Curcumin prevents cardiac remodeling secondary to chronic renal failure through deactivation of hypertrophic signaling in rats

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Abstract

Background: The prevalence of left ventricular hypertrophy (LVH) is frequent in patients with end stage renal disease following chronic renal failure (CRF). We investigated the therapeutic efficacy of curcumin, the principal curcuminoid of the Indian curry spice turmeric, in attenuation of LVH and sought to delineate the associated signaling pathways in blunting the hypertrophic response in nephrectomized rats.

Methods and Results: Adult Sprague-Dawley rats underwent nephrectomy (Nx) by removal of 5/6 of the kidneys. Four groups were studied for 7 weeks: 1- Control (sham), 2- Nx, 3- Nx + curcumin (150 mg/kg; bid), 4- Nx + enalapril (15 mg/kg; bid) as positive control. Subtotal nephrectomy caused renal dysfunction, as evidenced by gradual increase in proteinuria, elevation in blood urea nitrogen and plasma creatinine. Nx rats showed a significant hypertrophic response and increased diameter of inferior vena cava at inspiration which was inhibited by treatment with curcumin or enalapril. Moreover, the Nx rats demonstrated changes in the signaling molecules critically involved in the hypertrophic response. These include increased GSK-3β phosphorylation, β-catenin expression, calcineurin, pNFAT, pERK and pAKT. Both curcumin and enalapril variably but effectively deactivated these pathways.

Conclusion: Curcumin attenuates cardiac hypertrophy and remodeling in nephrectomized rats through deactivation of multiple hypertrophic signaling pathways. Considering the safety of curcumin, these studies should facilitate future clinical trials in suppressing hypertrophy in patients with CRF.
Introduction

Cardiac hypertrophy is one of the most common cardiac abnormalities in uremic patients and represents a leading cause of death (38). Experimental studies have also shown similar cardiovascular abnormalities in nephrectomized rats, the animal model of chronic uremia (16). The classical hypertrophy inducers including endothelin, catecholamines and angiotensin II (Ang II) are known to play a major role in clinical and experimental uremia (3, 16). We and others have previously shown that uremic rats with chronic renal failure develop significant cardiac hypertrophy (2, 16). Two important mechanisms of cardiac hypertrophy have been described, which include glycogen synthase kinase-3β (GSK-3β)β-catenin and calcineurin/nuclear factor of activated T cells (NFAT) pathways (43, 46). It has been shown that hypertrophic stimuli promote phosphorylation (inactivation) of GSK-3β via Akt (48) and ERK phosphorylation (14). Constitutively active GSK-3β remains dephosphorylated and prevents hypertrophic growth of cardiac myocytes by inhibiting transcriptional regulators, including β-catenin and NFAT (43). GSK-3β acts by both the non-canonical pathway, such as calcineurin/NFAT, and canonical Wnt signaling involving β-catenin (46). Calcineurin regulates pathological hypertrophy through dephosphorylation of NFAT which results in its nuclear translocation and modulation of gene expression (7). GSK-3β is also capable of modulating NFAT phosphorylation and subsequent nuclear migration of the transcription factor (6, 33).
Curcumin (diferuloylmethane) is a yellow pigment in the spice turmeric (also called curry powder) which has been used for centuries as a treatment for inflammatory diseases. Extensive research within the past two decades has shown that curcumin exerts its anti-inflammatory effects through the downregulation of inflammatory transcription factors (e.g., nuclear factor κB), enzymes (e.g., cyclooxygenase 2 and 5 lipoxygenase) and cytokines (e.g., tumor necrosis factor, interleukin 1 and interleukin 6) (4). Recent studies also show that curcumin blocks cardiac hypertrophy in Dahl salt sensitive rats (32). Moreover, we demonstrated that curcumin ameliorated chronic renal failure which was comparable to the angiotensin converting enzyme inhibitor (ACEI), enalapril (17). One of the downstream targets of Akt is GSK-3β. In leukemic cells, curcumin has been shown to inhibit GSK-3β phosphorylation by blocking Akt (45). Moreover, β-catenin is regulated by GSK-3β and curcumin has been shown to induce degradation of β-catenin (25). In addition, it has been demonstrated that cardioprotection with curcumin was mediated by interruption of p300- histone acetyltransferase (HAT) activity-dependent signaling pathways, resulting in protection against the deleterious effects of cardiac hypertrophy, inflammation, and fibrosis (32).

In the present study, we hypothesized that chronic renal failure induced by partial nephrectomy causes cardiac hypertrophy through mechanisms involving deactivation of multiple signaling pathways. These include GSK-3β/β-catenin, calcineurin/NFAT, Akt, and ERK1/2. Since several of the downstream targets of Akt phosphorylation can be inhibited by curcumin (39, 50), we further conjectured that curcumin would be effective in blunting hypertrophy in this model. Since Ang II is a known mediator of cardiac
hypertrophy and ACEIs are known to reduce cardiac hypertrophy (2, 9), we concurrently compared the effect of enalapril in attenuating hypertrophy and the associated signaling pathways.

**MATERIALS AND METHODS**

**Chemicals:** Curcumin was purchased from Biomol. All antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA.). Phospho-GSK-3β was purchase from Cell Signaling (Cell Signaling Technology, Danvers, MA).

**Animals:** The animal procedures were approved by the Institutional Animal Care and Use Committees of the Virginia Commonwealth University.

**Surgical procedures:** All surgical procedures were carried out using sterile conditions under isoflurane anesthesia. Chronic renal failure was induced in rats by performing five-sixths (5/6) nephrectomy as described previously by Ghosh et al (16). Rats weighing between 150-200 g were anesthetized and a left flank incision was made to expose the left kidney. The renal artery was temporarily occluded and the upper and lower thirds of the kidney were ligated and excised thereby leaving only 1/3 of the mass of the left kidney intact. Bleeding was controlled by compression until it stopped. The muscle and skin incisions were sutured with polypropylene suture. The animals were returned to the vivarium for recovery. One week later, a right flank incision was made,
the renal vessels and ureter were tied, and the right kidney was excised. The animals were allowed to recover and the drug treatment was initiated 7 days later.

Experimental Groups: Rats were randomized into four groups (n=6/group) as shown in Figure 1. The control group underwent sham surgery, and the other 3 groups underwent five-sixth nephrectomy as described above. Prior to surgery, the animals were kept overnight in metabolic cages and urine was collected for determining proteinuria. The animals were kept in the metabolic cages at the beginning of weeks 3, 5, and 7 for urine collection.

Treatment protocol: Suspension of curcumin and enalapril were prepared in 0.5% carboxymethylcellulose (CMC). Due to the instability of curcumin in the aqueous solution, it was prepared fresh and was administered within 10 minutes of the preparation. As shown in Fig 1, the nephrectomized (Nx) rats were randomized to receive the following treatments. 1. Vehicle (0.5% CMC) which was administered by oral gavage; 2. Curcumin at a dose of 150 mg/kg daily. 3. Enalpril (10 mg/kg, suspended in CMC). The control group consisted of sham operated rats where the capsule was removed. On the ninth week, the animals were sacrificed and blood as well as heart tissue were collected for analyses.

Determination of cardiac mass
Cardiac mass was assessed by measuring right and left ventricle wet weight (RVW and LVW, respectively). The RVW or LVW to body weight (BW) ratio was calculated.
Echocardiography was performed using the Vevo770™ imaging system (VisualSonics Inc., Toronto, Canada) prior to surgery (baseline) and 8 weeks after surgery prior to sacrificing the animal. Pentobarbital (30 mg/kg; \textit{ip}) was used for anesthesia and the procedure was carried out as previously described (37) to measure LV end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD). Inferior vena cava (IVC) diameter (in mm) was measured at end-inspiration by echocardiography using a subcostal approach. This technique provides simple and useful measurements of IVC size and function in cardiac disease (36), and is reflective of right atrial pressure which increases with heart failure.

Measurement of blood urea nitrogen and creatinine:

Blood urea nitrogen (BUN) and creatinine were measured by a NOVA16 autoanalyzer (NOVA Biomedical, Waltham, MA).

Longitudinal measurement of arterial pressure by tail plethysmography:

Arterial blood pressure (BP) was determined by tail plethysmography as stated before (17), using the CODA 2 system (Kent Scientific Corporation, Torrington, CT). CODA 2 utilizes volume pressure recording sensor technology to measure rat tail blood pressure. This is a computerized, non-invasive tail cuff acquisition system which can simultaneously measure systolic, diastolic and mean arterial pressure without operator intervention. Before surgery rats were trained for 3 days and were kept in a restraining holder for 5-10 min period. On the fourth day blood pressure (BP) was recorded (week
0). During this period, 25 sequential readings were obtained. Values within a range of 10 mm Hg were averaged. Two weeks after the second surgery, the animals were retrained and BP was recorded (week 2). Similar BP recording was done on weeks 5 and 8 post surgery.

Preparation of LV homogenate:

Left ventricle was dissected and immediately frozen in liquid nitrogen and stored at -70°C until use. The frozen ventricle was ground to a powder and distributed into 3 batches. One batch was used for RNA extraction (given blow), the other two batches were mixed in ice-cold HEPES buffer in presence and absence of phosphatase inhibitors (10 mM HEPES, 0.2% Triton X-100, 50 mM NaCl, 0.5 mM sucrose, 0.1 mM EDTA, protease inhibitors, with or without phosphatase inhibitors) and homogenized with ice-chilled dounce homogenizer at 4°C. Preparations with phosphatase inhibitors were used to measure phosphorylated proteins. An aliquot of the homogenate was stored and the rest was used to prepare cytosolic and nuclear extracts. The homogenate was spun at 10,000 rpm for 10 minutes and the supernatant was aliquoted and stored at -70°C as cytosolic extract. The pellet was suspended in ice cold buffer (10 mM HEPES, 500 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% IGEPAL and protease inhibitors, with or without phosphatase inhibitors) and vortexed at 4°C for 15 minutes and centrifuged for 10 minutes at 14,000 rpm. The resulting supernatant was aliquoted and stored as nuclear extract at -70°C. Absence of cross reactivity with β-actin in western blots confirmed the purity of nuclear extracts. A small aliquot of kidney homogenate, cytosol and nuclear extract was used for protein estimation.
**Immunoblotting:**

The cytosol (75–100 µg total protein) and nuclear extracts (50 µg total protein) were separated on a 4–20% SDS-PAGE gel, and proteins were transferred to a PVDF membrane as described previously (16). After washing briefly in phosphate-buffered saline containing 1% Tween-20 (PBS-T) and blocking in 5% nonfat dry milk, blots were incubated with appropriate antibodies in 5% nonfat dry milk overnight at 4°C. The membranes were washed 3-5 times in PBS-T, and subsequently incubated with appropriate secondary antibody diluted in 5% nonfat dry milk for 1 h at room temperature. After 3-5 washes in TBS, blots were developed using Lightning Chemiluminescence Reagent Plus and exposed to X-ray film.

**Quantitative Real Time RT-PCR Analysis:**

Total RNA was extracted from the LV with the RNeasy Mini Kit and analyzed by real time RT-PCR as described before (16). Briefly, 2 µg RNA was reverse transcribed with Thermoscript RT-PCR System (Invitrogen), and first strand cDNA was used to perform real-time PCR using Stratagene Mx3000p real-time PCR system with TaqMan Gene Expression Assays for GSK-3β (Rn00583429), β-catenin (Rn00584431), calcineurin (Rn00820912), NFAT (Rn01426728) and β-actin obtained from Applied Biosystems (Foster City, CA). The amount of mRNA was calculated by ΔΔCT method and normalized to β-actin.

*Activation of NFAT*
NFAT DNA-binding activity was assessed with TransAM™ NFAT transcription factor assay kit (Active Motif) using nuclear extracts from the kidney. Briefly, nuclear extracts were added to each well of a 96-stripwell plate to which the consensus NFAT binding site oligonucleotide had been immobilized. A primary antibody specific for an epitope on the bound and active form of the transcription factor was then added, followed by subsequent incubation with secondary antibody and developing solution. Intensity of the developed color was quantified and is reported as a measure of activated and DNA-bound NFAT in the tested nuclear extract.

**Statistical Analysis:**
Statistical comparisons among groups were performed using ANOVA followed by Tukey's Multiple Comparison Test. Groups were considered to be significantly different with a P value equal to, or less than, 0.05.

**RESULTS**
*Effect of curcumin and enalapril on systolic blood pressure, proteinuria, plasma creatinine and blood urea nitrogen:*

As shown in table 1, the systolic blood pressure (SBP) of Nx animals measured at 3, 5, and 7 weeks was significantly higher than control rats (p<0.01). Longitudinal measurement of SBP showed that enalapril treatment effectively curtailed the increase in SBP (p<0.01). Curcumin had no significant effect on SBP until the seventh week. Subtotal nephrectomy resulted in renal dysfunction, as evidenced by gradual increase in
proteinuria (Table 1), elevation in BUN and plasma creatinine measured at 8 weeks (Table 2). Moreover, Nx animals demonstrated progressive increase in proteinuria. Both curcumin and enalapril treatment significantly reduced proteinuria by 40-60%. The proteinuria in curcumin and enalapril cohorts was not significantly different. BUN and plasma creatinine were almost 4 fold higher in Nx animals than the vehicle control at 8 weeks. Curcumin was as effective as enalapril in reducing both the BUN and creatinine levels (p<0.01). These data suggest that curcumin is as effective as enalapril in attenuating renal dysfunction in NX animals.

Effect of nephrectomy on kidney weight:
Nephrectomized animals had their right kidney removed therefore the left kidney of the control was compared with the 2/3 excised left kidney of the nephrectomized animals. As shown in table 2 there was no significant difference in kidney weight between the groups. However, it is to be noted that the control animals had intact kidney and the partially ablated left kidney of the Nx group and treated animals (whose left kidney was also partially ablated) had hypertrophied to compensate for the nephron loss. Therefore there was no significant difference in the weight.

Effect of curcumin and enalapril on cardiac remodeling:
The body weight (BW) of curcumin and enalapril treated rats were lower by 17% and 21%, respectively, as compared with the corresponding vehicle control group (p<0.001, Table 2). LV weight of control and Nx rats were not different i.e., 1.13±0.14 versus 1.34±0.24 g, p>0.05. LV weight in rats treated with curcumin and enalapril were lower as compared with Nx (p<0.01). The LV/BW ratio for curcumin and enalapril-treated
animals were not different from control, but they were significantly lower than Nx animals (p<0.001). The average RV/BW of Nx animals was 1.7 fold higher than the control (p<0.01). Both curcumin and enalapril treatment significantly improved RV/BW ratio (p<0.05).

Figure 2A shows representative M-mode images from sham operated and Nx rats at 8 weeks. These images clearly show more dilatation and wall thickening in Nx rats as compared with sham control. The average data (in mm) showed a significant increase in LV mass in Nx rats (1.47±0.19) as compared with controls (0.98±0.04, P<0.01, Fig 2B). Curcumin and enalapril-treated rats had LV mass of 0.95±0.1 (P<0.01) and 0.93±0.3 (P<0.05), respectively. LV end diastolic diameter (EDD, in mm) was higher in Nx rats (7.2±0.2) as compared to controls (6.4±0.2, P=0.01). Curcumin and enalapril attenuated LV dilatation as shown by EDD of 6.3±0.5 and 6.2±0.5, respectively (P=0.01, Fig 2C).

Using a subcostal approach, we also measured changes in inferior vena cava (IVC) diameter at end-inspiration, which is a diagnostic tool for identification of increased right atrial pressure and congestive heart failure. As shown in Fig 3A, IVC diameter decreased with inspiration in both the control and Nx rats. However, the IVC diameter at end-inspiration was significantly higher in the Nx rats as compared to the sham control. Furthermore, rats treated with curcumin or enalapril demonstrated significantly smaller IVC diameter as compared to the untreated Nx controls (Fig 3B).

Effect of curcumin and enalapril on GSK-3β:

GSK-3β is among the serine/threonine kinases. It is inactivated by hypertrophic stimuli through phosphorylation, and inactive GSK-3β is responsible for cardiac hypertrophy
Our results show no significant changes in GSK-3β mRNA (not shown) and non-phosphorylated GSK3β (total) protein between the groups (Fig 4A). However, pGSK-3β in Nx hearts was almost 2 fold higher as compared with the controls (Fig 4B p<0.001). Moreover, pGSK3β/GSK3β ratio was 2.2 fold higher in Nx (p<0.001) as compared to controls demonstrating inactivation of the protein (Fig 4C). A significantly lower pGSKβ was observed in curcumin (44%, p<0.01) and enalapril (30%, p<0.05) treated animals.

Effect of curcumin and enalapril on β-Catenin expression:

β-catenin is a downstream target of GSK-3β and its overexpression is known to induce cardiomyocyte growth in vivo and in vitro (20). β-catenin mRNA, measured by real time PCR, was increased in Nx animals as compared to controls (p<0.05). Curcumin and enalapril treatment had no effect on the mRNA levels (Fig 5A). The cytosolic β-catenin was significantly higher in Nx animals as compared with controls (p<0.01), and was reduced by curcumin treatment (p<0.05, Fig 5B). Similarly, β-catenin was significantly higher in the nuclear fractions as compared with controls (p<0.01, Fig 5C), and was decreased by curcumin. In the Nx animals, the β-catenin levels in total homogenates were 1.4 fold higher than the controls but this was not statistically significant (data not shown). Enalapril treatment had no significant effect on β-catenin protein expression.

Effect of curcumin and enalapril on calcineurin:

The calcium activated protein phosphatase, calcineurin, is a serine-threonine-specific phosphatase which plays a central role in the development of pathological cardiac hypertrophy (31). Ang II has been shown to activate calcineurin in various settings (40, 41). Calcineurin mRNA (measured by real time PCR) and protein expression were
increased in Nx animals (Fig 6A and 6B), and decreased following treatment with enalapril. However, curcumin had no effect on calcineurin mRNA or protein expression.

**Effect of curcumin and enalapril on NFAT:**

Dephosphorylation of NFAT facilitates its nuclear migration and enhanced DNA binding leading to hypertrophy. Both calcineurin and GSK-3β are known to regulate NFAT phosphorylation and hypertrophy (34, 47). In the present study, we did not observe a significant difference in non-phosphorylated cytosolic NFAT between the groups (data not shown). However, cytosolic phosphorylated NFAT (p-NFAT) was significantly decreased in Nx animals compared to control (Figure 7A), and was significantly attenuated by enalapril and curcumin (p<0.05). The NFAT in the nuclear fraction of Nx animals was significantly higher as compared to the control (Figure 7B, p<0.01), and was decreased by curcumin (p<0.05) and enalapril (p<0.01). There was no significant difference in the cytosolic and nuclear NFAT between curcumin and enalapril. We also confirmed these results by using NFAT-DNA binding assay (TransAm™ transcription factor ELISA). As shown in figure 7C, NFAT DNA binding was nearly two fold higher than the control (p<0.01) and both curcumin and enalapril significantly decreased it (p<0.05).

Both AKT and ERK modulate the phosphorylation of GSK3β and activation of calcineurin-NFAT pathway (30, 43). We investigated the role of these signaling molecules in the Nx hearts and to see if curcumin and enalapril can modify their phosphorylation state. The ratios of pERK/ERK (Fig 8A) and pAKT/AKT was significantly higher in the Nx animals as compared to the controls (p<0.01, Fig 8B).
Curcumin and enalapril significantly reduced the pERK and pAkt (p<0.01) without affecting the non-phosphorylated proteins (Fig 8).

**DISCUSSION**

Cardiac hypertrophy is an adaptive response which occurs as a result of increased workload. Although the initial hypertrophic response is considered to be beneficial, sustained cardiac hypertrophy often leads to heart failure (22, 24, 28). Left ventricular hypertrophy is seen in up to 72% of adults and children with chronic renal failure and end stage renal disease (18). Cardiac hypertrophy is frequently observed in five-sixth nephrectomy, an animal model for chronic renal failure (16, 27). The present investigation was designed to show the effect of curcumin on attenuation of hypertrophy and to delineate the associated signaling pathways in blunting the hypertrophic response in Nx rats. Our results show that subtotal nephrectomy resulted in renal dysfunction, as evidenced by a gradual increase in proteinuria and elevation in BUN and plasma creatinine. Nx rats showed a significant hypertrophic response and increased end-inspiration IVC diameter, which were inhibited by treatment with curcumin and enalapril. Moreover, the Nx rats demonstrated changes in the signaling molecules that are critically involved in the hypertrophic response. These included increased GSK-3β phosphorylation, β-catenin expression, calcineurin, pNFAT, pERK as well as pAKT. Both curcumin and enalapril effectively deactivated most of these pathways triggered by nephrectomy in the heart. Taken together, these results demonstrate excellent protective
effect of curcumin, similar to enalapril, in attenuating cardiac hypertrophy in rats with chronic renal failure.

In the present study we utilized 150 mg/kg/day curcumin which effectively reduced the biochemical markers of renal dysfunction in the Nx group, similar to enalapril treatment. However, curcumin did not have a significant effect on lowering SBP although both gravimetric and echo analyses demonstrated a significant decrease in cardiac hypertrophy. These data suggest that curcumin modulated cardiac hypertrophy without lowering SBP in Nx mice. Although pressure overload plays an important role in inducing hypertrophy, there can be pressure-independent enhancement of cardiac hypertrophy as well (1, 26), which is clearly ameliorated by curcumin in the uremic rats in this study.

Our results provide strong evidence that curcumin blunted the action of several key signaling molecules implicated in the hypertrophic response. GSK-3β is unique among serine/threonine kinases in that it exists in its active form even in un-stimulated cells and gets inactivated by phosphorylation. Active GSK-3β is an endogenous negative regulator of cardiac hypertrophy (21). Increased inactive GSK-3β has been observed in the myocardium of diabetic mice with cardiac hypertrophy (19). Non-phosphorylated GSK-3β controls positive mediators of hypertrophy, including β-catenin (20) and NFAT (6), thereby imposing a negative constraint on the pro-hypertrophic mechanisms. Our results show a significant increase in the ratio of pGSK-3β/GSK-3β in the LV of untreated Nx animals indicating an abundance of the inactive form of this protein. The pGSK-3β was reduced by curcumin as well as enalapril suggesting an important role of GSK-3β in
regulating hypertrophy with these drugs. It has been shown that Ang II receptor antagonist losartan abrogates hypertrophy by antagonizing GSK-3β inactivation (19). Curcumin or enalapril treatment did not affect GSK-3β mRNA, which suggests that these drugs are affecting hypertrophy by post transcriptional modification of GSK-3β. Moreover, curcumin has been shown to prevent phosphorylation of GSK-3β by Akt (45).

The role of Akt and ERK in the development of cardiac hypertrophy has been established with the use of transgenic mice overexpressing Akt and ERK (10, 13). Both Akt and ERK regulate cardiac hypertrophy by modulating phosphorylation of downstream proteins including GSK-3β, β-catenin and NFAT (23). A significant increase in the phosphorylation of both Akt and ERK in the Nx animals was observed which was effectively blocked by curcumin and enalapril. Hypertrophic stimuli stabilize β-catenin in cardiomyocytes and overexpression of β-catenin induced hypertrophic growth both in vitro and in vivo (20). Moreover, GSK-3β inhibition via phosphorylation of Ser-9 by Akt appears to be the mechanism by which β-catenin is stabilized (20). The cytoplasmic stabilized β-catenin enters the nucleus and regulates hypertrophic gene expression (8, 42). It has been shown that targeted deletion of β-catenin in the heart leads to a blunted hypertrophic response to pathological stress-induced growth (11). Increased cytosolic β-catenin is also observed in renal tubular epithelial cells following injury initiated by unilateral ureteral obstruction (44). In the present study increased GSK-3β in the Nx animals was associated with augmentation in cytosolic β-catenin suggesting stabilization of the protein. This was followed by increased nuclear translocation of β-catenin in Nx animals. Since curcumin did not affect β-catenin mRNA levels, it probably
decreased the accumulation of cytosolic β-catenin and its subsequent migration to the nucleus by blunting phosphorylation of GSK-3β. Furthermore, curcumin-mediated breakdown of β-catenin (25) might also contribute to the reduced level of this protein in the curcumin treated animals. Enalapril did not have any effect on β-catenin although it reduced GSK-3β phosphorylation. The exact reason is not clear, but it has been shown that Ang II was able to induce cardiac hypertrophy when inducible cardiomyocyte specific deletion of the β-catenin gene was used. Accordingly it was suggested that β-catenin is not required for induction of cardiac hypertrophy induced by Ang II (5). Moreover, it has been proposed that the effect of β-catenin on cardiac hypertrophy might be dependent on the hypertrophic stimulus (7).

We also observed a significant increase in calcineurin mRNA and protein expression in the LV of Nx animals, which was inhibited by enalapril. Calcineurin is a protein phosphatase which regulates gene expression associated with pathological hypertrophy. Inhibitors of calcineurin have been reported to prevent cardiac hypertrophy in several experimental models (31). Ang II binds to the AT-1-receptor and increases the intracellular Ca²⁺ content. Elevated intracellular Ca²⁺-concentration, in turn, activates the cytoplasmic calcineurin which dephosphorylates NFAT (30, 35). In the present study, there was a significant increase in both calcineurin mRNA and protein expression in the Nx animals which was reduced by enalapril but not curcumin. Ang II is known to induce de novo synthesis of calcineurin in neutrophils(15). Moreover, modulation of renal failure by enalapril or Ang II receptor blocker has been shown to decrease cardiac
Therefore, it is likely that blunting of Ang II formation by enalapril reduces Ang II-stimulated calcineurin synthesis as well.

NFAT usually reside in the cytosol and is constitutively phosphorylated by active GSK-3β. Dephosphorylation of NFAT by calcineurin results in the nuclear translocation and activation of hypertrophic response (12). Phosphorylation of GSK-3β by Akt and or ERK can inhibit GSK-3β. This reduction in GSK3β activity allows NFAT to remain in the nucleus for a longer period of time, thereby promoting increased activation of hypertrophic genes (12, 47). A significant decrease of pNFAT in the cytosol with corresponding increase in nuclear NFAT in Nx animals was observed. The increase of dephosphorylated cytosolic NFAT in Nx animals appears to be due to increased calcineurin and pGSK-3β seen in this group. Curcumin and enalapril increased the levels of pNFAT in the cytosol and decreased its nuclear migration. It is to be noted that enalapril blocked both calcineurin and phosphorylation of GSK-3β whereas curcumin only affected GSK-3β. Consequently, we anticipated that the levels of pNFAT in enalapril group would be higher than the curcumin-treated group. Although, the pNFAT in the cytosol of enalapril animals was 21% higher than the curcumin, it was statistically insignificant. Moreover, there was no significant change in nuclear translocation of NFAT between the groups. Curcumin and enalapril neither affected the mRNA nor the nonphosphorylated GSK-3β and NFAT suggesting that these compounds might affect cardiac hypertrophy by post translational modification of these proteins. As summarized in figure 8, curcumin and enalapril both blunt phosphorylation of Akt and ERK which in turn keeps GSK-3β activated thereby increasing pNFAT in the cytosol and preventing its
nuclear translocation. Enalapril also increases cytosolic pNFAT by blocking the phosphatase calcineurin. However, curcumin but not enalapril can prevent β-catenin induced hypertrophy.

In summary, for the first time, we have demonstrated that curcumin attenuates cardiac hypertrophy and remodeling in nephrectomized rats independent of SBP reduction. Moreover, our results show that curcumin deactivates multiple hypertrophic signaling pathways including the GSK-3β/catenin, calcineurin/NFAT, Akt, and ERK1/2 as summarized in Fig. 9. Our findings are of tremendous clinical interest given the high prevalence of ventricular hypertrophy in adults and children with chronic renal failure and end stage renal disease. Because of the safety of curcumin, we believe that these studies would facilitate future clinical trials with this compound in the treatment of hypertrophy in patients with chronic renal failure.
Figure Legends:

Figure 1:  **Experimental protocol.** Arrows indicate time points for treatment, performance of surgical procedures and measurement of various parameters.

Figure 2:  A.  **Representative M-mode images of LV from sham operated and 5/6 nephrectomized (Nx) rats at 8 weeks.** Note that there is more LV dilatation and wall thickening in Nx rats as compared with sham control. B.  Average data showed a significant increase in LV mass and end-diastolic diameter (EDD, mm) in Nx rats as compared with sham control. Curcumin and enalapril-treated rats had significantly lower LV mass dilatation.

Figure 3:  **Changes of diameter of the inferior vena cava (IVC) at end-inspiration, as measured by echocardiography using a subcostal approach.** A: Representative images of sham control and nephrectomized (Nx) rats. B: Average IVC diameter data. Note that end-inspiration IVC diameter is significantly higher in Nx rats as compared to the sham control. Treatment with curcumin or enalapril significantly attenuated the increase in end-inspiration IVC diameter, reflective of lower right atrial pressure.

Figure 4. **Western blot showing GSK-3β expression in LV from the experimental groups.** Note the enhancement in GSK-3β phosphorylation with 5/6 nephrectomy and its attenuation by treatment with curcumin and enalapril. The summary results are shown as the ratio of total GSK-3β/GAPDH, pGSK-3β/GAPDH and pGSK-3β/GSK-3β.
Figure 5. The effect of curcumin and enalapril on changes in β-catenin in LV from sham and 5/6 nephrectomized rats. A. mRNA levels; B. cytosolic, and C. nuclear levels, normalized with lamin. Note that 5/6 nephrectomy caused significant increase in mRNA and cytosolic as well as nuclear levels of β-catenin. Curcumin significantly reduced β-catenin in the cytosol and nuclear without having any effect on the mRNA. Enalapril had no effect on β-catenin. The nuclear fraction was devoid of GAPDH signal suggesting that the nuclear extracts were free from cytosolic contamination.

Figure 6. The effect of curcumin and enalapril on changes in calcineurin in LV from sham and 5/6 nephrectomized rats. A. mRNA levels; B. cytosolic, and C. nuclear levels, normalized with lamin. Note that 5/6 nephrectomy caused significant increase in mRNA and cytosolic levels of calcineurin which were significantly reduced by enalapril but not curcumin.

Figure 7. The effect of curcumin and enalapril on changes in pNFAT and nuclear NFAT in the cytosolic and nuclear fractions of LV from sham and 5/6 nephrectomized rats. A. cytosolic, B. nuclear levels, normalized with lamin and C. DNA binding of NFAT determined by TransAmTM Assay. Note that 5/6 nephrectomy caused significantly reduced cytosolic levels of pNFAT and increased nuclear NFAT translocation and activity determined by western blots and DNA binding assay respectively. Both curcumin and
enalapril reversed this trend.

Figure 8. The effect of curcumin and enalapril on changes in pERK and pAKT from LV of sham and 5/6 nephrectomized rats. The summary results are shown as the ratio of pERK/ERK and pAkt/Akt. Note that pERK and pAKT were significantly increased 5/6 nephrectomy, which were reduced by curcumin and enalapril.

Figure 9. Summary of signaling pathways by which curcumin and enalapril attenuate LV hypertrophy 5/6 nephrectomized rats.
REFERENCES


Table 1: Changes in systolic blood pressure and proteinuria in sham-operated control rats, 5/6 nephrectomized (Nx), Curcumin treated 5/6 nephrectomized (Curc) and enalapril (Enap) treated 5/6 nephrectomized rats.

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<th>Weeks</th>
<th>Systolic Blood Pressure (mm Hg)</th>
<th>Proteinuria (mg/24 hours)</th>
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<td></td>
<td>Control</td>
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<tr>
<td>3</td>
<td>117 ± 4.4</td>
<td>146 ± 6.3†</td>
</tr>
<tr>
<td>5</td>
<td>122 ± 3.9</td>
<td>151 ± 7.9†</td>
</tr>
<tr>
<td>7</td>
<td>124 ± 7.8</td>
<td>157 ± 7.2†</td>
</tr>
</tbody>
</table>

The values are mean ± SD of 6-8 animals per group. †p<0.001 compared to control; ** p<0.01 compared to Nx; * p<0.05 compared to Nx
Table 2
Changes in physiological and biochemical parameters in sham-operated control (Cont) rats, 5/6 nephrectomized (Nx), Curcumin treated 5/6 nephrectomized (Curc) and enalapril (Enap) treated 5/6 nephrectomized rats. The values are mean ± SD of 6-8 animals per group.

<table>
<thead>
<tr>
<th></th>
<th>Cont</th>
<th>Nx</th>
<th>Curc</th>
<th>Enap</th>
<th>Cont vs Nx</th>
<th>Cont vs Curc</th>
<th>Cont vs Enap</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight</strong></td>
<td>409.6</td>
<td>318.3</td>
<td>339.8</td>
<td>324.8</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td><strong>(BW) (gms)</strong></td>
<td>±29.6</td>
<td>±13.8</td>
<td>±25.4</td>
<td>±26.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Left Ventricular Weight</strong></td>
<td>1.13 ±0.14</td>
<td>1.34 ±0.24</td>
<td>0.83 ±0.10</td>
<td>0.89 ±0.09</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td><strong>(LV) (gms)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LV/BW</strong></td>
<td>0.0028 ±0.0004</td>
<td>0.0042 ±0.0009</td>
<td>0.0025 ±0.0004</td>
<td>0.0028 ±0.0003</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td><strong>Right Ventricular Weight</strong></td>
<td>0.15 ±0.04</td>
<td>0.20 ±0.04</td>
<td>0.13 ±0.03</td>
<td>0.14 ±0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>(RV) (gms)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RV/BW</strong></td>
<td>0.00037 ±0.00010</td>
<td>0.00064 ±0.00014</td>
<td>0.00040 ±0.00010</td>
<td>0.00042 ±0.00014</td>
<td>p &lt; 0.01</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td><strong>Creatinine</strong></td>
<td>0.49 ±0.13</td>
<td>1.92 ±0.18</td>
<td>0.95 ±0.14</td>
<td>0.88 ±0.1</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td><strong>(mg/dL)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BUN</strong></td>
<td>17.6 ±1.13</td>
<td>66.6 ±15.6</td>
<td>39.8 ±4.7</td>
<td>42.5 ±7</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
<td>p&lt; 0.001</td>
</tr>
<tr>
<td><strong>(mg/dl)</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Left Kidney weight</strong></td>
<td>1.2 ±0.09</td>
<td>1.5 ±0.25</td>
<td>1.3 ±0.42</td>
<td>1.3 ±0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

Isoflurane

Curcumin (150 mg/kg; bid)
Enalapril (15 mg/kg; bid)
CMC 0.5% (volume-matched; bid)

Gavage

Wk 1  Wk 2  Wk 3  Wk 4  Wk 5  Wk 6  Wk 7  Wk 8  Wk 9

2/3 Left Nx  3/3 Right Nx

5/6 Nx

Echocardiography
BUN
Creatinine
SBP
Cardiac Hypertrophy
Figure 2

A. 

Sham
LV mass = 0.77 mg

Nephrectomy
LV mass = 1.12 mg

B. 

C.

LV mass (g)
Figure 3

A.

Inspiration

Control

Nephrectomy

B.

IVC (mm) *

P=0.001

P=0.050

P=NS

Sham

Nx

Nx + Curcumin

Nx + Enalapril
Figure 6

A

calcineurin mRNA

B

Calcineurin

GAPDH

Calcineurin/Actin

<0.01
<0.05

Calcineurin/GAPDH

<0.05
<0.05

Control  Nx  Curcumin  Enalapril

Control  Nx  Curcumin  Enalapril
Figure 7

A
Cytosolic p-NFAT
GAPDH

B
Nuclear NFAT
Lamin

C
NFAT DNA-binding Activity

Cytosolic p-NFAT

Nuclear NFAT

% Control
Figure 8

A

pERK

ERK

B

pAKT

AKT

pERK/ERK

pAKT/AKt

Control

Nx

Curcumin

Enalapril

Control

Nx

Curcumin

Enalapril

<0.01

<0.01

<0.01

<0.01

<0.01

<0.01

<0.01

<0.01
Figure 9

5/6 Nephrectomy

↑ pAkt / pERK

Enalapril

Calcineurin

↑ pGSK-3β

Curcumin

B-Catenin

↑ pNFAT Cytosol

NFAT Nucleus

P

Hypertrophy