FGF23 receptor blocker as opposed to an ANGII blocking drug. The results also have the potential to show that in order to decrease ANGII, one might be able to administer an FGF23 receptor blocker. Our study provides major insight for pharmaceutical companies and the research world so that LVH may one day be alleviated in CKD patients.

Introduction

Chronic kidney disease (CKD) is the progressive loss of renal function. Renal function is commonly measured by glomerular filtration rate (GFR)(1). CKD is categorized into five stages (1-5), with each increasing stage characterized by worsening kidney function measured as a drop in GFR (1-2). CKD stage 1, which affects one in ten Americans, is characterized by a GFR of 60-89 mL/min. In its fifth and final stage, CKD is associated with a GFR of less than 15 mL/min. Patients in this last stage live, on average, 4.25 years after diagnosis (2). Research shows that 50% of patient deaths and 30% of patient hospitalizations result not from CKD but cardiovascular disease (4). We hope to elucidate this connection.

Cardiovascular Disease in CKD Patients

Some of the most common cardiovascular events to emerge in CKD patients include myocardial infarction, coronary artery thickening, angina, cardiovascular arrest, and arrhythmia, among many others (5). The cardiovascular event we focus on is left ventricular hypertrophy (LVH). Left ventricular hypertrophy (LVH) is one of the most common cardiovascular diseases that develops in CKD patients, as it is present in 50-75% of CKD stage 2-4 patients, and 90% of stage 5 CKD patients (6).

Left Ventricular Hypertrophy

LVH is a disorder characterized by excessive enlargement of the left ventricle. This enlargement can lead to a decrease in pumping efficiency, arrhythmia, excessive myocardial stress, and myocardial infarction resulting in death (5). LVH develops when additional sarcomeres attach to cardiomyocytes. This increases cardiomyocyte surface area as a response to elevated hemodynamic burden such as hypertension. This increase in cell surface area due to the

additional parallel sarcomeres leads to an increase in cardiomyocyte width, and thus leads to wall thickening and aortic stress (5). In CKD patients affected by LVH, it has been shown that LVH regresses by up to 75% after kidney transplant. This finding corroborates the conclusion that the diseased kidney is the root cause of LVH in CKD patients (6). In 2011, Faul et. al found that the endocrine growth factor, fibroblast growth factor 23 (FGF23), induces LVH in animal models of CKD (6).

Fibroblast Growth Factor 23

Fibroblast growth factors (FGF) regulate cellular differentiation, proliferation, and migration of cardiomyocytes, and FGF receptors (FGFR) are expressed in different types of heart cells, including cardiomyocytes and fibroblasts. Additionally, FGFs induce a change in cardiac gene expression via utilization of nuclear factor of activated T-cells (NFAT). This change leads to an increase in surface area in order to induce cardiac growth (5). In cardiomyocytes, the NFAT pathway is regulated by calcium signaling (6). FGF23 is the most recently identified member of the FGF family.

In 2011, Faul et al. found that serum levels of FGF23 directly correlates with an increase in LV mass and CKD stage. Most importantly, the investigators found that FGF23 can directly induce LVH. FGF23 treatment caused an activation of prohypertrophic gene programs in isolated neonatal rat ventricular myocytes (NRVMs), including decreased alpha myosin heavy chain (α-MHC), and increased fetal beta myosin heavy chain (β-MHC) expression in contrast to control NRVMs that had been not treated. The low expression levels of α-MHC, and high levels of β-MHC indicate a shift from adult to fetal gene programs and an induction of pathological cardiac remodeling.

In addition, FGF23 mice had an average of a 7mm thicker left ventricular wall then the control mice. It was found that FGF23 utilizes FGFR isoforms 4 (FGFR4) to activate the NFAT pathway in the heart (5). Additionally, FGF23 binds in the kidney and parathyroid glands to FGFR1. Therefore, we hypothesize that since FGF23 binds in the kidney, and stresses the heart similarly to angiotensinogen II (ANGII), it is likely that ANGII and FGF23 are related.

Upon approaching my mentor with this idea, he disclosed that the calcineurin/NFAT signaling pathway in cardiomyocytes can be triggered directly by ANGII, which is regulated by the renin-angiotensin system (RAS). ANGII's receptor AT1R utilizes the NFAT pathway and upon activation induces hypertrophic growth of cardiomyocytes, similar to the described effects of FGF23-induced activation of FGFR4. Since circulating levels of ANGII and FGF23 are significantly elevated in CKD, and both factors can directly induce LVH by activating calcineurin/NFAT signaling in cardiomyocytes, we designed the hypothesis that AngII/AT1R and FGF23/FGFR4 signaling synergistically contribute to the development of LVH in CKD.

Renin-Angiotensin-Angiotensinogen System Function and Specifics

The renin-angiotensin system (RAS) is another mechanism found within the kidney that may also help to explain the increase in cardiovascular disease in CKD patients. The RAS is regulated by the juxtaglomerular apparatus (JGA), which is located near the afferent arteriole, behind a dense section of the nephron called the macula densa. When JGA blood volume drops due to malfunctioning nephrons from CKD and osmolarity decreases, the JGA releases the enzyme renin that yields angiotensinogen, which proceeds to yield ANGI, which in turn yields ANG II.

ANGII is a peptide hormone that acts as an arteriole constrictor and raises blood pressure by decreasing blood flow to capillaries including kidney capillaries (1). ANGII also stimulates

the adrenal glands to release aldosterone that targets distal tubes in the nephron by increasing the Na+ absorbance rate. After blood pressure is regulated once renin is released, there is normally negative feedback to RAS signals the JGA to stop producing ANGII. In CKD patients, these signals are not regulated properly, and thus the patient develops increased blood pressure, increased low-density-lipoprotein cholesterol, and an inflammatory response (4). ANGII has been found to target the heart via the angiotensinogen type 1 receptor (AT1R) pathway and the NFAT pathway (5).

Since AT1R and FGFR4 both utilize the NFAT pathway in cardiomyocytes, *it would be valuable to find a relationship between ANGII/AT1R and FGF23/FGFR4 to further clarify the CKD-CVD connection so that the progression of cardiovascular disease may be attenuated as early as possible in CKD diagnosis*. ANGII and RAS system over-activation can be attenuated by the use of AT1R blockers (ARB). Repurposing these drugs for blocking LVH progression could lead to a dramatic decrease in LVH development in CKD patients, and thus a much lower death rate due to cardiovascular events. Therefore, it is critical to test ANGII/AT1R interference in NVRMs to see if hypertrophy may be decreased by not only blocking AT1R utilization of the NFAT pathway, but also by blocking FGF23/FGFR4 utilization of the NFAT pathway. We hypothesize that combined pharmacological interference with RAS and FGFR4 might have cardio-protective effects in NVRMs, as seen in figure 1.

AT1R Blockers and FGFR4 blockers

AT1R blockers (e.g. Losartan) prevent ANGII from utilizing the AT1R pathway, thereby preventing ANGII from actually binding to cells in the heart and taking effect. Therefore, AT1R and FGFR4 blockers can be used to determine if ANGII/AT1R interference could result in decreased levels of FGF23/FGFR4 signaling, and if FGF23/FGFR4 interference could result in

decreased levels of ANGII/AT1R. If ANGII is blocked with AT1R blockers and FGF23 decreases, AT1R blockers and other ANGII blockers may be able to be utilized in CKD patients as a treatment for blocking the pathologic effects of FGF23, thus leading to decreased occurrence of LVH in CKD patients. Additionally, blocking the accumulation of ANGII via utilization of anti-FGFR4 blockers may have cardioprotective effects in a CKD setting.

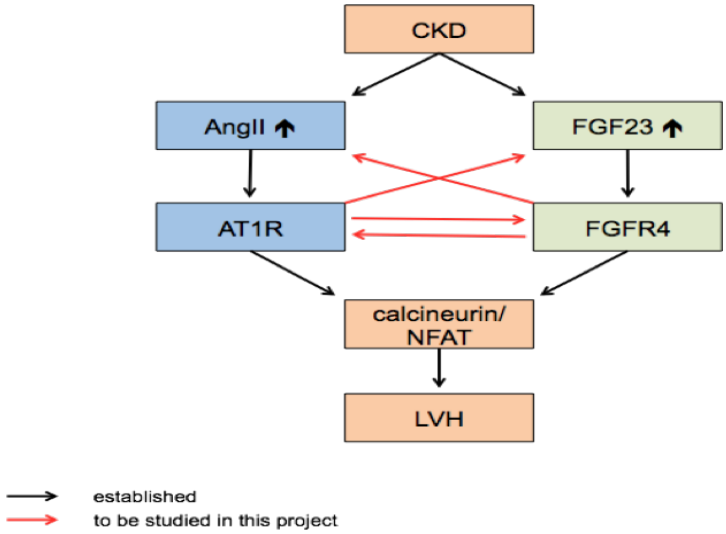

Figure 1
Chart describing CKD-LVH pathway and clarifying novel connections to be studied in project. This study clarifies if the ANGII's receptor triggers a higher production of FGF23 and vice versa. (Faul et. al, 2016)

Hypotheses

H_0: ANGII/AT1R and FGF23/FGFR4 do not crosstalk.

H_1: AngII/AT1R and FGF23/FGFR4 signaling contribute synergistically to the induction of cardiac hypertrophy.

H_2: Combined pharmacological interference with RAS and FGFR4 has cardioprotective effects in patients with CKD.

Objectives

1. Confirm that AngII induces calcineurin/NFAT signaling and hypertrophy in cultured neonatal rat ventricular myocytes (NRVM).
2. Determine whether ANGII induces FGF23 expression in NRVM with qPCR analysis.
3. Determine whether FGF23 induces ANGII expression in NRVM with qPCR analysis.
4. Determine if FGFR4 inhibition reduces AngII expression and/or AngII-induced calcineurin/NFAT signaling and hypertrophy in NRVM.

My Role in The Study

After studying ANGII for two years and reading 45 journal articles, I brought the original idea of researching FGF23 in conjunction with ANGII to my mentor. I proposed two objectives: to determine if ANGII induces FGF23 expression, and to determine if FGF23 induces ANGII expression. Together, my mentor and I collaborated to design the remaining two objectives.

I spent 240 hours at The Miller School of Medicine at Miami University learning how to perform various laboratory techniques including qPCR tray loading and medium changing. After being instructed by Dr. Alexander Grabner, Dr. Brian Czaya, and Dr. Christopher Yauncil on how to use the appropriate kits, I independently changed all cell medium. Additionally, I made cDNA out of RNA of half of my cell plates, and lysed half of my cell plates using a RNeasy Kit. Dr. Grabner and Dr. Yauncil performed the other half of the cDNA translations and lysings.

For QPCR analysis, I worked with Dr. Czaya to design plate wells to run, one of which is shown in Figure 2. Together, we decided which primers to run. I translated the data into the bar graphs shown in this paper and provided the discussion and conclusion.

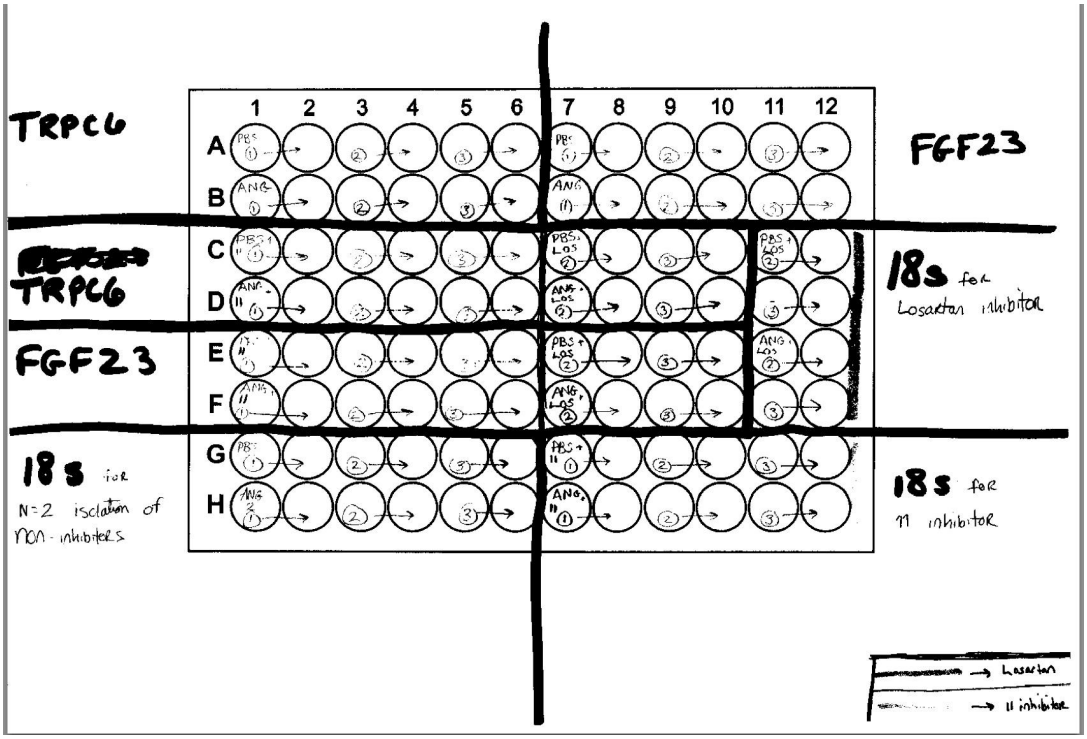

Figure 2
Outline of inhibitor well plate for QPCR analysis (Competition entrant, 2016)

Methods

Inhibitors

A human monoclonal FGFR4-specific blocking antibody (anti-FGFR4; U3Pharma, Martinsried, Germany) was used to inhibit FGF23-mediated activation of FGFR4. Losartan (Sigma-Aldrich) was used as an ARB to prevent ANGII from binding to AT1R.

Primers

Transient receptor potential channel 6 (TRPC6) is an established NFAT target gene, and TRPC6 expression levels were analyzed as a readout for NFAT activity (table 1). FGF23 was measured for expression of itself. For ANGII expression, mRNA levels of its precursor form, angiotensinogen, were measured. As a housekeeping gene, 18s was used.

Table 1: List of primer sequences (Karp, 2016)

Primer	FW Sequence	Rev Sequence
TRPC6	5’ -GTCGGTGGTCATCAACTACAATC-3′	5’-CCACATCCGCATCATCCTCAATT-3′
18s	5’ -CATTCGAACGTCTGCCCTAT-3’	5’-GTTTCTCAGGCTCCCTCTCC -3’
Angiotensinogen	5'-TTGTTGAGAGCTTGGGTCCCTTCA-3’	5'-CAGACACTGAGGTGCTGTTGTCCA-3'
FGF23	5′-GACGGAACACCCCATCAGACTATC-3′	5′-CGGGCTGAAGTGATACGAT-3′

Isolation and Culture of NRVMs

All animals were euthanized prior to my arrival at the lab, and were not sacrificed solely for my project. NRVMs were isolated using a standard isolation system (Worthington Biochemical Corporation) (7). Hearts from one to three day-old rats were harvested and minced in calcium- and magnesium-free HBSS, and the tissue was digested with 50 μg/ml trypsin at 4°C for 20 to 24 hours. Soybean trypsin inhibitor in HBSS was added, and the tissue was further digested with collagenase (in Leibovitz L-15 medium) under slow rotation (0.1 *g*) at 37°C for 45 minutes. Cells were released by gently triturating the suspension 20 times with a standard 10-ml plastic serological pipette and filtering it twice through a cell strainer (70 μm, BD Falcon). Cells were incubated at room temperature for 20 minutes and spun at 100 *g* for five minutes. The cell pellet was resuspended in plating medium (340 DMEM, 85 mL M199, 75 mL FBS (15% f.c.), and 5 mL PenStrep).

Cells were plated on plastic six well plates with 10 cm^2 surface area/well. Cells were seeded at a concentration of 1 x 10^6 cells/well. Cells were left undisturbed in plating medium at 37°C for 72 hours and then cultured in maintenance medium (400 mL DMEM, 100 mL M199, 5 mL PenStrep, and 5 mL ITS) for four days. Cells were plated in the presence of 1 μM ANGII, 1 μM of isoproterenol or 25ng/ml FGF23 for 48 hours. PBS-treated cells served as controls. QPCR was run on these plates. For protein analysis of FGF23, one well was plated with 2 μm PBS, one with 2 μm ANGII, and 1 with .5 μm IGF. Cells were left undisturbed in maintenance media.

Six more 6-well plates were cultured and treated with inhibitors. Losartan was used as an ARB blocker and anti-FGFR4 was used as a FGFR4 receptor blocker.

RNA Isolation and Quantification

Cells were washed in PBS after 24 hours, and lysed in RLT buffer. RNA was purified from isolated NRVMs using the RNeasy Kit. Prior to RT-PCR, total RNA samples were digested with DNase I, and RNA was transcribed into cDNA using SuperScript II.

QPCR

100ng of cDNA was used in the first run with sequence-specific outside primers (n=1). The total reaction was analyzed by agarose gel electrophoresis. Gene expression was analyzed by real-time PCR using the SYBR Green PCR Master Mix (Applied Biosystems), with the ABI PRISM 7700 Sequence Detection System. Relative expression values were evaluated using *18s* as housekeeping gene.

Results/Discussion

FGF23 and ANGII induce cardiac hypertrophy via utilization of the NFAT pathway. Real-time PCR results show ANGII treatment of NRVMs (n=1) results in a 1.6 times greater fold change in TRPC6 mRNA expression compared to the PBS control. In addition, FGF23 treatment (n=1) resulted in a 1.7 times greater fold change in TRPC6 compared to the control (Figure 3). TRPC6 is a marker of NFAT activity. Since NFAT activity in myocytes results in cardiac hypertrophy, TRPC6 was used as a measurement for cardiac hypertrophy.

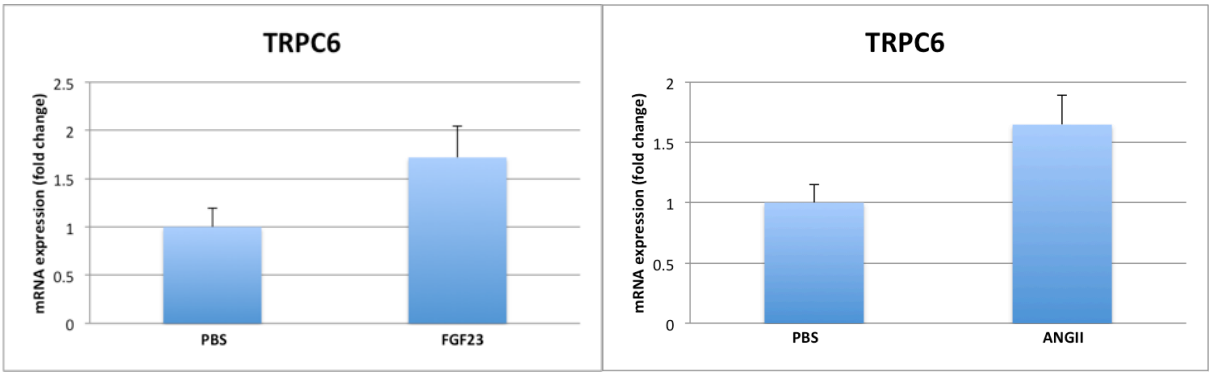

Figure 3 NRVMS treated with PBS, FGF23, and ANGII undergo PCR for TRPC6 fold change measurement. Both FGF23 and ANGII-treated cells demonstrate significantly higher TRPC6 mRNA expression than PBS-treated cells (Karp, 2016)

ANGII does not increase FGF23 expression. We tested the hypothesis that ANGII increases FGF23 expression via real time-PCR. The results show that ANGII (n=1) does not increase mRNA expression of FGF23, and in fact decreases FGF23 expression. ANGII-treated cells demonstrate .57 times less mRNA concentration than the control (Figure 4). *This is a novel finding in NRVMS.*

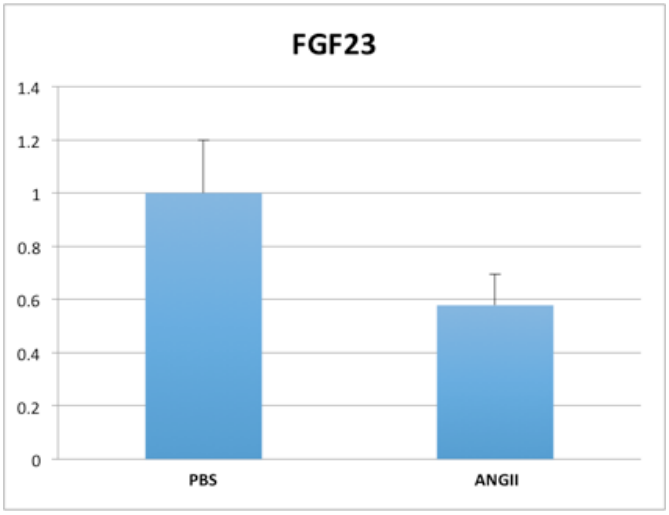

Figure 4 NRVMS treated with PBS and ANGII undergo PCR for FGF23 fold change measurement. ANGII-treated NRVMS demonstrated a fold change of 0.57 compared to the control of 1.0. (Karp, 2016)

FGF23 increases angiotensinogen levels in NRVMs. We tested the hypothesis that FGF23 increases ANGII expression via real time-PCR. Since angiotensinogen utilizes renin to produce ANGI, which utilizes ACE to produce ANGII, we used angiotensinogen as an upstream marker of ANGII. Results show that FGF23 increases ANGII expression by a fold change of 2.7 compared to the control (Figure 5). *This is a novel finding in the ANGII-FGF23 crosstalk.* Additionally, angiotensinogen was confirmed to be elevated in the presence of isoproterenol, a common inducer of hypertrophy, by a fold change of 4.2.

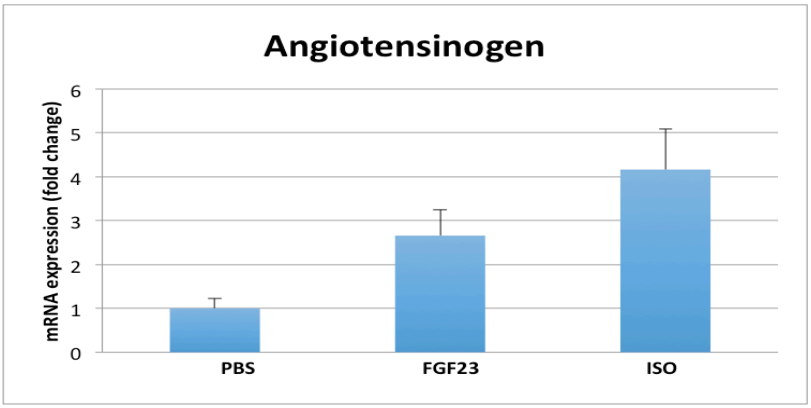

Figure 5 NRVMs treated with PBS, Isoproterenol, and FGF23 undergo PCR analysis for angiotensinogen expression. FGF23 yielded an mRNA fold change expression 2.7 time that of the PBS. (Competition entrant, 2016)

11 Does not block ANGII-triggered NFAT activity. FGFR4 blocker treatment of NRVMs previously treated with ANGII (n=1) resulted in increased TRPC6 mRNA expression relative to the control. *This is a novel finding, as the FGFR4-ANGII crosstalk has not been previously explored.* Fold change in mRNA expression of TRPC6 in ANGII and anti-FGFR4 treated NRVMs (n=1) was 1.7 times higher than that of the control (Figure 6).

Losartan blocks ANGII-triggered NFAT activity. AT1R blocker treatment of NRVMS previously treated with ANGII (n=1) decreased TRPC6 expression relative to the control. This finding is consistent with previous literature. Fold change of TRPC6 in ANGII and Losartan treated NRVMs (n=1) was .7 times as great as the control, and .4 times as great as TRPC6 expression in ANGII alone treated cells (Figure 6).

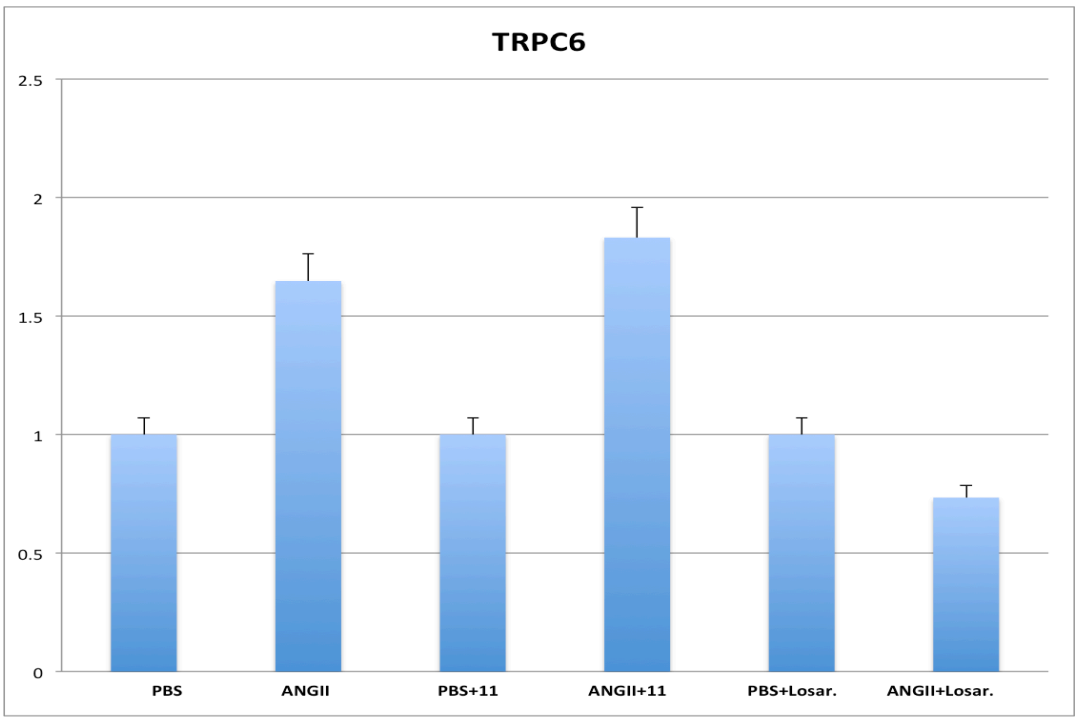

Figure 6

NRVMs treated with PBS, ANGII, PBS and anti-FGFR4, ANGII, anti-FGFR4 PBS and Losartan, and ANGII and Losartan are measured for TRPC6 expression. All treatments administered to PBS-treated cells had a fold change of 1.0. ANGII expressed significantly increased TRPC6 expression by 1.6.The anti-FGFR4 treatment The anti-FGFR4 increased TRPC6 expression even more so in ANGII treated cells by 1.8. Losartan significantly decreased mRNA expression of TRPC6 in ANGII-treated cells to 0.7 the fold change of PBS.

Conclusion

Heretofore unreported in the literature, this study presents the finding that angiotensin II triggers LVH independently from FGF23 in NRMVs. This finding was evident in the .4 times lower FGF23 fold change compared to the control. This conclusion was further corroborated by the failure of Losartan to reduce FGF23 levels as well as failure of the FGFR4 inhibitor 11 to reduce TRPC6 in the presence of angiotensin (Figure 7).

The angiotensinogen PCR results suggest that while ANGII does not trigger FGF23 production, it is possible that FGF23 triggers ANGII production (Figure 7). This finding leads to the question: can Losartan decrease FGF23 NFAT activity?

We confirmed previous reports that ANGII and FGF23 trigger LVH via NFAT activation. This was shown via TRPC6 mRNA expression, where both ANGII and FGF23 had a

TRPC6 fold change expression .7 higher than the control. Isoproterenol was also shown to increase angiotensinogen by 4.2 mRNA fold change. Since isoproterenol is a major inducer of LVH and TRPC6 indicates NFAT activity leading to hypertrophy, these findings support that conclusion that ANGII induces LVH. Furthermore, Losartan was shown to reduce TRPC6 expression by .4 fold change, showing that Losartan can be used to reduce LVH. These findings have the potential to improve treatment for CKD patients suffering from LVH, by showing that in order to decrease FGF23, one must administer an FGF23 receptor blocker as opposed to an ANGII blocking drug. The results also have the potential to show that in order to decrease ANGII, one might be able to administer an FGF23 receptor blocker. Our study provides major insight for pharmaceutical companies and the research world so that LVH may one day be alleviated in CKD patients.

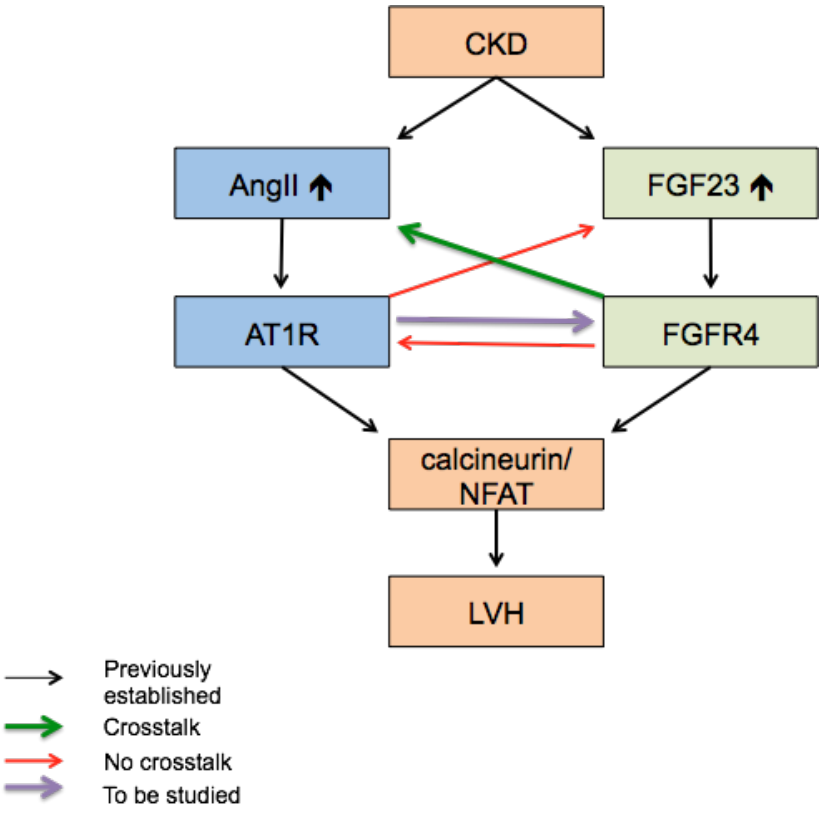

Figure 7
Chart clarifying findings in study. FGF23 independently causes LVH. However, ANGII is upregulated by the presence of FGF23 binding to its receptor, FGFR4 (Karp, 2016)

The finding that ANGII and FGF23 independently trigger LVH poses a potential aid to CKD patients diagnosed with LVH. Since this study shows that Losartan does not lower FGF23 levels, it suggests that in order to reduce both ANGII and FGF23, both an FGFR4 and AT1R inhibitor must be used. This is significant information because it not only contributes to the understanding of how to treat LVH patients, but also clarifies the ANGII-NFAT-FGF23 pathway by showing that there is not a crosstalk present. This finding entails a potential crosstalk in the reverse direction: FGF23-NFAT-ANGII. Additionally, the finding that FGF23 increases angiotensinogen levels calls for further investigation of FGFR4 blockers and angiotensinogen II. The findings in this study should be confirmed, as there were only n=1 trials performed. This study is a stepping stone in developing more effective therapy for aiding CKD patients with LVH. Specifically, our results aid future research in determining the efficacy of using Losartan and/or FGF23 blockers to attenuate LVH. Our hope is that through a better understanding of the crosstalk between FGF23 and ANGII, the death rate among CKD patients due to cardiovascular disease may be curtailed.

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