MicroRNAs mediate the cardioprotective effect of angiotensin converting enzyme inhibition in acute kidney injury

Indrajeetsinh Rana aψ, Elena Velkoska bψ, Sheila K Patel b, Louise M Burrell b*, Fadi J Charchar a*

a School of Science and Technology, Federation University Australia, Ballarat, Victoria, Australia.
b Department of Medicine, Austin Health, The University of Melbourne, Victoria, Australia.

ψ First authors contributed equally to the study
* Senior authors contributed equally to the study

Corresponding author:
Prof. Fadi Charchar
Building Y, University Drive.
Federation University Australia.
Mt Helen, VIC 3350, Australia.
E-mail: f.charchar@federation.edu.au

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STNx, subtotal nephrectomy; ACEi, angiotensin converting enzyme inhibitor; RAS, renin angiotensin system; TGF-β1, transforming growth factor-beta; Fn1, fibronectin 1; CollA1, collagen 1a1; FoxO3, forkhead box proteins O3; BCL-2, B-cell lymphoma 2; PTEN, Phosphatase and tensin homolog.

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Abstract:
Cardiovascular disease including cardiac hypertrophy is common in patients with kidney disease, and can be partially attenuated using blockers of the renin angiotensin system (RAS). It is unknown whether cardiac microRNAs contribute to pathogenesis of cardiac hypertrophy or to the protective effect of RAS blockade in kidney disease. Using subtotal nephrectomy rat model of kidney injury, we investigated changes in cardiac microRNAs that are known to have direct target genes involved in regulation of apoptosis, fibrosis and hypertrophy. The effect of treatment with the angiotensin converting enzyme inhibitor (ACEi), ramipril on cardiac microRNAs was also investigated. Kidney injury led to a significant increase in cardiac microRNA-212 and microRNA-132 expression. Ramipril reduced cardiac hypertrophy, attenuated the increase in microRNA-212 and microRNA-132 and significantly increased microRNA-133 and microRNA-1 expression. There was altered expression of Caspase-9, B-cell lymphoma 2, transforming growth factor-beta, fibronectin 1, collagen 1a1 and forkhead box proteins O3, all known to be involved in the regulation of apoptosis, fibrosis and hypertrophy in cardiac cells, whilst being targets for the above microRNAs. ACEi treatment increased expression of microRNA-133 and microRNA-1. Inhibitory action of ACEi treatment on increased cardiac NOX1 expression after STNx surgery suggest that inhibition of oxidative stress is also one of mechanism of ACEi mediated cardioprotection. These finding suggests the involvement of microRNAs in the cardioprotective action of ACEi in acute renal injury, which is mediated through an inhibitory action on pro-fibrotic and pro-apoptotic target genes, and stimulatory action on anti-hypertrophic and anti-apoptotic target genes.

Introduction:
Cardiovascular disease is the major cause of death in patients with kidney disease (12, 33, 41). We have previously reported that kidney disease secondary to subtotal nephrectomy (STNx) causes increased blood pressure, impaired cardiac function, activation of the cardiac renin angiotensin system (RAS) and marked cardiac hypertrophy and fibrosis (6, 7). Blockade of the RAS, a mainstay of therapy in patients with kidney disease (25, 54, 58), attenuated the increase in blood pressure and reduced cardiac hypertrophy and collagen accumulation in experimental kidney damage (6). It is however not known whether blockade of the RAS causes changes in cardiac microRNA.
MicroRNAs are a class of small noncoding RNAs that modulate gene expression at the post-transcription level (47, 52), and are involved in cardiac hypertrophy, fibrosis, and remodelling (15, 32, 51) through key epigenetic control of genes involved in these processes. To date the role of microRNAs in the pathogenesis of these cardiac complications of kidney disease has not been investigated. Studies in null mice and a model of pressure-overload induced heart failure indicate that microRNA-212 and microRNA-132 regulate cardiac hypertrophy (51). Others have reported that microRNA-1 and microRNA-133 are associated with cardiovascular pathologies (2, 3, 10, 21, 42) and regulate genes involved in cardiac fibrosis and apoptosis (9, 21).

In addition to being potent regulators of gene expression in the disease process, microRNA may serve beneficially during therapeutic interventions (13). We hypothesize that microRNAs may be important players both in the development and prevention (after angiotensin converting enzyme (ACE) inhibitor treatment) of cardiac hypertrophy, fibrosis and apoptosis associated with kidney disease. In this study, we examined the effect of acute kidney injury on cardiac microRNA-1, microRNA-133, microRNA-212 and microRNA-132 and their target mRNA genes involved in fibrosis (transforming growth factor-beta (TGF-β1), fibronectin 1 (Fn1), collagen 1a1 (Col1A1)), hypertrophy (forkhead box proteins O3 (FoxO3)) and apoptosis (B-cell lymphoma 2 (BCL-2), Caspase-9).

To understand molecular mechanisms of cardiac pathology associated with STNx, we also examined expression of mRNA for Fas Ligand (Fas L), BCL2L11 (Bim-1), Phosphatase and tensin homolog (PTEN), all known to play an important role in regulation of cell death pathways. Oxidative stress has been increasingly linked to the high incidence of cardiovascular events in patients with chronic kidney disease (CKD), and expression of NADPH Oxidase isoform-1 (NOX-1) in particular has been linked with angiotensin (Ang) II signalling (14, 28). Expression of NOX-1 and cellular antioxidant enzyme manganese superoxide dismutase (MnSOD) were also measured. We also investigated the effect of treatment with the ACE inhibitor (ACEi) ramipril on the regulation of these microRNAs and genes. In vitro experiments on cultured H9c2 cells were performed to understand mechanisms leading to altered microRNA expression.

**Methods:**

**Animal surgeries and cardiovascular assessment:**
Experimental procedures were performed in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation and were approved by the Animal Ethics Committee, Austin Health. Rats were housed in a 12h/12h light/dark cycle, with ad libitum food containing 0.4–0.6% NaCl (Norco, Lismore, NSW, Australia) and water. STNx or sham surgery was performed in Sprague Dawley rats (body weight of 200–250 g) as described previously (6). In brief, rats were anaesthetized by an intraperitoneal (i.p.) injection of sodium pentobarbitone (60 mg/kg body weight; Boehringer Ingelheim, Artarmon, NSW, Australia), and STNx was performed by nephrectomy of the right kidney, followed by infarction of approximately two-thirds of the left kidney with selective ligation of all but one extra-renal branches of the left renal artery.

Following STNx or sham surgery, animals were randomly allocated to vehicle (Sham+Veh, n=9; STNx+Veh, n=9) or the ACE inhibitor treatment group (Sham+Ram, n=10; STNx+Ram, n=9). Ramipril was given daily by oral gavage at a dose of 1mg/kg/day for 10 days. On day 9, rats were housed in metabolic cages for 24 h, and urine volume measured. For urinary protein measurement, 50 μl urine samples were diluted 10-fold in lysis buffer (2 M Tris HCl, 5 M NaCl and Triton X-100), and urinary protein was determined using the bicinchoninic acid (BCA) method with a commercially available BCA protein assay kit (Pierce, Rockford, IL, USA). On day 10, rats were anaesthetized (i.p. sodium pentobarbionate, 60 mg/kg body weight), and the mean arterial pressure (MAP), heart rate and left ventricular end-diastolic pressure (LVEDP) were determined using a microtipped pressure transducer catheter (Millar, 1.5F) inserted into the left carotid artery and advanced into the LV (left ventricle). Data were analysed using Millar conductance data acquisition and analysis software.

**Plasma Ang II radioimmunoassay**

Blood samples for measurement of Ang II were collected into tubes containing 20 μl/ml of blood of an inhibitor cocktail [50 mM EDTA, 0.2 M N-ethyl- maleimide and 1–2 TIU (trypsin inhibitory units)/ml aprotinin made up in saline] and plasma was snap-frozen and stored at −80 °C. The radioimmunoassay for Ang II has been described previously (55, 56). The anti-rabbit antibody and the specific radioisotope, 125I-Ang II, were made by Prosearch. The intra- and inter-assay coefficients of variation were 7.6 and 8.3%.

**In vitro experiments:**

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H9c2 cardiac myoblast cells were cultured by method described earlier (4) with some modification. In brief, cells were grown in Dulbecco’s Modified Eagle’s Medium (Sigma-Aldrich, USA) supplemented with 1.5 g/L sodium bicarbonate, 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma-Aldrich, USA) and 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich, USA), in 75-cm² tissue-culture flasks at 37 °C in a humidified atmosphere of 5 % CO₂. Cells were fed every 2–3 days and subcultured once they reached 70–80 % confluence. For differentiation to cardiomyocytes, H9c2 cells were plated at a density of 50,000 cells per well in 24 well culture dishes and allowed to adhere the dish for one day. Afterward, the concentration of serum in the media was reduced to 1 %, and cells were cultured for 5 more days in the presence of all-trans retinoic acid (RA, 1 μM, Sigma-Aldrich, USA). Addition of RA was performed daily for 5 consecutive days. RA was prepared in DMSO and stored at −20 °C in the dark. Earlier studies have demonstrated that H9c2 cell differentiated in low serum plus RA exhibits higher expression for cardiomyocytes specific markers (4, 39), suggesting transformation of cardiomyoblast to cardiomyocytes. Ramipril is metabolized to ramiprilat in the liver, which then exerts its inhibitory effect on ACE. Cells were exposed to hypoxia (2% oxygen) in presence and absence of ramiprilat (100 nM, Santa Cruz Biotechnology, USA) for 8 hours. Control cells were treated with vehicle DMSO. To confirm up-regulated RAS signalling in these cells, ACE mRNA expression was measured from both control and hypoxia group cells. To test effect direct exposure to Ang II, after 5 days of incubation in media with RA and 1% serum, cells were incubated with Ang II (100nM, Sigma-Aldrich, USA) for 12 hrs. After 12 hrs cells were used for RNA extraction.

**RNA Extraction:**

RNA was extracted using Qiagen RNeasy kits (Qiagen, Hilden, Germany) from tissue collected from STNx rats and from similar region of the heart from sham rats, as described earlier (6). Then cDNA synthesis was performed using microRNA-specific primers (Applied Biosystems, USA), by method provided by manufacturer. Standard cDNA synthesis protocol was followed to obtain cDNA for gene assays (37).

H9c2 cardiomyocytes were lysed and total RNA was extracted using TRIzol (Life Technologies, USA) according manufacturer’s instructions. All RNA samples were quantified by spectrophotometry using Nanodrop (Thermo Fisher). Then cDNA synthesis was performed using microRNA-specific primers...
(Applied Biosystems, USA), by the method provided by manufacturer. Standard cDNA synthesis protocol was followed to obtain cDNA for gene assays.

Quantitative PCR for microRNAs:
Quantitative PCR experiments were performed using TaqMan assays (Applied Biosystems, USA) by method described previously (48). In brief, reverse transcription carried out (at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then to 4 °C) using 10ng/µl RNA, and then PCRs were conducted in triplicate for each sample (95 °C for 1 min and 40 cycles of 95 °C for 15 s and 60 °C for 30 s). All PCR amplifications were performed on ViiA 7 Real-Time PCR system (Applied Biosystems, USA) using ViiA 7 RUO Software. mRNA was normalized to U87 endogenous control and the relative fold difference in expression was calculated using the 2-ΔΔCt method (62). Information on TaqMan Assays used in this study is found in table 2.

Quantitative PCR for genes:
Quantitative PCR experiments were performed using TaqMan assays (Applied Biosystems, USA) by method described previously (37). mRNA was normalized to GAPDH endogenous control and the relative fold difference in expression was calculated using the 2-ΔΔCt method (62). All PCR amplifications were performed on ViiA 7 Real-Time PCR system (Applied Biosystems, USA) using ViiA 7 RUO Software. Information on TaqMan Assays used in this study given in table 2.

Quantification of apoptotic cell death by TUNEL assay:
Cardiac (LV) paraffin sections 4µm thick sections were used to perform TUNEL staining by the method provided by manufacturer (TACS.XL DAB In Situ Apoptosis Detection Kit, Catalogue number #4828-30-DK). The kit is designed to measure nuclear DNA fragmentation, which is a widely accepted method to assay for apoptosis. We stained 3 sections from each animal and three images were taken from random fields of each section. Then TUNEL positive cells were counted on computer screen by looking at images. Average number of cells counted from all three fields from each section used for analysis.

Determination of cardiac collagen and TGF-β1 proteins:
Cardiac (LV) paraffin sections 4µm thick were deparaffinised, rehydrated, and then stained with 0.1% Sirius Red (Polysciences Inc) in saturated picric acid (picrosirius red) for 1 hour, differentiated in 0.01% HCl for 30 seconds, and rapidly dehydrated. Interstitial collagen volume fraction was determined by measuring the area...
of stained tissue within a given field, excluding vessels, artefacts, minor scars or incomplete tissue fields; 15- 20 fields were analysed per animal in a blinded manner. The area stained was then calculated as a percentage of the total area within a given field.

Tissue expression of TGF-β was assessed immunohistochemically using a polyclonal anti-TGF-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) by a method described previously previously (34). In brief, cardiac (LV) paraffin sections 4µm thick were were incubated with antibody overnight at 4 °C. The following day the sections were thoroughly washed in PBS and incubated with goat anti-rabbit (1:200) IgG Fab2 tagged with Alexa Flour-647 (Molecular Prob, USA). For comparison of intensity and staining, sections from all three groups were imaged using Nikon Eclipse Ti inverted confocal microscope with the same settings.

**Statistical analysis:**

Statistical analysis was performed using Graph Pad Prism version 5 software. Data were compared using one-way analysis of variance (ANOVA) with post hoc test to determine if there were overall significant effects between groups. If a statistical difference was obtained, subsequent comparisons were made using student’s unpaired t-test. The presence of correlations between variables was tested using Pearson’s correlation analysis.

**Results:**

**Kidney and cardiovascular parameters:**

The kidney and cardiac measurements are presented in Table 1. Rats with STNx had kidney impairment as indicated by a 3.5-fold increase in urine output and 2-fold increase in urinary protein levels compared with the vehicle- treated sham animals (P<0.001 for both, Table 1). Ramipril had no effect on proteinuria in the STNx rats and caused proteinuria in sham rats (Table 1). This effect of ACE inhibition can also occur clinically early after commencing treatment, but does not persist long-term (8). Compared with vehicle- treated sham rats, the vehicle-treated STNx rats had an increased heart rate (P<0.05, Table 1). Heart rate in the ramipril-treated STNx rats was comparable to those of the vehicle-treated sham rats and significantly lower than those of the vehicle-treated STNx rats (P<0.05, Table 1). MAP was significantly increased in
STNx rats (P<0.001 vs. vehicle-treated sham rats) and was reduced with ramipril (P<0.001 vs. vehicle-treated STNx, Table 1). Cardiac dysfunction was present with a significant increase in LVEDP in STNx rats (Table 1). Ramipril reduced MAP (P<0.001 vs. vehicle-treated STNx, Table 1) and improved LVEDP (P<0.01, Table 1) in STNx rats. STNx rats had left ventricular hypertrophy (LVH) compared to vehicle-treated sham rats (P<0.001), which was reduced by ramipril (P<0.001, Table 1). Ramipril treated sham rats had 13% lower MAP and LVEDP compared to vehicle-treated sham rats (both P<0.05, Table 1). Heart rate and LVH were not significantly different between vehicle treated and ramipril treated sham rats (Table 1).

**Plasma Angiotensin II measurements:**

Compared to vehicle-treated sham rats, plasma Ang II levels were elevated in STNx rats (P<0.01, Table 1) and were not reversed with ramipril. Ramipril in sham rats increased plasma Ang II (P<0.001 compared to vehicle-treated sham, Table 1). It has previously been suggested that this could be due to Ang II escape following ACEi treatment (53).

**Quantification of LV microRNAs:**

We compared LV microRNA-1, microRNA-133, microRNA-212 and microRNA-132 levels in all vehicle-treated and ramipril-treated STNx rats with vehicle-treated sham rats. Levels of microRNA-1 and microRNA-133 mRNA were not increased in STNx compared to vehicle-treated sham, but they were increased in the ramipril-treatment STNx (P<0.01 and P<0.001 respectively, compared to vehicle-treated sham and vehicle-treated STNx rats, Fig 1a and 1b). Cardiac expression of microRNA-133 and microRNA-1 was higher even in sham rats treated with ramipril (P<0.05 and P<0.01 compared to respective vehicle treated sham rats, Fig 1a and 1b). MicroRNA-212 and microRNA-132 levels were increased in STNx compared to vehicle-treated sham rats (P<0.05 for both), and this increase was significantly attenuated by ramipril (P<0.05, compared with vehicle-treated STNx, Fig 1c and 1d).

**Quantification of cardiac cells undergoing apoptosis by TUNEL assay:**

There were more cells showing dark brown staining in cell nucleus in vehicle treated STNx rats as compared to other two groups of cells (Fig 2a). Quantification of number of TUNEL positive cells showed 3.8 fold more apoptotic cells in vehicle treated STNx rats as compared to vehicle-treated sham rats. The number of apoptotic cells in ramipril treated STNx rats was not significantly different from vehicle-treated sham rats (Fig 2a and 2b).
Quantification of LV mRNA for apoptosis:

Anti-apoptotic gene BCL-2 mRNA level was not altered by STNx surgery (Fig 2c) but there was significant increase in the ramipril treatment group (P<0.001 compared with vehicle-treated sham and P<0.05 compared with the vehicle-treated STNx group, Fig 2c). Compared to vehicle-treated sham, mRNA for pro-apoptotic genes Fas L and Caspase-9 was significantly higher in the vehicle-treated STNx rats (P<0.01 for both), which was reversed by ramipril (Fig 2d and 2e). Caspase-9 but not Fas L was significantly reduced in ramipril-treated Sham rats. We report that compared to vehicle treated sham rats, Bim-1 expression significantly decreased in STNx group (P<0.05, Fig 2f) and was almost restored by ramipril (P=0.09, Fig 2f). Expression of PTEN was increased in STNx+Veh rats (P<0.05 compared to vehicle treated sham, Fig 2g). This STNx mediated increase in PTEN expression was lowered by ramipril (P<0.05 Fig 2g).

Cardiac collagen and TGF-β1 proteins:

Compared to vehicle-treated sham, there was significant increase in TGF-β1 immunoreactivity in vehicle treated STNx rats as evident by higher intensity and labelling (Fig 3a). Also, both intensity and TGF-β1 labelled area appeared less in ramipril treated STNx rats. STNx was associated with a 0.6 fold increase in total collagen in the heart as assessed by picrosirius red staining (P<0.05), which was reversed with ramipril (P<0.05, Fig 3b).

Quantification of LV mRNA for fibrosis:

The LV from STNx rats had upregulated activity of TGF-β1, Smad 3 and Smad 4 genes as evident from increased mRNA levels (P<0.01 for TGF-β1 and P<0.05 for both Smad 3 and Smad 4, compared to vehicle-treated sham). This increase in TGF-β1 mRNA was significantly attenuated by ramipril treatment (P<0.05, Fig 3c). Similarly, increases in Smad 3 and Smad 4 mRNA were significantly attenuated by ramipril treatment (Table 1). Ramipril treatment in sham rats did not alter TGF-β1, Smad 3 and Smad 4 mRNA expression (Fig 3c and Table 1). We studied mRNA level for extracellular matrix protein Col1A1 and Fn1 as markers of fibrosis. There was a 4.5-fold increase in mRNA for Col1A1 and a 5.5-fold increase in Fn1 mRNA in vehicle-treated STNx rats (P<0.001 compared with vehicle-treated sham, Fig 3d and3e). Treatment with ramipril in STNx rats caused a significant reduction in mRNA level of both Col1A1 and Fn1 compared to vehicle treated STNx rats (P<0.01 and P<0.001 respectively). Expression of both Col1A1 and Fn1 was also significantly higher in ACEi treated sham rats compared to vehicle treated sham rats (P<0.05
for both, Fig 3d and Fig 3e), however it was lower than in STNx vehicle rats (Fig 3d and Fig 3e).

**Quantification of LV mRNA for hypertrophy**

Antihypertrophic gene FoxO3 mRNA levels were 40% lower in STNx rats but were not significantly reduced compared to vehicle-treated sham (P=0.071, Fig 4). There was significant increase in expression of FoxO3 mRNA in ramipril-treated STNx rats (P<0.05 compared with vehicle-treated sham; P<0.01 compared with vehicle-treated STNx group, Fig 4).

**Expression of NOX-1 and MnSOD:**

Expression of NOX-1 was increased in vehicle treated STNx group (P<0.05 compared to vehicle treated sham group, Fig 5a). Ramipril treatment was able to attenuate this increase in NOX-1 mRNA expression (P<0.05 compared to STNx+Veh, Fig 5a). MnSOD expression was not altered by STNx and/or ramipril treatment (Fig 5b).

**In vitro work on H9c2 cells:**

Compared to control cells, level of ACE mRNA was higher in cells treated with Hypoxia (Data not shown), confirming that 8 hours of hypoxia treatment was sufficient to increase RAS signalling in these cells.

Compared to control cells, expression of both microRNA-133 and microRNA-1 was not different in hypoxic cells. We found a significant increase in expression of both microRNA-133 and microRNA-1 in hypoxic cells treated with ramiprilat (P<0.01 and P<0.05, respectively compared to control cells, Fig 6a and 6b).

Compared to hypoxic cells, expression of microRNA-133 but not microRNA-1 was significantly different in hypoxic cells treated with ramiprilat (P<0.01, Fig 6a and 6b). Hypoxia was able to cause 0.8 fold increase in mRNA for TGF-β1 (P<0.05, Fig 6c), which was (non-significantly) lowered by ramiprilat treatment (P=0.09, Fig 6c). Level of mRNA for Fn1 and Col1A1 were not altered by hypoxia (Fig 6d and 6e).

Direct exposure to Ang II for 12 hrs was able to cause a small but significant reduction in expression of microRNA-133 and microRNA-1 in H9c2 cardiomyocytes (P<0.05 for both, Fig 7a and 7b). Expression of microRNA-212 and microRNA-132 was increased (P<0.05 for both, Fig 7c and 7d).

**Discussion:**

Acute kidney injury results in cardiac structural changes that are attenuated by ACE inhibition. In the current study we examined whether the cardiac changes induced by kidney injury may be triggered by dysregulation of cardiac microRNAs that lead to aberrant control of fibrotic, apoptotic and hypertrophy pathways. In
particular, we found that the cardiac expression of the pro-hypertrophic microRNA-212 and microRNA-132 were upregulated after kidney injury, which resulted in suppression of the anti-hypertrophic FoxO3 target gene. Treatment with an ACEi resulted in attenuation of the expression of these two microRNAs and as a result, a restoration of FoxO3 mRNA levels. The cardioprotective microRNA-133 and microRNA-1 did not change with kidney injury; however, ACE inhibition resulted in the up regulation of both these microRNAs. In parallel to microRNA-133, mRNA for its target genes, pro-fibrotic Col1A1 and apoptotic Caspase-9, were both upregulated in STNx rats and down regulated with ramipril treatment. Similarly, the ACEi mediated increase in microRNA-1 resulted in inhibition of its profibrotic target gene Fn1 and up-regulation of anti-apoptotic gene BCL-2.

We found a significant increase in number of cells with nuclear DNA fragmentation in vehicle treated STNx rats, which is consistent with the earlier finding in this model reporting increased cardiac apoptosis (1). A study by Amann et al. in STNx rat model reported that ramipril treatment attenuated cardiomyocytes loss and abnormal increase in cardiomyocytes volume (1), suggesting cardioprotective effect of ACEi. However, the study did not provide any information on the molecular mechanism leading to the inhibition of cell death. Our data shows significant attenuation of STNx mediated increase in apoptotic genes Caspase-9 and Fas L expression by ramipril treatment. Caspase-9 is a pro-apoptotic gene (16), shown to be associated with various cardiac pathological conditions. Capsase-9 is a part of a “mitochondrial” apoptotic pathway activated by mitochondrial cytochrome c release. Apart from Caspase-9, we report increased expression of another pro-apoptotic gene Fas L, in STNx rats. Fas L belong to the family of TNF-related cytokines and induce apoptosis via “death receptor” signalling pathway. Thus, our data suggests inhibition of both mitochondrial and death receptor apoptotic pathways by ACE inhibition in STNx rats.

Interestingly, we found that kidney failure induced by STNx did not alter microRNA-133 in the heart, however, ACEi treatment after STNx caused a significant increase in expression of this microRNA. Luciferase gene reporter assays performed by an earlier study have demonstrated Caspase-9 as a direct target of microRNA-133 (21). In parallel, we found a significant increase in cardiac mRNA level for Caspase-9 in vehicle-treated STNx rats. Injection of a microRNA-133 mimic into the heart resulted in the prevention of ischemia reperfusion injury-induced apoptosis of cardiac cells; inhibition of Caspase-9 was suggested as a mechanism of inhibition of cardiac apoptosis (21). Our results suggest that increased microRNA-133 in
ACEi treatment group attenuate Caspase-9 mRNA to inhibit STNx activated mitochondrial apoptotic pathway. However, involvement of microRNAs in ACEi mediated inhibition of death receptor pathways is not clear. No change in microRNA-133 expression in non-treated STNx rats suggests blood pressure independent regulation of this microRNA in heart, further experiments are required to validate this hypothesis.

BCL-2 has been shown to inhibit apoptosis in cardiac (35, 49) and other cell types (22, 46). An earlier study has demonstrated BCL-2 as a direct target of microRNA-1 by performing luciferase reporter experiments and also reported down regulatory effect of microRNA-1 on BCL-2 expression (49). Our results show that kidney injury itself was not able to alter cardiac expression of either microRNA-1 or BCL-2 mRNA, but ramipril treatment after STNx surgery causes significant increase in both microRNA-1 and cardiac BCL-2 mRNA. The role of microRNA-1 in regulating cardiomyocyte apoptosis is controversial; some studies have reported that microRNA-1 induces apoptosis (49, 64) while others have reported an anti-apoptotic role for microRNA-1 (20, 21). An earlier study on H9c2 cardiac cells showed that ACE inhibition in cells exposed to anoxia/regeneration led to increased BCL-2 expression (59). This cell culture finding suggests that ACE inhibition can be a trigger for reported increased BCL-2 expression in our ramipril treated STNx rats. Also, this effect of ACE inhibition on BCL-2 may be independent of microRNA-1.

Earlier studies on STNx rats have also demonstrated the involvement of TGF-β1-Smad signalling in both cardiac and kidney fibrosis (29, 63). We have previously reported activation of cardiac RAS in STNx rats (6, 56), which is associated with significant cardiac fibrosis affecting both the interstitium and perivascular area (6, 7). Our findings in this study suggest that inhibition of TGF-β1-Smad signalling by ramipril treatment may be the mechanism by which ACEi treatment reduces fibrosis.

TGF-β1 can also increase extracellular matrix proteins collagen and fibronectin synthesis by promoting mRNA expression of genes, including Col1A1 and Fn1 (38, 57). We found a significant increase in cardiac mRNA for extracellular matrix protein Col1A1 and Fn1 by STNx that was attenuated by ramipril treatment. Luciferase gene reporter assays performed by earlier studies have demonstrated TGF-β1 and Col1A1 as direct targets of microRNA-133 (9, 44). Fn1 is a direct target of microRNA-1 (61). Treatment with an angiotensin receptor blocker, irbesartan upregulates microRNA-133, which in turn mediates the inhibition of Col1A1 and the attenuation of myocardial fibrosis in a rat model of Ang II-dependent hypertension (9).
results suggest that increased microRNA-133 and microRNA-1 after ramipril treatment may be the molecular switch responsible for the inhibition of TGF-β1-Smad signalling, which in turn inhibits Col1A1 and Fn1 mRNA expression.

We are the first to report increased cardiac expression of microRNA-212 and microRNA-132 after STNx. Both these microRNAs exhibit similar mature sequences and share the same seed region; hence, they are expected to target the same mRNAs (60). Increased cardiac expression of microRNA-212 and microRNA-132 have been reported in a rat model of Angiotensin II induced hypertension (17). Anti-hypertrophic and pro-autophagic FoxO3 transcription factor has been shown as a common target gene for both microRNAs -212 and 132 (51). Phosphorylated form of FoxO3 is retained in cytoplasm but its dephosphorylation leads to translocation to the nucleus, which then promotes expression of its target genes. A study on mesangial cells has shown that TGF-β1 has inhibitory effect on FoxO3 transcription and apoptosis (26). FoxO3 transcription factor also activates expression of atrogin-1 that inhibits calcineurin dependent cardiac hypertrophy (30, 40, 43). A recently published study has demonstrated attenuated trans-aortic constriction-induced cardiac hypertrophy in microRNA-212/132 null mice (51). The same study reported up regulated cardiac mRNA for atrogin-1 in microRNA-212/132 null mice and suggested increased FoxO3 activity as a mechanism for inhibition of cardiac hypertrophy. In our study we found lowered expression of FoxO3 mRNA after STNx (P<0.071 compared with controls, Fig 6), which was increased by ramipril treatment. Our data on cardiac hypertrophy in STNx rats and its attenuation by ACE inhibitor treatment are consistent with earlier findings in this model (1, 50). Our results suggest that increase in FoxO3 expression due to ACE inhibition mediated attenuation of microRNA-212 and microRNA-132 in STNx rats is the mechanism for inhibition of cardiac hypertrophy.

Using miR-212/132 over expression and Ang II treatment as tool, a study on cardiac fibroblast cells has demonstrated that their 22 validated target genes are involved in Ang II signalling (18). It clearly suggests a complex role of these microRNAs in regulating Ang II signalling pathway. The same study found increased PTEN expression in cardiac fibroblast cells overexpressing miR-212/132 (18). In contrast, another study by Jin et al. has demonstrated that Ang II induced expression of miR-132 results in the reduction of PTEN expression in vascular smooth muscle cells (24). In the current study we have found increased expression of PTEN along with increased levels of microRNA-132/212. STNx mediated increased expression of
microRNA-212 but not microRNA-132 were normalise by ACEi treatment to the same level as of sham rats. Compared to (both vehicle and ACEi treated) sham rats Expression of microRNA-132 were significantly higher even after ACEi treatment in STNx rats.

Using cultured mesangial cells, an earlier report by Kato et al., (2006) has demonstrated that TGF-β1 induces FoxO3a phosphorylation which then decreases the expression of a key FoxO target genes, the proapoptotic Bim and an antioxidant gene MnSOD(26). In our study we have found significant increase in TGF-β1 expression and significant reduction in expression of Bim-1 expression in STNx rats, which was attenuated by ACEi treatment. There was no difference in expression of MnSOD but we observed a significant increase in expression of NOX1 in STNx rats suggesting an increase in cardiac oxidative stress. ACEi treatment had no effect on MnSOD expression but attenuated the increase in NOX1 observed after STNx. There is emerging evidence suggesting that TGF-β1 activates NOX to promote production of reactive oxygen species (ROS) (23). Three NADPH isoforms NOX1, NOX2 and NOX4 are known to play key role in cardiac pathologies associated with cardiovascular diseases (5). Using A549 and H1437 cancer cells, Singh et al. demonstrated that increased expression of microRNA-1 can inhibit NADPH oxidise activity, suggesting that microRNA-1 prevents the production of nucleotide precursors by inhibiting the expression of pentose phosphate pathway genes (45). Our data shows an increased microRNA-1 expression in ACEi treatment group, which also has reduced NOX-1 expression. These data suggest that inhibition of oxidative stress may be one of the mechanisms of ACEi mediated cardioprotection in experimental kidney injury.

Further detailed investigations are required to understand involvement of other NOX isoforms and mechanisms in how microRNAs contribute to oxidative stress in the heart after renal damage.

Our in vitro data on H9c2 cardiomyocytes demonstrates direct exposure to Ang II is sufficient to induce expression of microRNA-212 and microRNA-132. This is in agreement with an earlier finding that has reported that hypertrophic stimuli including Ang II up-regulated expression of microRNA-212 and microRNA-132 in primary neonatal cardiomyocytes (51). Therefore, lowered expression of microRNA-212 and microRNA-132 in STNx rats treated with ACEi reflects reduced Ang II signalling in heart. Hypoxia is also one the stimuli that can induce hypertrophy in cultured cardiomyocytes cells (11). We report that
hypoxia treatment was able to increase ACE mRNA expression which is consistent with earlier reports showing chronic hypoxia mediated activation of tissue specific renin angiotensin system activity (19, 36, 62). A study on pulmonary artery smooth muscle cells has demonstrated that hypoxia inducible factor-1a (HIF-1a) can bind and transactivate the ACE promoter directly (65). A study on human pulmonary artery adventitial fibroblasts cells demonstrated increased ACE activity after exposure to hypoxia (27). Exposure to hypoxia increased collagen synthesis in cultured lung fibroblasts by increasing ACE expression and Ang II release (31). Anoxia/reoxygenation treatment in H9c2 cells has been shown to induce apoptosis that was attenuated by inhibition of ACE (59). Our data on cultured H9c2 cells demonstrate that inhibition of ACE in cells stimulated with hypertrophic stimuli causes increase in expression of microRNA-133 and microRNA-1.

To our knowledge, this is the first study to report ACE inhibitors may mediate its cardioprotective effect by up-regulating expression of microRNAs. However, the detail molecular mechanism of how ACE inhibition alters expression of these microRNAs is still not clear and requires further investigation.

In summary, an activated cardiac RAS in STNx rats contributes to cardiac pathology via mechanisms involving microRNAs. Our study demonstrates for the first time an important role of microRNAs in ACE inhibitor mediated cardioprotection after acute kidney damage. Therapies targeting microRNA-133, microRNA-1 and microRNA-212/132 may provide beneficial outcomes in the treatment of kidney diseases.

Acknowledgment:

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Disclosure:

The authors declare that there is no conflict of interest regarding the publication of this paper.
References:


Table and Figure Legends:

**Table 1:** Cardiorenal parameters from Sham+Veh, Sham+Ram, STNx+Veh and STNx+Ram rats. Data presented as mean±SEM. *P<0.05, **P<0.01, ***p<0.001 vs Sham+Veh, +P<0.05, +++p<0.001 vs Sham+Ram, #P<0.05, ###P<0.001 vs STNx+Veh. STNx+Veh indicates vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats. Data analysed by performing one-way ANOVA statistical test followed by t test.

**Table 2:** Table shows Applied biosystem assay ID for all the primers used in this study.

**Fig 1:** Quantitative analysis of microRNA-133a (a), microRNA-1 (b), microRNA-212 (c) and microRNA-132 from Sham+Veh (N=9), Sham+Ram (N=10), STNx+Veh (N=9) and STNx+Ram (N=9) rats. Data presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs Sham+Veh. +P<0.05, ++p<0.01 vs Sham+Ram, #P<0.05, ###P<0.001 vs STNx+Veh. STNx+Veh indicates vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats.

**Fig 2:** Figure shows dark brown staining in cells undergoing apoptosis in subtotal nephrectomy rats, (A) Sham+Veh (B) STNx+Veh (C) STNx+Ram. All images were taken at 400x magnification. Scale bar=100µm (a) Quantitative analysis of number of apoptotic cells from sham (N=9), STNx+Veh (N=9) and STNx+Ram (N=9) group of rats (b). Quantitative analysis of mRNA for BCL-2 (c), Fas L (d), Caspase 9 (e), BIM-1 (f), and PTEN (g), from Sham+Veh (N=9), Sham+Ram (N=10), STNx+Veh (N=9) and STNx+Ram (N=9) rats. Data presented as mean±SEM. *P<0.05, **P<0.01, ***P<0.001 vs Sham+Veh. +P<0.05, ++P<0.01, +++p<0.001 vs Sham+Ram, #P<0.05, ###P<0.001 vs STNx+Veh. STNx+Veh indicates vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats.

**Fig 3:** Figure shows TGF-β1 immunoreactivity in rat heart. (A) Sham+Veh (B) STNx+Veh (C) STNx+Ram. All images were taken at 200x magnification, Scale bar = 200µm (a). Quantitative analysis of picrosirius red staining from Sham+Veh (N=9), STNx+Veh (N=9) and STNx+Ram (N=9) rats (b). Quantitative analysis of mRNA for TGF-β1 (c), collagen 1a1 (Col1A1; d) and fibronectin (Fn1; e) mRNA from Sham+Veh (N=9), Sham+Ram (N=10), STNx+Veh (N=9) and STNx+Ram (N=9) rats (b). Data presented as mean±SEM.
*P<0.05, **P<0.01, ***P<0.001 vs Sham+Veh, ++P<0.01, +++P<0.001 vs Sham+Ram, #P<0.05, ##P<0.01, ###P<0.001 vs STNx+Veh. STNx+Veh indicate vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats.

Fig 4: Quantitative analysis of FoxO3 mRNA from Sham+Veh (N=9), Sham+Ram (N=10), STNx+Veh (N=9) and STNx+Ram (N=9) rats. Data presented as mean±SEM. **P<0.01 vs Sham+Veh. #P<0.05 vs STNx+Veh. STNx+Veh indicates vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats.

Fig 5: Quantitative analysis of mRNA for NOX1 (a) and Manganese Superoxide dismutase (MnSOD) (b) from Sham+Veh (N=9), Sham+Ram (N=10), STNx+Veh (N=9) and STNx+Ram (N=9) rats. Data presented as mean±SEM. *P<0.05, ***P<0.001 vs Sham+Veh. +P<0.05 vs Sham+Ram, ##P<0.01, ###P<0.001 vs STNx+Veh. STNx+Veh indicates vehicle treated subtotal nephrectomy rats and STNx+Ram indicates ramipril treated subtotal nephrectomy rats.

Fig 6: Quantitative analysis of microRNA-133a, micoRNA-1, TGF-β1, Fibronectin-1 and Collagen 1A1 from Vehicle treated Control, Vehicle treated Hypoxia, Ramiprilat treated Control and Ramiprilat treated Hypoxia group of cells. Data presented as mean±SEM of three independent experiments performed in triplicate. **P<0.01 vs vehicle treated Control, *P<0.05 vs vehicle treated Control. ##P<0.01 vs Hypoxia+Ramiprilat. Cont – Control Cells, Cont+Ramt – Control + Ramiprilat group, Hypo – Hypoxia treated cells, Hypo+Ramt- Hypoxia+Ramiprilat group of cells.

Fig 7: Quantitative analysis of microRNA-133a, micoRNA-1, microRNA-212 and microRNA-132 from Vehicle treated Control and Ang II treated H9c2 cells. Data presented as mean±SEM of three independent experiments performed in triplicate. **P<0.01 vs vehicle treated Control.
Table 1: Cardiorenal parameters from Sham+Veh, Sham+Ram STNx+Veh and STNx+Ram rats.

<table>
<thead>
<tr>
<th></th>
<th>Sham+Veh (N=9)</th>
<th>Sham+Ram (N=10)</th>
<th>STNx+Veh (N=9)</th>
<th>STNx+Ram (N=9)</th>
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</thead>
<tbody>
<tr>
<td><strong>Kidney Parameters</strong></td>
<td></td>
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<tr>
<td>Urine Output (ml/24hr/100g)</td>
<td>4±1</td>
<td>6±1**</td>
<td>14±2***+++</td>
<td>14±1****+++</td>
</tr>
<tr>
<td>Urinary Protein (mg/24hr)</td>
<td>5.5±0.1</td>
<td>12.7±0.6**</td>
<td>11.6±0.9***</td>
<td>12.3±1.7**</td>
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<tr>
<td><strong>Cardiac Parameters</strong></td>
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<tr>
<td>Heart Rate (bpm)</td>
<td>357±6</td>
<td>366±11</td>
<td>386±2*</td>
<td>359±10#</td>
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<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>99±5</td>
<td>86±3*</td>
<td>151±9***+++</td>
<td>85±5***</td>
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<td>Left Ventricle End Diastolic pressure (LVEDP)</td>
<td>5.9±0.6</td>
<td>3.2±0.8*</td>
<td>7.7±0.2***+++</td>
<td>2.7±0.8***##</td>
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<td>Left Ventricular Hypertrophy (g/100g)</td>
<td>0.22±0.006</td>
<td>0.22±0.002</td>
<td>0.31±0.011***</td>
<td>0.23±0.004###</td>
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<tr>
<td>BNP mRNA (fold change)</td>
<td>1.0±0.13</td>
<td>0.40±0.04***</td>
<td>4.43±1.10***+++</td>
<td>1.15±0.27###++</td>
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<tr>
<td>Smad 3 mRNA (fold change)</td>
<td>1.02±0.12</td>
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<td>0.94±0.23#</td>
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<td>Smad 4 mRNA (fold change)</td>
<td>1.08±0.27</td>
<td>1.10±0.2</td>
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<td><strong>Plasma Parameters</strong></td>
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<td>Ang II levels (pg/ml)</td>
<td>90.2±5.8</td>
<td>434.3±122.1***</td>
<td>201.3±48.8**</td>
<td>259.3±62.4*</td>
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Table 2: Table shows Applied Biosystems TaqMan Assay ID for microRNAs and genes used in this study.

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<thead>
<tr>
<th>Gene / microRNA</th>
<th>Applied biosystem TaqMan assay ID</th>
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<tr>
<td>microRNA-1</td>
<td>002064</td>
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<td>microRNA-133</td>
<td>002246</td>
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<td>microRNA-212</td>
<td>002551</td>
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<td>microRNA-132</td>
<td>000457</td>
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<td>Collagen 1A1 (Col1A1)</td>
<td>Rn01463848_m1</td>
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<td>FoxO3</td>
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<td>Fas L</td>
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<td>Caspase 9</td>
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<td>BCL-2</td>
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<td>Smad 4</td>
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<td>TGF-β1</td>
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<td>Control Gene (Gapdh)</td>
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<td>Bim-1</td>
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<td>PTEN</td>
<td>Rn00477208_m1</td>
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<td>MnSOD</td>
<td>Rn00690588_g1</td>
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<td>NOX-1</td>
<td>Rn00586652_m1</td>
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<td>Control microRNA (U87)</td>
<td>001712</td>
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</tbody>
</table>
Fig 1:

(a) 

(b) 

(c) 

(d) 

Fold Change in miR-133

Fold Change in miR-1

Fold Change in MicroRNA-212

Fold Change in MicroRNA-132
Fig 2:

(a) 

(b) 

(c) 

(d) 

(e) 

(Figures showing different conditions and their effects on apoptosis, BCL-2, Fas L, and Caspase 9 with statistical comparisons)

** Sh + Veh

** Sh + Ram

** STNx+Veh

** STNx+Ram

## Sh + Veh

## Sh + Ram

## STNx+Veh

## STNx+Ram

* Sh + Veh

* Sh + Ram

* STNx+Veh

* STNx+Ram

### Sh + Veh

### Sh + Ram

### STNx+Veh

### STNx+Ram

---
Fold Change in Bim-1

Fold Change in PTEN

(f)  
(g)
Figure 3:

(a) Interstitial Fibrosis (% picrosirius red stained area)

(b) Fold change in TGF-β

(c) Fold change in Col1A1

(d) Fold change in Fn1

(e)
Fig 4:

![Graph showing fold change in FoxO3 for different groups: Sham+Veh, Sham+Ram, STNx+Veh, STNx+Ram. Notations: *, **, and *** indicate statistical significance.](image-url)
Fig 5:

(a)  
Sham + Veh  
Sham + Ram  
STN + Veh  
STN + Ram

(b)  
Fold Change in NOX1

Sham + Veh  
Sham + Ram  
STN + Veh  
STN + Ram

Fold Change in MnSOD
Fig 6:

(a) Cont Cont+Ramt Hypo Hypo+Ramt
0.0 0.5 1.0 1.5 2.0 2.5 Fold Change in TGF-β mRNA

(b) Cont Cont+Ramt Hypo Hypo+Ramt
0.0 0.5 1.0 1.5 2.0 Fold Change in Fibronectin-1 mRNA

(c) Cont Cont+Ramt Hypo Hypo+Ramt
0 1 2 Fold change in microRNA-133

(d) Cont Cont+Ramt Hypo Hypo+Ramt
0 1 2 Fold change in microRNA-1

(e) Cont Cont+Ramt Hypo Hypo+Ramt
0 1 2 Fold change in Col1A1 mRNA
Fig 7:

(a) Control Ang II
(b) Fold Change in miR-1

(c) Control Ang II
(d) Fold Change in miR-212