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

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MicroRNAs mediate the cardioprotective effect of angiotensin-converting enzyme inhibition in acute kidney injury. Am J Physiol Renal Physiol 309: F943–F954, 2015. First published September 23, 2015; doi:10.1152/ajprenal.00183.2015.—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 the pathogenesis of cardiac hypertrophy or to the protective effect of RAS blockade in kidney disease. Using a subtotal nephrectomy rat model of kidney injury, we investigated changes in cardiac microRNAs that are known to have direct target genes involved in the regulation of apoptosis, fibrosis, and hypertrophy. The effect of treatment with the angiotensin-converting enzyme (ACE) inhibitor 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-β1, fibronectin 1, collagen type 1A1, and forkhead box protein O3, which are all known to be involved in the regulation of apoptosis, fibrosis, and hypertrophy in cardiac cells while being targets for the above microRNAs. ACE inhibitor treatment increased expression of microRNA-212 and microRNA-1. The inhibitory action of ACE inhibitor treatment on increased cardiac NADPH oxidase isoform 1 expression after subtotal nephrectomy surgery suggests that inhibition of oxidative stress is also one of mechanism of ACE inhibitor-mediated cardioprotection. These findings suggest the involvement of microRNAs in the cardioprotective action of ACE inhibition in acute renal injury, which is mediated through an inhibitory action on profibrotic and proapoptotic target genes and stimulatory action on antiapoptotic and antiapoptotic target genes.

microRNA; microRNA-133; microRNA-1; microRNA-212/132; cardiorenal cross talk; angiotensin-converting enzyme inhibitors; subtotal nephrectomy

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 posttranscription level (47, 52) and are involved in cardiac hypertrophy, fibrosis, and remodeling (15, 32, 51) through 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. A study (51) in null mice and a model of pressure-overload induced heart failure has indicated that microRNA-212 and microRNA-132 regulate cardiac hypertrophy. 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 hypothesized that microRNAs may be important players in both the development and prevention [after angiotensin-converting enzyme (ACE) inhibitor treatment] of cardiac hypertrophy, fibrosis, and apoptosis associated with kidney disease. In the present 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 (TGF)-β1, fibronectin (Fn), collagen type 1A1 (Col1A1)], hypertrophy [forkhead box protein (Fox)O3], and apoptosis [B cell lymphoma (BCL)-2 and caspase-9].

To understand the molecular mechanisms of cardiac pathologies associated with STNx, we also examined mRNA expression for Fas ligand, BCL2L11 (Bim-1), and phosphatase and tensin homolog (PTEN), which are all known to play an important role in the regulation of cell death pathways. Oxidative stress has been increasingly linked to the high incidence of cardiovascular events in patients with chronic kidney disease, and expression of NADPH oxidase isoform 1 (NOX-1), in particular, has been linked with ANG II signaling (14, 34a). Expression of NOX-1 and the cellular antioxidant enzyme MnSOD were also measured. We also investigated the effect of treatment with the ACE inhibitor ramipril on the regulation of these microRNAs and genes. In vitro experiments on cultured H9c2 cells were performed to understand the mechanisms leading to altered microRNA expression.

METHODS

Animal surgeries and cardiovascular assessment. Experimental procedures were performed in accordance with National Health and Medical Research Council of Australia guidelines for animal experimentation and were approved by the Animal Ethics Committee of...
Austen Health. Rats were housed on a 12:12-h light-dark cycle with ad libitum food containing 0.4–0.6% NaCl (Noro, Lismore, NSW, Australia) and water. STNx or sham surgery was performed in Sprague-Dawley rats (body weight: 200–250 g) as previously described (6). In brief, rats were anesthetized by an intraperitoneal injection of pentobarbitone sodium (60 mg/kg body wt, 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 extrarenal branch of the left renal artery.

After STNx or sham surgery, animals were randomly allocated to vehicle (vehicle-treated sham group: n = 9 and vehicle-treated STNx group: n = 9) or ACE inhibitor-treated (ramipril-treated sham group: n = 10 and ramipril-treated STNx group: n = 9) groups. Ramipril was given daily by oral gavage at a dose of 1 mg·kg⁻¹·day⁻¹ for 10 days. On day 9, rats were housed in metabolic cages for 24 h, and urine volume was 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 method with a commercially available bicinchoninic acid protein assay kit (Pierce, Rockford, IL). On day 10, rats were anesthetized (pentobarbitone sodium 60 mg/kg body wt ip), and mean arterial pressure (MAP), heart rate, and left ventricular (LV) end-diastolic pressure (LVEDP) were determined using a micromanipulated pressure transducer catheter (Millar, 1.5-Fr) inserted into the left carotid artery and advanced into the LV. Data were analyzed 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 mM EDTA, 0.2 M NaCl, and 1–2 trypsin inhibitory units/ml aprotinin made up in -ethyl-2-amino-2-methyl-1-propanol (EMAP) buffer (5 mM NaCl, 100 mM Na₂HPO₄, 100 μg/ml streptomycin, and 10% heat-inactivated FBS (Sigma-Aldrich)). The addition of RA was performed daily for 5 consecutive days. RA was prepared in DMSO and stored at -80°C. The intra- and interassay coefficients of variation were 7.6% and 8.3%.

In vitro experiments. H9c2 cardiac myoblast cells were cultured by previously described methods (4) with some modifications. In brief, cells were grown in DMEM (Sigma-Aldrich) supplemented with 1.5 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% heat-inactivated FBS (Sigma-Aldrich) 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/well in 24-well culture dishes and allowed to adhere the dish for 1 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). The addition of RA was performed daily for 5 consecutive days. RA was prepared in DMSO and stored at -20°C in the dark. Previous studies (4, 39) have demonstrated that H9c2 cells differentiated in low serum plus RA exhibit higher expression for cardiomyocyte-specific markers, suggesting transformation of cardiomyoblasts to cardiomyocytes. Ramipril is metabolized to ramiprilat in the liver, which then exerts its inhibitory effect on ACE. Cells were exposed to hypoxia (2% O₂) in the presence and absence of ramiprilat (100 nM, Santa Cruz Biotechnology) for 8 h. Control cells were treated with vehicle (DMSO). To confirm upregulated RAS signaling in these cells, ACE mRNA expression was measured from both control and hypoxic cells. To test the effect of direct exposure to ANG II, after 5 days of incubation in media with RA and 1% serum, cells were incubated with ANG II (100 nM, Sigma-Aldrich) for 12 h. After 12 h, 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 regions of the heart from sham-operated rats, as previously described (6). cDNA synthesis was then performed using microRNA-specific primers (Applied Biosystems) by methods provided by the manufacturer. A 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) according the manufacturer’s instructions. All RNA samples were quantified by spectrophotometry using Nanodrop (Thermo Fisher). cDNA synthesis was then performed using microRNA-specific primers (Applied Biosystems) by methods provided by the manufacturer. A 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) by previously described methods (48). In brief, reverse transcription was carried out (at 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then to 4°C) using 10 ng/μl RNA, and PCRs were then 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 a ViiA 7 Real-Time PCR system (Applied Biosystems) using ViiA 7 RUO software. mRNA was normalized to the U87 endogenous control, and the relative fold difference in expression was calculated using the 2⁻ΔΔCt method (where Ct is threshold cycle) (62).

Information on the TaqMan assays used in this study is shown in Table 1.

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

Quantification of apoptotic cell death by TUNEL assay. Cardiac (LV) paraffin 4-μm-thick sections were used to perform TUNEL staining by methods provided by the manufacturer (TACS.XL DAB In Situ Apoptosis Detection Kit, catalog no. 4828-30-DK). The kit is designed to measure nuclear DNA fragmentation, which is a widely accepted method to assay for apoptosis. We stained three sections from each animal, and three images were taken from random fields of each section. TUNEL-positive cells were then counted on the computer screen by examination of the images. The average number of cells counted from all three fields from each section was used for analysis.

Determination of cardiac collagen and TGF-β1 proteins. Cardiac (LV) paraffin 4-μm-thick sections were deparaffinized, rehydrated, and genes used in this study

<table>
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<tr>
<th>Gene/MicroRNA</th>
<th>Applied Biosystem TaqMan Assay Identifier</th>
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<tr>
<td>MicroRNA-1</td>
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<tr>
<td>MicroRNA-133</td>
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<td>MicroRNA-132</td>
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<td>Collagen type 1A1</td>
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<td>Control microRNA (U87)</td>
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Table 1. Applied biosystem assay identifiers for microRNAs and genes used in this study
and then stained with 0.1% Sirius red (Polysciences) in saturated picric acid (microsirius red) for 1 h, differentiated in 0.01% HCl for 30 s, and rapidly dehydrated. The 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 analyzed 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 polyclonal anti-TGF-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) by previously described methods (34). In brief, cardiac (LV) paraffin 4-μm-thick sections were incubated with antibody overnight at 4°C. The following day, sections were thoroughly washed in PBS and incubated with goat anti-rabbit (1:200) IgG Fab2 tagged with Alexa fluor-647 (Molecular Probes). For comparison of intensity and staining, sections from all three groups were imaged using a 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 ANOVA with a post hoc test to determine if there were overall significant effects between groups. If a statistical difference was present, subsequent comparisons were made using a Student’s unpaired t-test. The presence of correlations between variables was tested using Pearson’s correlation analysis.

RESULTS

Kidney and cardiovascular parameters. Kidney and cardiac measurements are shown in Table 2. 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 vehicle- treated sham animals (P < 0.001 for both; Table 2). Ramipril had no effect on proteinuria in STNx rats and caused proteinuria in sham rats (Table 2). 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, vehicle-treated STNx rats had an increased heart rate (P < 0.05; Table 2). Heart rate in ramipril-treated STNx rats was comparable with those of vehicle-treated sham rats and significantly lower than those of vehicle-treated STNx rats (P < 0.05; Table 2). 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 rats; Table 2). Cardiac dysfunction was present with a significant increase in LVEDP in STNx rats (Table 2). Ramipril reduced MAP (P < 0.001 vs. vehicle-treated STNx rats; Table 2) and improved LVEDP (P < 0.01; Table 2) in STNx rats. STNx rats had LV hypertrophy compared with vehicle-treated sham rats (P < 0.001), which was reduced by ramipril (P < 0.001; Table 2). Ramipril-treated sham rats had 13% lower MAP and LVEDP compared with vehicle-treated sham rats (both P < 0.05; Table 2). Heart rate and LV hypertrophy were not significantly different between vehicle-treated and ramipril-treated sham rats (Table 2).

Table 2. Cardiorenal parameters from sham + Veh, sham + Ram, STNx + Veh, and STNx + Ram rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham + Veh Group</th>
<th>Sham + Ram Group</th>
<th>STNx + Veh Group</th>
<th>STNx + Ram Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of rats/group</td>
<td>9</td>
<td>10</td>
<td>9</td>
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<tr>
<td>Kidney parameters</td>
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<tr>
<td>Urinary output, ml · 24 h · 100 g⁻¹</td>
<td>4 ± 1</td>
<td>6 ± 1**</td>
<td>14 ± 2**††††</td>
<td>14 ± 1**††††</td>
</tr>
<tr>
<td>Urinary protein, mg/24 h</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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<td>Cardiac parameters</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>357 ± 6</td>
<td>366 ± 11</td>
<td>386 ± 2*</td>
<td>359 ± 10†</td>
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<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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<tr>
<td>Left ventricular end-diastolic pressure, mmHg</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>
</tr>
<tr>
<td>Left ventricular hypertrophy, g/100 g</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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<td>Brain natriuretic peptide mRNA, fold change</td>
<td>1.0 ± 0.13</td>
<td>1.0 ± 0.16</td>
<td>2.02 ± 0.36††‡</td>
<td>1.15 ± 0.27**†††</td>
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<td>Smad 3 mRNA, fold change</td>
<td>1.02 ± 0.12</td>
<td>1.20 ± 0.16</td>
<td>2.27 ± 0.42‡‡</td>
<td>0.88 ± 0.24‡′</td>
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<td>Smad 4 mRNA, fold change</td>
<td>1.08 ± 0.27</td>
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<td>Plasma parameters</td>
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<tr>
<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>

Data are presented as means ± SE. Sham, sham operated; Veh, vehicle treatment; Ram, ramipril treatment; STNx, subtotal nephrectomy. Data were analyzed by a one-way ANOVA statistical test followed by a t-test. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. sham + Veh rats; †P < 0.05, ††P < 0.01 and †††P < 0.001 vs. sham + Ram rats; ‡P < 0.05, ‡‡P < 0.01 and ‡‡‡P < 0.001 vs. sham + Veh rats.
number of apoptotic cells in ramipril-treated STNx rats was not significantly different from vehicle-treated sham rats (Fig. 2, A and B).

**Quantification of LV mRNA for apoptosis.** Antiapoptotic gene BCL-2 mRNA levels were not altered by STNx surgery (Fig. 2C), but there was a significant increase in the ramipril-treated group ($P < 0.001$ compared with the vehicle-treated sham group and $P < 0.05$ compared with the vehicle-treated STNx group; Fig. 2C). Compared with vehicle-treated sham rats, mRNA for the proapoptotic genes Fas ligand and caspase-9 was significantly higher in vehicle-treated STNx rats ($P < 0.01$ for both), which was reversed by ramipril (Fig. 2, D and E). Caspase-9 but not Fas ligand was significantly reduced in ramipril-treated sham rats. We report that compared with vehicle-treated sham rats, Bim-1 expression was significantly decreased in the STNx group ($P < 0.05$; Fig. 2F) and was almost restored by ramipril ($P = 0.09$; Fig. 2F). Expression of PTEN was increased in vehicle-treated STNx rats ($P < 0.05$ compared with vehicle-treated sham rats; 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 with vehicle-treated sham rats, there was a significant increase in TGF-β1 immunoreactivity in vehicle-treated STNx rats, as evident by higher intensity and labeling (Fig. 3A). In addition, both intensity and TGF-β1-labeled 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 with vehicle-treated sham rats). 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 2). Ramipril treatment in sham rats did not alter TGF-β1, Smad 3, and Smad 4 mRNA expression (Fig. 3C and Table 2). We studied mRNA levels for the extracellular matrix protein ColIα1 and Fn1 as markers of fibrosis. There was a 4.5-fold increase in mRNA for ColIα1 and a 5.5-fold increase in Fn1 mRNA in vehicle-treated STNx rats ($P < 0.001$ compared with vehicle-treated sham rats; Fig. 3, D and E). Treatment with ramipril in STNx rats caused a significant reduction in mRNA levels of both ColIα1 and Fn1 compared with vehicle-treated STNx rats ($P < 0.01$ and $P < 0.001$, respec-
tively). Expression of both Col1A1 and Fn1 was also significantly higher in ACE inhibitor-treated sham rats compared with vehicle-treated sham rats ($P < 0.05$ for both; Fig. 3, D and E); however, it was lower than in vehicle-treated STNx rats (Fig. 3, D and E).

**Quantification of LV mRNA for hypertrophy** Antihypertrophic gene FoxO3 mRNA levels were 40% lower in STNx rats but were not significantly reduced compared with vehicle-treated sham rats ($P = 0.071$; Fig. 4). There was a significant increase in the expression of FoxO3 mRNA in ramipril-treated STNx rats ($P < 0.05$ compared with vehicle-treated sham rats and $P < 0.01$ compared with vehicle-treated STNx rats; Fig. 4).

**Expression of NOX-1 and MnSOD.** Expression of NOX-1 was increased in the vehicle-treated STNx group ($P < 0.05$ compared with the vehicle-treated sham group; Fig. 5A). Ramipril treatment was able to attenuate this increase in NOX-1 mRNA expression ($P < 0.05$ compared with the vehicle-treated STNx group; Fig. 5A). MnSOD expression was not altered by STNx and/or ramipril treatment (Fig. 5B).

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**Fig. 3.** A: images showing transforming growth factor (TGF)-β1 immunoreactivity in the rat heart [sham + Veh group (a), STNx + Veh group (b), and STNx + Ram group (c)]. All images were taken at $\times 200$ magnification. Scale bar = 200 μm. B: quantitative analysis of picrosirius red staining from sham + Veh ($n = 9$), STNx + Veh ($n = 9$), and STNx + Ram ($n = 9$) rats. C–E: quantitative analysis of mRNA for TGF-β1 (C), collagen type 1A1 (Col1A1; D), and fibronectin 1 (Fn1; E) mRNA from sham + Veh ($n = 9$), sham + Ram ($n = 10$), STNx + Veh ($n = 9$), and STNx + Ram ($n = 9$) rats. Data are presented as mean ± SE. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. sham + Veh rats; #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ vs. STNx + Veh rats.
In vitro work on H9c2 cells. Compared with control cells, the level of ACE mRNA was higher in cells treated with hypoxia (data not shown), confirming that 8 h of hypoxia treatment was sufficient to increase RAS signaling in these cells. Compared with control cells, expression of both microRNA-133 and microRNA-1 was not different in hypoxic cells. We found a significant increase in the expression of both microRNA-133 and microRNA-1 in hypoxic cells treated with ramiprilat ($P < 0.01$ and $P < 0.05$, respectively, compared with control cells; Fig. 6, A and B). Compared with hypoxic cells, expression of microRNA-133 but not microRNA-1 was significantly different in hypoxic cells treated with ramiprilat ($P < 0.01$; Fig. 6, A and B). Hypoxia was able to cause a 0.8-fold increase in mRNA for TGF-$\beta_1$ ($P < 0.05$; Fig. 6C), which was (nonsignificantly) lowered by ramiprilat treatment ($P = 0.09$; Fig. 6C). mRNA levels for Fn1 and Col1A1 were not altered by hypoxia (Fig. 6, D and E).

Direct exposure to ANG II for 12 h was able to cause a small but significant reduction in the expression of microRNA-133 and microRNA-1 in H9c2 cardiomyocytes ($P < 0.05$ for both; Fig. 7, A and B). Expression of microRNA-212 and microRNA-132 was increased ($P < 0.05$ for both; Fig. 7, C and D).

**DISCUSSION**

Acute kidney injury results in cardiac structural changes that are attenuated by ACE inhibition. In the present 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 cardiac expression of prohypertrophic microRNA-212 and microRNA-132 were upregulated after kidney injury, which resulted in suppression of the antihypertrophic FoxO3 target gene. Treatment with an ACE inhibitor resulted in attenuation of the expression of these two microRNAs and, as a result, a restoration of FoxO3 mRNA levels. Cardioprotective microRNA-133 and microRNA-1 did not change with kidney injury; however, ACE inhibition resulted in the upregulation of both these microRNAs. In parallel with microRNA-133, mRNA for its target genes, profibrotic Col1A1 and apoptotic caspase-9, were both upregulated in STNx rats and downregulated with ramipril treatment. Similarly, the ACE inhibitor-mediated increase in microRNA-1 resulted in inhibition of its profibrotic target gene Fn1 and upregulation of the antiapoptotic gene BCL-2.

We found a significant increase in the number of cells with nuclear DNA fragmentation in vehicle-treated STNx rats, which is consistent with an earlier finding in this model reporting increased cardiac apoptosis (1). A study by Amann et al. (1) in the STNx rat model reported that ramipril treatment attenuated cardiomyocytes loss and abnormal increase in cardiomyocyte volume, suggesting cardioprotective effect of ACE inhibition. However, the study did not provide any information on the molecular mechanism leading to the inhibition of cell death. Our data show a significant attenuation of the STNx-mediated increase in the expression of the apoptotic genes caspase-9 and Fas ligand by ramipril treatment. Caspase-9 is a proapoptotic gene (16) and has been shown to be associated with various cardiac pathological conditions. Caspase-9 is a part of a “mitochondrial” apoptotic pathway activated by mitochondrial cytochrome c release. Apart from caspase-9, we found increased expression of another proapoptotic gene, Fas ligand, in STNx rats. Fas ligand belongs to the family of TNF-related cytokines and induces apoptosis via a “death receptor” signaling pathway. Thus, our data suggest 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, ACE inhibitor treatment after STNx caused a significant increase in the expression of this microRNA. Luciferase gene reporter assays performed in a previous study (21) determined caspase-9 as a direct target of microRNA-133. In parallel, we found a significant increase in the 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 the ACE inhibitor-treated group attenuates caspase-9 mRNAs to inhibit a STNx-activated mitochondrial apoptotic pathway. However, the involvement of microRNAs in ACE inhibitor-mediated inhibition of death receptor pathways is not clear. The fact that there was no change in microRNA-133 expression in nontreated STNx rats suggests blood pressure-independent regulation of this microRNA in the heart; further experiments are required to validate this hypothesis.

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

Previous studies (29, 63) in STNx rats have also demonstrated the involvement of TGF-β1-Smad signaling in both cardiac and kidney fibrosis. We have previously reported activation of the 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 signaling by ramipril treatment may be the mechanism by which ACE inhibitor treatment reduces fibrosis.
TGF-β1 can also increase the extracellular matrix protein 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 the extracellular matrix protein Col1A1 and Fn1 by STNx, which was attenuated by ramipril treatment. Luciferase gene reporter assays performed in previous studies (9, 44) have demonstrated TGF-β1 and Col1A1 as direct targets of microRNA-133. Fn1 is a direct target of microRNA-1 (61). Treatment with an ANG 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). Our 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 signaling, 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 ANG II-induced hypertension (17). The antihypertrophic and proautophagic FoxO3 transcription factor has been shown as a common target gene for both microRNA-212 and microRNA-132 (51). The phosphorylated form of FoxO3 is retained in the cytoplasm, but its dephosphorylation leads to translocation to the nucleus, which then promotes expression of its target genes. A study (26) in mesangial cells has shown that TGF-β1 has an inhibitory effect on FoxO3 transcription and apoptosis. The FoxO3 transcription factor also activates the expression of atrogin-1, which inhibits calcineurin-dependent cardiac hypertrophy (30, 40, 43). A recently published study (51) has demonstrated attenuated transaortic constriction-induced cardiac hypertrophy in microRNA-212/132-null mice. The same study reported upregulated cardiac mRNA for atrogin-1 in microRNA-212/132-null mice and suggested increased FoxO3 activity as a mechanism for the 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 results suggest that the increase in FoxO3 expression due to the ACE inhibition-mediated attenuation of microRNA-212 and microRNA-132 in STNx rats is the mechanism for the inhibition of cardiac hypertrophy.

Using miR-212/132 overexpression and ANG II treatment as tools, a study (18) in cardiac fibroblast cells has demonstrated that 22 validated target genes are involved in ANG II signaling. It clearly suggests a complex role of these microRNAs in regulating the ANG II signaling pathway. The same study found increased PTEN expression in cardiac fibroblast cells overexpressing microRNA-212/132 (18). In contrast, another study by Jin et al. (24) demonstrated that ANG II-induced expression of microRNA-132 results in a reduction of PTEN expression in vascular smooth muscle cells. In the present study, we found increased expression of PTEN along with increased levels of microRNA-212/132. The STNx-mediated increased expression of microRNA-212 but not microRNA-132 was normalized by ACE inhibitor treatment to the same level as in sham rats. Compared with (both vehicle and ACE inhibitor treated) sham rats, expression of microRNA-132 was significantly higher even after ACE inhibitor treatment in STNx rats.

Using cultured mesangial cells, a previous report by Kato et al. (26) demonstrated that TGF-β1 induces FoxO3a phosphorylation, which then decreases the expression of key FoxO target genes, the proapoptotic gene Bim and the antioxidant AJP-Renal Physiol • doi:10.1152/ajprenal.00183.2015 • www.ajprenal.org

Fig. 7. Quantitative analysis of microRNA-133a, microRNA-1, microRNA-212, and microRNA-132 from vehicle-treated control and ANG II-treated H9c2 cells. Data are presented as means ± SE of three independent experiments performed in triplicate. *P < 0.05 vs. vehicle-treated control cells.
gene MnSOD. In our study, we found significant increase in TGF-β1 expression and a significant reduction in Bim-1 expression in STNx rats, which was attenuated by ACE inhibitor treatment. There was no difference in the expression of MnSOD, but we observed a significant increase in the expression of NOX-1 in STNx rats, suggesting an increase in cardiac oxidative stress. ACE inhibitor treatment had no effect on MnSOD expression but attenuated the increase in NOX-1 observed after STNx. There is emerging evidence suggesting that TGF-β1 activates NOX to promote the production of ROS (23). Three NADPH isoforms (NOX-1, NOX-2, and NOX-4) are known to play key roles in cardiac pathologies associated with cardiovascular diseases (5). Using AS49 and H1437 cancer cells, Singh et al. (45) demonstrated that increased expression of microRNA-1 can inhibit NOX activity, suggesting that microRNA-1 prevents the production of nucleotide precursors by inhibiting the expression of pentose phosphate pathway genes. Our data show increased microRNA-1 expression in the ACE inhibitor-treated group, which also had reduced NOX-1 expression. These data suggest that inhibition of oxidative stress may be one of the mechanisms of the ACE inhibitor-mediated cardioprotection in experimental kidney injury. Further detailed investigations are required to understand the 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 demonstrate that 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 hypertrophic stimuli, including ANG II, upregulated expression of microRNA-212 and microRNA-132 in primary neonatal cardiomyocytes (51). Therefore, the lowered expression of microRNA-212 and microRNA-132 in STNx rats treated with ACE inhibitor reflects reduced ANG II signaling in heart. Hypoxia is also one stimulus 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 previous reports (19, 36, 62) showing chronic hypoxia-mediated activation of tissue-specific RAS activity. A study (65) in pulmonary artery smooth muscle cells demonstrated that hypoxia inducible factor-1α can bind and transactivate the ACE promoter directly. A study (27) in human pulmonary artery adventitial fibroblasts cells demonstrated increased ACE activity after exposure to hypoxia. 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, which 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 an increase in the expression of microRNA-133 and microRNA-1. To our knowledge, this is the first study to report that ACE inhibitors may mediate cardioprotective effects by upregulating the expression of microRNAs. However, the detail molecular mechanism of how ACE inhibition alters the 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.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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