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p90 Ribosomal S6 Kinase Regulates Activity of the Renin-Angiotensin System: a Pathogenic Mechanism for Ischemia/Reperfusion Injury

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Abstract

Increasing evidence suggests that local Renin-Angiotensin System (RAS) plays an important role in cardiac diseases. Elevated p90 ribosomal S6 Kinase (RSK) activity has been observed in diabetic animal, as well as in human failing hearts. We hypothesize that RSK mediates cardiac dysfunction by up regulating local RAS signaling. In the present study, we show that the prorenin mRNA level was significantly increased (~5.6-fold) in transgenic mouse hearts with cardiac specific expression of RSK (RSK-Tg). The RSK-Tg mice were more vulnerable to ischemia/reperfusion (I/R) injury than non-transgenic littermate controls (NLC). To further understand the direct contribution of cardiac renin to I/R injury, we used a Langendorff system to evaluate the effect of renin inhibition by aliskiren in RSK-Tg mouse hearts. In the vehicle-perfused group, I/R significantly decreased left ventricular developed pressure (LVDP) in RSK-Tg hearts compared to NLC (7% versus 60% of the baseline). However, aliskiren perfusion significantly increased LVDP in RSK-Tg (7% to 61%, $p<0.01$) but not in NLC hearts (60% to 62%, n.s.). The protective effect of aliskiren in RSK-Tg hearts was further demonstrated with positive (contraction) dp/dt (6.5% to 63%, $p<0.01$) and rate pressure product (RPP) (5% to 51%, $p<0.01$). Moreover, aliskiren significantly decreased I/R induced infarction in RSK-Tg (60% to 32%, $p<0.01$), compared to NLC hearts (37% to 32%, n.s.). These results suggest that RSK plays a crucial role in regulating local cardiac renin, which contributes to I/R induced cardiac injury and dysfunction. Thus, renin inhibition may provide an alternative therapeutic strategy under conditions of increased RAS.

Keywords

Renin-Angiotensin System; RSK; Aliskiren; Ischemia-Reperfusion

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1. Introduction

The Renin-Angiotensin System (RAS) is a circulating endocrine system that plays a key role in regulating blood pressure homeostasis. Renin was first identified in kidney and it catalyzes the rate-limiting step of RAS signaling by cleaving liver-secreted angiotensinogen (AGN) to generate angiotensin I (AngI). AngI is subsequently cleaved by angiotensin converting enzyme 1 (ACE1) to produce AngII, which triggers downstream signaling mainly through binding to AngII type 1 and type 2 receptors (AT1R, AT2R). Increased AngII acts through a negative-feedback loop systemically to prevent over production of renin by the kidney. However, this negative-feedback loop may be disrupted under some pathological conditions. Over-activation of RAS causes pathological remodeling and restructuring of various tissues, leading to functional impairment.

Blocking RAS signaling with ACE inhibitors (ACEi) or AT1 receptor blockers (ARB) is effective for treating hypertension, preventing cardiac remodeling, as well as delaying the onset of diabetes mellitus. In 2007, aliskiren became the first oral active renin inhibitor approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of human hypertension [1]. However, renin inhibition did not provide much greater improvement on reducing cardiovascular morbidity and mortality in hypertensive patients as anticipated. One explanation could be the involvement of increased local RAS in pathological conditions.

The concept of “local RAS” was adopted recently as RAS components were identified in isolated tissues including heart [2] brain and eye. It has been reported that the majority of AngI and AngII detected in the heart was produced locally [3]. The most direct supporting evidence for local RAS is from the study of transgenic rats with cardiac-specific over-expression of mouse renin (Ren-Tg). Treatments with ACEi or ARB decreased myocardial hypertrophy at dosages that did not significantly reduce blood pressure in those Ren-Tg rats [4]. These findings suggest that local RAS plays a crucial role in regulating cardiac remodeling and injury. Importantly, the local RAS is highly up regulated under pathological conditions because of the lack of a negative-feedback regulation. Hence, it is very crucial to investigate the role of RAS inhibitors in pathological conditions.

The p90 ribosomal S6 kinase (RSK) family members are mitogen-activated serine threonine kinases that play an important role in cell growth, proliferation and differentiation. Transgenic mice with cardiac specific over-expression of p90RSK (RSKTg) develop cardiac dysfunction with increased fibrosis and hypertrophied cardiomyocytes at 10 months of age. Young (~2–3 month) RSK-Tg mice are more vulnerable to I/R injury than NLC [5]. In this study, we report that prorenin and ACE1 mRNA levels were significantly increased in RSK-Tg mouse hearts. Treatment with renin inhibitor aliskiren significantly improved cardiac functional recovery and decreased infarct size in isolated RSK-Tg mouse hearts after I/R. Our results further demonstrate the induction of local cardiac RAS by RSK plays a pivotal role in I/R injury. Renin inhibition could be an alternative treatment for patients under ischemic conditions.

2. Materials and Methods

2.1 Measurement of left ventricular function by Langendorff preparation

RSK-Tg mice were generated as we have described before [5]. Mice were maintained by breeding with FVB strain F1 animals (Jackson Laboratory, Bar Harbor, Me). All mice were used in accordance with Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the University of Rochester Animal Care Committee.

Hearts isolated from RSK-Tg and NLC mice (10–12 weeks of age) were subjected to I/R by langendorff system as we've described before [5]. Isolated hearts were subjected to 20 minutes perfusion with KH buffer to wash out plasma renin, and then perfused for 25 minutes with vehicle (KH buffer) or aliskiren (25 μM) in KH buffer. This was followed by 20 minutes of no-flow normothermic global ischemia and 60 minutes reperfusion.

2.2 Analysis of infarct in the hearts

Following treatment, hearts were disconnected from the Langendorff system and sliced horizontally to have the maximum exposure to 2,3,5-triphenyltetrazolium chloride (TTC) staining. Sections were incubated in phosphate buffer (0.1M Na_2HPO_4 and 0.1M NaH_2PO_4, pH 7.4 at 37°C) containing TTC (10 mg/ml) for 20 minutes. The living tissue was stained red while the infarcted dead tissue was white. The heart sections were fixed in 10% formaldehyde overnight, photographed, and infarct area analyzed with Scion Image.

2.3 Extraction of RNA from the heart tissuse and Reverse Transcription-PCR

Perfused hearts were ground in liquid nitrogen. Total RNA was extracted using TRIzol (Invitrogen) and treated with DNase I (Promega). First strand cDNA was synthesized using Reverse transcription kit (Promega). cDNAs were amplified using the following primers with GoTaq (Promega) for 36 cycles of 94° C for 45 seconds, 55° C for 45 seconds, and 72° C for 1 minute. mRNA levels were normalized to GAPDH mRNA expression (PCR for 24 cycles). Primer sequences are shown in the following. Prorenin: 5'ACCTTGCTTGTGGGATTCAC3' (forward); 5'CG CACAGCCTTCTTCACATA3' (reverse). ACE1: 5' TTGTGCTGCAGTTCCAGT TC3' (forward); 5' TGAGCTTGGCAATCTTGTTG3' (reverse). ACE2: 5' CCTCTTTCTGCT GCTCTGCT3' (forward); 5' TGAGCTTGGCAATCTTGTTG3' (reverse). AT1aR: 5'CA AAGCTTGCTGGCAATGTA3' (forward); 5'TCCAGCTCCTGACTTGTCCT3' (reverse). AT2R: 5'CAACTTCAGTTTTGCTGCCA3' (forward); 5' CCAGCAGACCAC TGAGCATA3' (reverse). GAPDH: 5'TCAAGAAGGTGGTGAAGCAG3' (forward); 5'TGGGAGTTGCTGTTGAAGTC3'(reverse).

2.4 Statistical Analysis

The animal numbers for each group (n=6) were determined by Power Calculation. All values are presented as mean ±SEM. Statistical differences between groups were determined by One-way ANOVA. Values of $P<0.05$ were considered statistically significant.

All authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

3. Results

3.1 Prorenin mRNA level is increased in the RSK-Tg mouse hearts

We hypothesized that there would be an elevation of RAS signaling in the cardiac specific RSK-Tg mice for two reasons: 1) there is increased prorenin-converting enzyme and evidence for increased renin in the hearts; and 2) there is more severe injury after I/R, which can be prevented by ACEi and ARB [5]. To test our hypothesis we examined mRNA levels of various components of the RAS (AGN, prorenin, ACE1, ACE2, AT1aR and AT2R) in mouse hearts from RSK-Tg and NLC mice. As shown in Table 1 there is a significant increase of prorenin and ACE1 mRNAs but not significant change of AGN, ACE2, AT1aR and AT2R mRNA levels. These results confirm the induction of RAS when RSK expression is increased, and support the hypothesis that there is increased renin in RSK-Tg mouse hearts.

3.2 Aliskiren improves cardiac function of RSK-Tg hearts after I/R injury

As there was a significant increase of prorenin in RSK-Tg mouse hearts, we hypothesized that renin inhibition would improve the recovery of cardiac function after I/R in RSK-Tg mice. To test directly the effect of a renin inhibitor on the local cardiac RAS, we used an *ex vivo* Langendorff model (Figure 1A). Briefly, isolated mouse hearts were perfused with KH buffer in a constant non-circulating flow (4 ml/min) for 20 minutes to wash out plasma renin. Hearts then were perfused with KH buffer or aliskiren for 25 minutes, followed by global ischemia for 20 minutes and reperfusion for 60 minutes. In preliminary experiments we compared the recovery of LVDP and positive dp/dt in response to 5, 25 or 50 μM aliskiren and found that the maximum recovery was observed at 25 μM aliskiren (data not shown). Therefore, 25 μM aliskiren was used for all subsequent experiments.

There was no significant difference in LVDP or positive dp/dt between RSK-Tg and NLC hearts after perfusion for 45 minutes (*in Figure 1A) which is the first data point in Figures 1B–C. In response to 20 minutes global ischemia and 60 minutes reperfusion, the LVDP of NLC hearts recovered to 52% of the basal level. In contrast, there was a dramatic worsening of the LVDP recovery (to only 7%) in RSK-Tg mouse hearts. These data are consistent with the previous finding of enhanced cardiac dysfunction after *ex vivo* I/R in RSK-Tg mice [5]. Treatment with 25 μM aliskiren before ischemia did not significantly change LVDP in NLC hearts (60% versus 52%). In contrast, there was a significant increase of LVDP in RSK-Tg hearts following aliskiren perfusion (61% versus 7%, Figure 1B). Similarly, post-I/R, positive dp/dt in RSK-Tg hearts was also increased significantly by aliskiren treatment (Figure 1C).

To further evaluate the cardiac function after I/R, we also calculated the rate pressure product (RPP). Consistent with LVDP and positive dp/dt, there was a significantly greater decrease in RPP in RSK-Tg compared to NLC hearts (5% versus 40% of baseline, $p<0.01$). Treatment with aliskiren dramatically improved the recovery in RSK-Tg (5% to 51%, $p<0.01$) compared to NLC hearts (40% to 47%, n.s.) (Figure 1D).

3.3 Aliskiren reduced infaction of RSK-Tg hearts after I/R injury

As shown in Figure 1E, top row is the grayscale of TTC stained heart sections represented from each experimental group. Grey and white area indicates the dead tissue. Dark area indicates the living part of the tissue. Shadowed area in bottom row was automatically outlined by the same defined grey threshold, which indicates the infarct area. Infarct size after I/R is significantly greater in RSK-Tg compared to NLC (60% versus 37%, $p<0.01$). Infusion with aliskiren decreased infarct size in RSK-Tg hearts (60% to 32%, $p<0.01$) but had no effect on infarct size in NLC hearts (37% to 32%, n.s.). The limited protective effect of aliskiren in NLC mice implies that increased cardiac RAS plays a crucial role in enhanced I/R injury in RSK-Tg mice.

4. Discussion

This study describes two major novel findings. Firstly, the mRNA expression levels of local cardiac RAS components such as prorenin and ACE1 are significantly increased in RSK-Tg mouse hearts with myocyte-specific expression of RSK. Previously, we also found that prorenin-converting enzyme was significantly increased in RSK-Tg mouse hearts as well as in streptozotocin induced diabetic mouse hearts [5]. These data suggest that cardiac RSK is a crucial mediator in up regulating local RAS under pathological conditions such as I/R. Secondly, we show that renin inhibitor aliskiren significantly improved cardiac functional recovery and decreased infarction size after I/R in isolated RSK-Tg mouse hearts. Overall, these data suggest that local cardiac RAS plays a critical role in I/R-induced cardiac damage

and dysfunction, and inhibiting the local cardiac renin is cardiac protective. Our findings agree with a previous observation that aliskiren, at a dose not affecting blood pressure, improves mouse ventricular remodeling and heart function after LAD ligation [6].

RSK is highly activated in failing human hearts with dilated cardiomyopathy [7]. *In vivo* I/R stimulates RSK activation at the ischemic area in mouse hearts [8]. Additionally, increased renin mRNA and protein expression has been detected in pathological conditions, including hyperthyroidism induced hypertrophy and LAD ligation. Our current study identified a novel pathway of an RSK-mediated up regulation of cardiac local RAS. Though the molecular mechanisms remain to be determined.

To date, 4 surrogate end-point trials involving 20,000 patients have been completed in which treatment with renin inhibitor aliskiren alone, or in combination with ACEis, ARBs or other antihypertensive drugs, have been compared in diabetic patients [9], patients with chronic heart failure [10], hypertensive patients with left ventricular hypertrophy [11], and aged hypertensive patients [12]. Aliskiren showed well-tolerated effective doses, prolonged effectual time, comparable antihypertensive outcome and cardiac functional improvement relative to the ACEis and ARBs in all of these trials. As renin inhibition targets the rate-limiting step of RAS signal, it was anticipated to have greater effect on reducing cardiac and renal morbidity and mortality than ACEis and ARBs, though only combined treatment provided enhanced improvements to cardiac output. Our study revealed a novel mechanism of an RSK-mediated up regulation of cardiac local renin signaling. This finding provides a rationale to investigate the effect of renin inhibition in certain pathological conditions, such as diabetes and heart failure. Three ongoing clinical trials are investigating the effect of aliskiren in type 2 diabetes [13], chronic and acute heart failure [14, 15].

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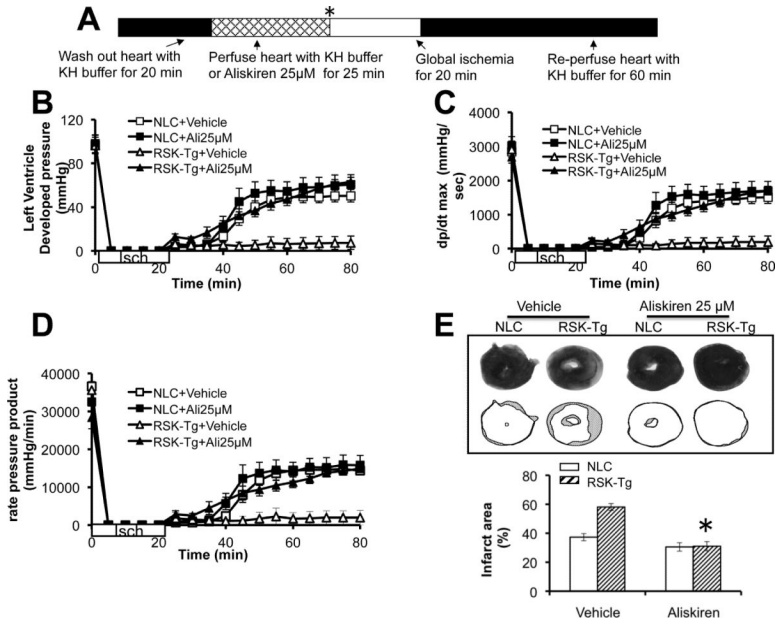

Figure 1.

The renin inhibitor aliskiren (25μM) protected RSK-Tg but not NLC hearts from I/R induced contractile dysfunction and injury.

A. Diagram of I/R experimental protocol. In brief, isolated mouse hearts were perfused with KH buffer for 20 minutes to wash out plasma renin. Hearts then were perfused with KH buffer or aliskiren (25μM) for 25 minutes, followed by global ischemia for 20 minutes and reperfusion for 60 minutes. * indicates the first data point in Figures 1B–D.

B. Measurements of left ventricular developed pressure (LVDP) and **C**. Positive dp/dt before and during I/R with vehicle or aliskiren treatment in NLC and RSK-Tg mouse hearts

D. Rate pressure product (RRP) was calculated based on the equation: RPP = LVDP (mmHg)×heart rate (bpm). (shown as mean ±SEM, n=6, $P<0.01$)

E. Representative TTC stained heart sections from each experimental group. Top row shows the grayscale of TTC stained heart sections; dark area indicates the living part of the tissue. Grey and white area indicates the dead tissue. Bottom row shows the stroked result from the top row using Adobe photoshop CS3 (by the same defined grey threshold). Shadow indicates the infarct area. Quantified infarct area is shown as mean ±SEM, n=6, $P<0.01$.

Table 1

genotype	AGN	prorenin	ACE1	ACE2	AT1aR	AT2R
WT-RSK	0.78±0.13	1.12±0.23	2.02±0.19	0.62±0.17	0.87±0.15	0.60±0.15
NLC	0.60±0.04	0.20±0.05	0.37±0.12	0.44±0.11	0.75±0.14	0.28±0.05
WT/NLC	1.30	5.60*	5.46*	1.41	1.16	2.14

mRNA levels of cardiac RAS components were measured by reverse transcription followed by PCR. Data was analyzed by normalizing the mRNA levels with GAPDH. Shown as mean ±SEM, n=4,

*P<0.01