ORIGINAL RESEARCH

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# Direct Renin Inhibition With Aliskiren Protects Against Myocardial Ischemia/Reperfusion Injury by Activating Nitric Oxide Synthase Signaling in Spontaneously Hypertensive Rats

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***Background*—**We tested the hypothesis that direct renin inhibition with aliskiren protects against myocardial ischemia/reperfusion (I/R) injury in spontaneously hypertensive rats (SHR), and examined the mechanism by which this occurs.

***Methods and Results*—**Male SHR were treated (orally, 4 weeks) with saline or aliskiren (30 or 60 mg $kg^{-1}$ $day^{-1}$) and subjected to 30 minutes of left anterior descending coronary artery occlusion followed by 6 or 24 hours of reperfusion. Only the higher dose significantly lowered systolic blood pressure, the lower dose causing a smaller apparent lowering that was nonsignificant. Despite this difference in blood pressure-lowering effect, both doses increased the ejection fraction and fractional shortening and reduced myocardial infarct size equally. I/R decreased cardiac expression of phosphatidylinositol 3-kinase (PI3K), phospho-Akt and phospho-endothelial nitric oxide synthase (phospho-eNOS), but increased expression of inducible nitric oxide synthase (iNOS); these changes were all abrogated by aliskiren. Moreover, aliskiren decreased superoxide anion generation and increased cyclic guanosine-3′,5′-monophosphate, an index of bioactive nitric oxide, in myocardium. It also decreased the expression of myocardial matrix metalloproteinase-2, matrix metalloproteinase-9, and tissue inhibitor of metalloproteinases-1 (TIMP-1) following I/R. In a Langendorff heart preparation, the detrimental cardiac effects of I/R were abrogated by aliskiren, and these protective effects were abolished by NOS or PI3K inhibition. In a parallel study, although specific iNOS inhibition reduced plasma malondialdehyde and myocardial superoxide anion generation, it did not affect the deleterious effects of I/R on myocardial structure and function.

***Conclusions*—**Direct renin inhibition protects against myocardial I/R injury through activation of the PI3K-Akt-eNOS pathway. **(*J Am Heart Assoc.* 2014;3:e000606 doi: 10.1161/JAHA.113.000606)**

**Key Words:** aliskiren • ischemia • myocardial infarction • nitric oxide synthase • reperfusion



Myocardial ischemia/reperfusion (I/R) injury is an important complication of acute coronary arterial occlusion and subsequent recanalization, whether it is spontaneous or therapeutic. Following acute myocardial infarction, therapeutic coronary artery recanalization by thrombolytic therapy or percutaneous coronary intervention is used to minimize infarct size. However, the reoxygenation of ischemic heart may lead to irreversible loss of myocardial function.[1] Many signaling molecules are postulated to modulate I/R injury, including reactive oxygen species and nitric oxide (NO). Many studies have demonstrated that NO plays a fundamental biological role in protecting the heart against I/R injury,[2] and most studies examining the role of NO in modulating the severity of I/R injury in non-preconditioned myocardium have concluded that NO has a protective effect.[3,4] Moreover, both L-arginine (the substrate for NO biosynthesis) and NO donors have been shown to ameliorate I/R injury in vivo.[3,4]

Aliskiren is the first orally effective direct renin inhibitor to be used clinically, and it may offer benefits in cardiovascular disease over and above those achieved with conventional renin-angiotensin system (RAS) blocking strategies.[5] The RAS plays a central role in regulation and maintenance of blood pressure. In hypertension and heart failure, RAS blockade is

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traditionally achieved through inhibition of production of angiotensin II (Ang II) using angiotensin converting-enzyme inhibitors, or inhibition of the effect of Ang II on its principal receptor using AT-1 receptor blockers. Both of these strategies increase plasma renin activity (PRA) and plasma renin concentration. Several studies have shown a direct link between level of PRA and risk of cardiovascular disease[6,7]; in particular, PRA exhibits a strong association with occurrence of cardiovascular events, especially myocardial infarction, among hypertensive patients.[7]

Recent evidence suggests that aliskiren improves NO bioavailability and hence limits atherosclerosis in animal models.[8,9] Moreover, aliskiren can upregulate vascular endothelial NO synthase (eNOS) expression and phosphorylation, as well as phosphorylation of the upstream protein kinase B (Akt), in Watanabe heritable hyperlipidemic rabbits.[8] Phosphatidylinositol 3-kinase (PI3K) and its downstream target Akt are a conserved family of signal transduction enzymes which are involved in regulating cellular activation, inflammatory responses, and apoptosis.[10] Activation of PI3K/Akt-dependent signaling has been shown to prevent cardiomyocyte apoptosis and protect the myocardium from I/R injury.[10,11]

We hypothesized that direct renin inhibition with aliskiren will protect against myocardial I/R injury in SHR, through activation of PI3K/Akt signaling leading to eNOS phosphorylation and activation with resultant increased NO biosynthesis.

## Methods

### Animals

Male, spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were purchased from Vital River Laboratories in Beijing, China. The animals were fed a laboratory diet with water and kept under constant environmental conditions, with 12-hour light/dark cycles. Animal experiments were performed in accordance with the guidelines for the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals. This study was approved by the Committee on Animal Care of Nanjing Medical University (approval no. NJMU-ERLAUA-20100112).

At the age of 8 to 10 weeks, WKY and SHR were assigned to sham operation or coronary artery ligation followed by reperfusion (see below); and in the latter case were also treated with vehicle (saline) or aliskiren (Novartis, Switzerland); aliskiren was administered at 2 different doses in SHR: 30 mg $kg^{-1}$ $day^{-1}$ (alis-L) or 60 mg $kg^{-1}$ $day^{-1}$ (alis-H). Aliskiren or vehicle were administered in equal volume, by gavage, once daily for 4 weeks. There were 15 to 20 rats in each WKY and SHR group studied (sham, vehicle, alis-L, alis-H). Systolic blood pressure (SBP) was measured by tail-cuff plethysmography (MedLab-U/4C501H, Medease Company), in conscious rats before initiation of treatment and at the end of the 4-week treatment period.

### In Vivo Myocardial I/R Model

Rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally [i.p.]), given atropine (0.1 mg/kg by subcutaneous injection) to reduce airway secretions, and artificially ventilated. Intraoperative monitoring of adequate anesthesia was done by toe pinching. Myocardial ischemia was induced by exposing the heart through a left thoracic incision and placing a 6/0 silk suture with a slipknot around the left anterior descending coronary artery. After 30 minutes ischemia, the slipknot was released and the myocardium reperfused for either 6 hours (for measurement of cardiac function) or 24 hours (for determination of infarct size, western blotting and biochemical assays). Sham-treated SHR underwent left thoracotomy only. The chest wall was closed 30 minutes after thoracotomy in the case of sham-treated SHR or immediately after slipknot release in the case of SHR undergoing I/R, and the animals allowed to recover. After 6 hours reperfusion, rats were anesthetized by inhalation of isoflurane (0.5% to 1.5%, Isoflurane Vaporizer, Matrx VIP3000) and anchored to a positionable platform in a supine position. Cardiac function was evaluated by echocardiography using a GE Vivid 7 (GE, USA) equipped with a 14-MHz phase array linear transducer S12; all measurements were made by one observer who was blinded to the treatments administered. All measurements were averaged over 5 consecutive cardiac cycles. Blood and cardiac tissue samples were obtained after 24 hours of reperfusion.

### Langendorff Perfusion Assay

Hearts were extracted from male Sprague–Dawley rats (weighing 220 to 250 g) after i.p. injection of heparin (1000 IU) followed by i.p. injection after 5 minutes of pentobarbital sodium (60 mg/kg). After removal, the ascending aorta was cannulated and the heart subjected to retrograde perfusion with modified Krebs-Henseleit buffer (NaCl 115 mmol/L, KCl 4.6 mmol/L, $KH_2PO_4$ 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 1.8 mmol/L, $NaHCO_3$ 25 mmol/L, and glucose 11 mmol/L) equilibrated with 95% $O_2$/5% $CO_2$ to pH 7.3 to 7.4. Perfusion was initiated in a non-recirculating Langendorff heart perfusion apparatus, reaching a basal perfusion pressure of ≈70 mm Hg. Both the perfusion solution and the heart were maintained at 37°C. Following a 20-minute stabilization period, hearts were subjected to 30 minutes perfusion with the NOS inhibitor $N^G$-nitro-L-arginine methylester (L-NAME 100 μmol/L; Sigma), the PI3K inhibitor wortmannin (100 nmol/L; Merck), aliskiren (200 ng/mL), aliskiren (200 ng/mL) plus L-NAME (100 μmol/L), aliskiren

(200 ng/mL) plus wortmannin (100 nmol/L), or Krebs-Henseleit buffer only (Figure 1). At the end of this time, I/R injury was produced by subjecting the hearts to 30 minutes normothermic ischemia, followed by reperfusion for 60 minutes with L-NAME (100 μmol/L), wortmanin (100 nmol/L), aliskiren (200 ng/mL), aliskiren (200 ng/mL) plus L-NAME (100 μmol/L), aliskiren (200 ng/mL) plus wortmannin (100 nmol/L), or Krebs-Henseleit buffer, respectively. A fluid-filled balloon was inserted into the left ventricle via the mitral valve and inflated to yield a left ventricular end-diastolic pressure of 4 to 10 mm Hg. The balloon was attached via polyethylene tubing (PE50) to a pressure transducer connected to a Heart Performing Analyzer (BL420, TME Technology), and left ventricular pressure was continuously measured. Positive and negative peak of the first derivative of left ventricular pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$, respectively) were obtained from the left ventricular pressure curve.

Thereafter, hearts were snap frozen before being cut into 2 mm-thick slices using a sharp blade. The slices were then incubated for a period of 15 minutes at room temperature in a solution of 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4).[12] Infarct size was expressed as the ratio of infarcted zone (lacking staining with tetrazolium) to the whole section area, as determined by computerized planimetry.

## Measurement of Plasma Creatine Kinase, Myeloperoxidase and Malondialdehyde

Blood samples were obtained from the right carotid artery, mixed with disodium EDTA (15 mg/mL) and centrifuged at 1000*g* for 15 minutes at 4°C. Creatine kinase (CK), myeloperoxidase (MPO), and malondialdehyde (MDA) were quantified in the supernatants thus obtained. CK, MDA, and MPO levels were determined spectrophotometrically at 340 nm (CK), 532 nm (MDA), and 460 nm (MPO), according to the manufacturer's instructions (kits from Genesys). Each measurement was performed in duplicate.

## Measurement of Cardiac Cyclic Guanosine-3′, 5′-Monophosphate

Cardiac tissue samples were homogenized in lysis buffer (Tris-HCl 50 mmol/L, NaCl 250 mmol/L, EDTA 10 mmol/L, NaF 4 mmol/L, phenylmethylsulfonyl fluoride 1.0 mmol/L, leupeptin 10 μg/mL, NP-40 0.5%, Triton X-100 1%). Homogenates were placed on ice for 40 minutes, and then centrifuged at 500*g* for 15 minutes at 4°C. The supernatants, comprising cardiac lysates, were used for measurement of cyclic guanosine-3′, 5′-monophosphate (cGMP) using a cGMP ELISA kit (R&D Systems).

## Myocardial Matrix Metalloproteinase Activity Determination

Myocardial levels of matrix metalloproteinase (MMP)-2 and MMP-9, as well as that of tissue inhibitor of metalloproteinases (TIMP)-1, were measured in cardiac tissue homogenates using MMP-2, MMP-9, and TIMP-1 ELISA kits, respectively (Cusabio Biotech Co Ltd), according to the manufacturer's instructions. Each measurement was performed in duplicate.

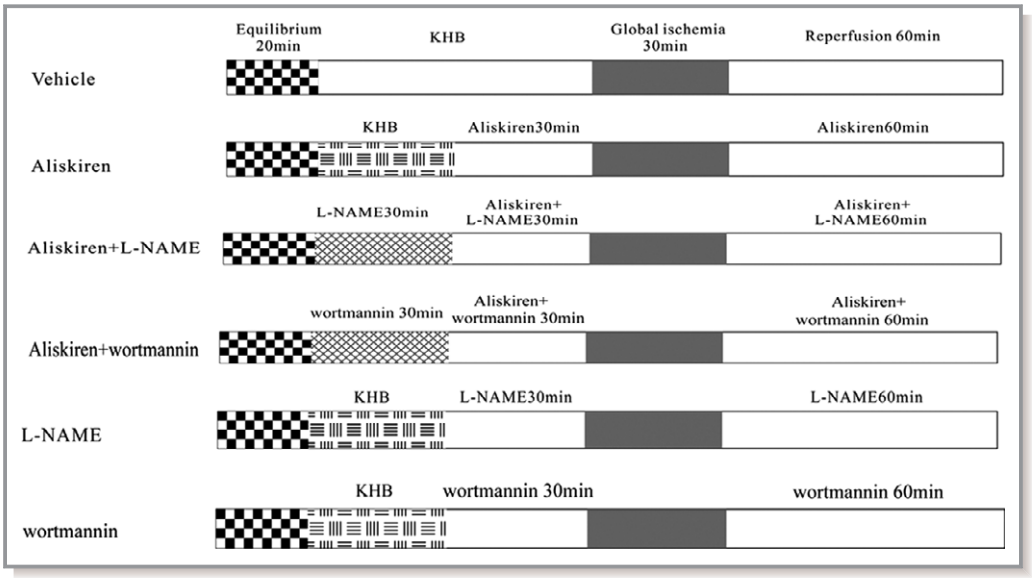


**Figure 1.** Schematic illustration of different experimental treatments in Langendorff perfusion experiments. L-NAME indicates $N^G$-nitro-L-arginine methylester; KHB, Krebs-Henseleit buffer.

## Measurement of Superoxide Anion Generation

Cardiac tissue samples were homogenized and centrifuged as described above for western blotting. The supernatant was used for measurement of superoxide anion production by lucigenin-enhanced chemiluminescence. The light reaction between superoxide and lucigenin (5 μmol/L) was detected in a 96-well microplate luminometer (GloMax, Promega) during incubation in a HEPES-modified Krebs buffer (pH 7.4). Additionally, hearts removed from SHR were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek), then cut into 5 μm-thick sections and placed on glass slides. Dihydroethidium (DHE, 2 μmol/L) was applied to each tissue section and the slides incubated in a light-protected humidified chamber at 37°C for 15 minutes. The slides were then examined by fluorescence microscopy (Olympus).

## Statistical Analysis

All data are expressed as mean±SD. They were analyzed by paired *t* tests or one-way ANOVA followed by post hoc tests. Nonparametric tests were used for data with heterogeneity of variance (Stata13.0). A value of $P<0.05$ (2-tailed) was considered statistically significant.

# Results

## Aliskiren at High But Not Low Dose Prevents the Age-related Increase in Blood Pressure in Spontaneously Hypertensive Rats

Table shows systolic blood pressure and body weight in each of the 7 treatment groups, before and at the end of 4 weeks of treatment. As expected, systolic blood pressure rose with advancing age in SHR, while it did not change in WKY rats. In the SHR-sham and SHR-vehicle groups, systolic blood pressure was higher at the end of treatment than the beginning. In SHR, high-dose aliskiren (60 mg $kg^{-1}$ $day^{-1}$; Alis-H) treatment prevented the age-related increase in SBP to a much greater extent than did low-dose aliskiren (30 mg $kg^{-1}$ $day^{-1}$; Alis-L), and indeed Alis-H decreased SBP significantly compared with SHR-vehicle whereas Alis-L did not. Body weight increased over the course of treatment in all 7 groups, and this was not affected by aliskiren treatment.

## Aliskiren Reduces Infarct Size and Protects Cardiac Function After Myocardial I/R

The effect of aliskiren on infarct size/area at risk (AR) ratio in SHR and WKY rats was determined by the Evans-blue/triphenyltetrazolium chloride dye method. Despite the aforementioned differences in effect on blood pressure between the SHR-Alis-H and the SHR-Alis-L groups, a similar reduction was observed in infarct size/AR ratio in both of these groups as compared with the SHR-vehicle-treated group (Figure 2A); similarly, Alis-L reduced the infarct size/AR and infarct size/left ventricular area (LV) ratios in WKY, despite no discernible effect on blood pressure (Figure 2B).

Cardiac function in aliskiren-treated SHR and WKY rats was measured by echocardiography, and typical M-mode traces for each of the 7 groups are shown in Figure 2C. Thirty minutes of ischemia followed by 6 hours of reperfusion resulted in a decrease in ejection fraction (EF) and in fractional shortening (FS) in vehicle-treated compared with sham-operated rats, both SHR and WKY. Aliskiren treatment improved myocardial function in both SHR and WKY rats (Figure 2D and 2E), even at the lower dose; and when specifically examined in SHR, the 2 doses of aliskiren improved EF and FS to equal degrees.

**Table.** Systolic Blood Pressure and Body Weight Before and After 4 Weeks of Treatment With Aliskiren in 7 Study Groups (n=6 to 8)

| | Systolic blood Pressure (mm Hg) | | Body Weight (g) | |
|---|---|---|---|---|
| | Before | After | Before | After |
| WKY-sham | 108.76±5.80 | 119.20±8.34 | 224.50±7.20 | 243.45±9.56 |
| WKY-vehicle | 101.29±9.60 | 102.10±7.66 | 223.33±4.84 | 243.83±10.83 |
| WKY-Alis-L | 115.30±7.68 | 120.56±8.20 | 230.65±8.52 | 270.64±11.63 |
| SHR-sham | 162.55±13.94 | 197.03±13.00* | 227.25±7.17 | 261.38±10.56 |
| SHR-vehicle | 168.60±13.49 | 187.85±9.63† | 227.25±9.17 | 276.00±18.03 |
| SHR-Alis-L | 164.92±5.29 | 176.84±17.04 | 231.38±9.68 | 259.88±9.16 |
| SHR-Alis-H | 159.92±7.70 | 158.88±7.70‡ | 229.63±7.96 | 266.75±8.92 |

Alis-H indicates high-dose aliskiren (60 mg $kg^{-1}$ $day^{-1}$); Alis-L, low-dose aliskiren (30 mg $kg^{-1}$ $day^{-1}$); ANOVA, analysis of variance; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

*$P<0.001$ vs SHR-sham group before treatment with paired *t* tests.

†$P<0.01$ vs SHR-vehicle group before treatment with paired *t* tests.

‡$P<0.01$ vs SHR-vehicle group after treatment analyzed with one-way ANOVA followed by post hoc tests.

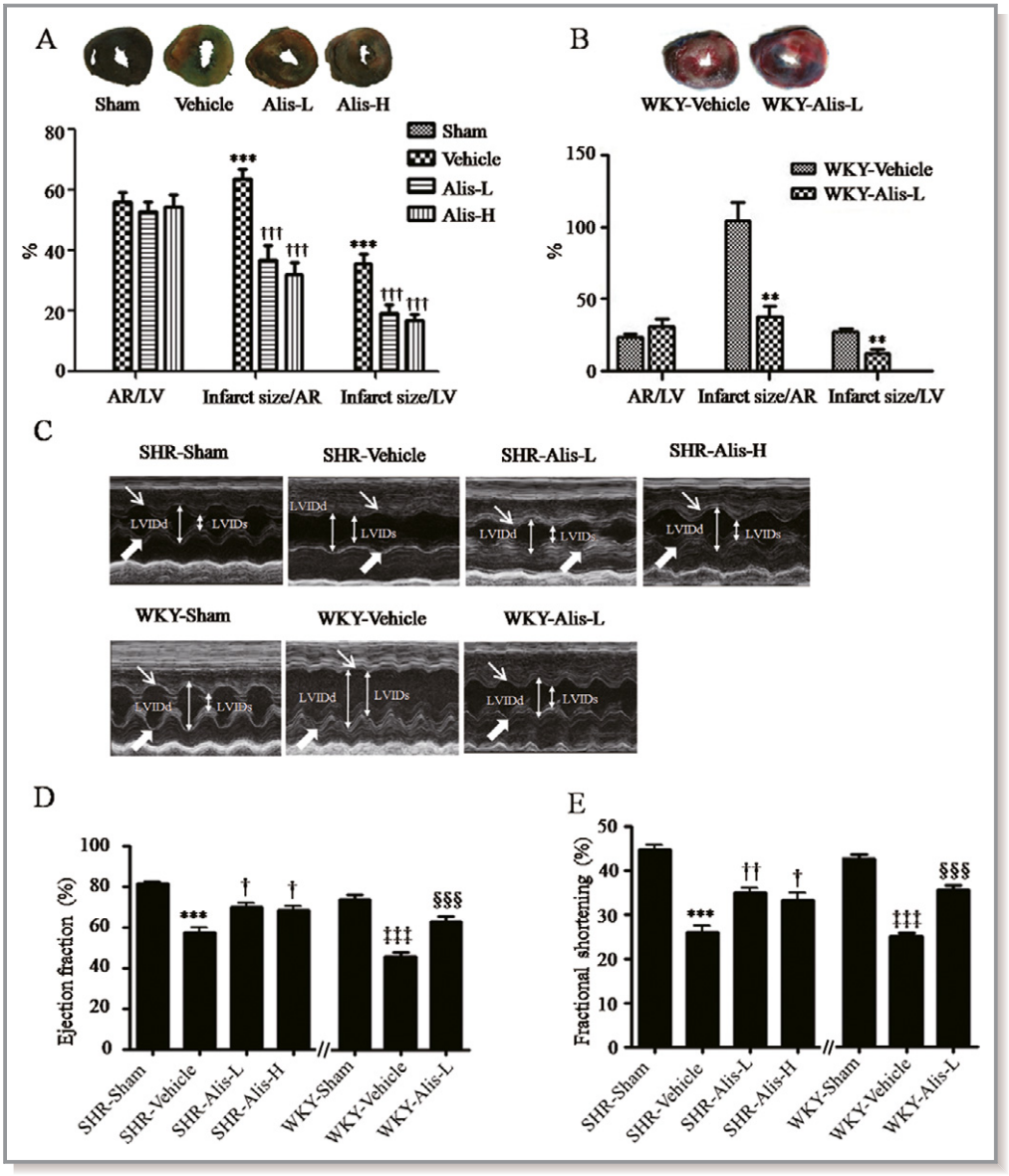


**Figure 2.** Effect of aliskiren on myocardial I/R injury in SHR and WKY rats. Aliskiren reduced infarct size post-myocardial I/R in both (A) SHR and (B) WKY. AR, area at risk; LV, left ventricular area (n=5 to 6). C, Typical M-mode traces on echocardiography indicating improved (→, endocardium in lateral wall; ⇒, endocardium in septal wall; ↔, left ventricular internal diameter). LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole; (D) ejection fraction and (E) fractional shortening in the left ventricles of SHR and WKY rats post-myocardial I/R (n=6 to 7). Sham, sham operation; Vehicle, I/R only; Alis-L, I/R and co-treatment with aliskiren 30 mg $kg^{-1}$ $day^{-1}$; Alis-H, I/R and co-treatment with aliskiren 60 mg $kg^{-1}$ $day^{-1}$. For A, D, and E, ***$P$<0.001 vs SHR-Sham; $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 vs SHR-Vehicle; $^{\ddagger\ddagger\ddagger}P$<0.001 vs WKY-Sham; $^{\S\S\S}P$<0.001 vs WKY-Vehicle. For B, **$P$<0.01 vs WKY-Vehicle. Alis-H indicates high-dose aliskiren; Alis-L, low-dose aliskiren; AR, area at risk; I/R, ischemia/reperfusion; LV, left ventricle; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

## Aliskiren Decreases Myocardial Injury and Systemic Oxidative Stress, and Favorably Modulates Cardiac Matrix Metalloproteinases, After Myocardial I/R in SHR

We measured plasma CK, because this is an important indicator of extent of myocardial I/R injury. Thirty minutes of ischemia followed by 24 hours of reperfusion increased CK, and this increase was entirely abrogated by treatment with either Alis-L or Alis-H (Figure 3A).

Tissue recruitment of neutrophils and their activation results in release of MPO into the circulation. Compared with the sham-operated group, plasma MPO activity was increased by myocardial I/R, as expected. This increase was entirely

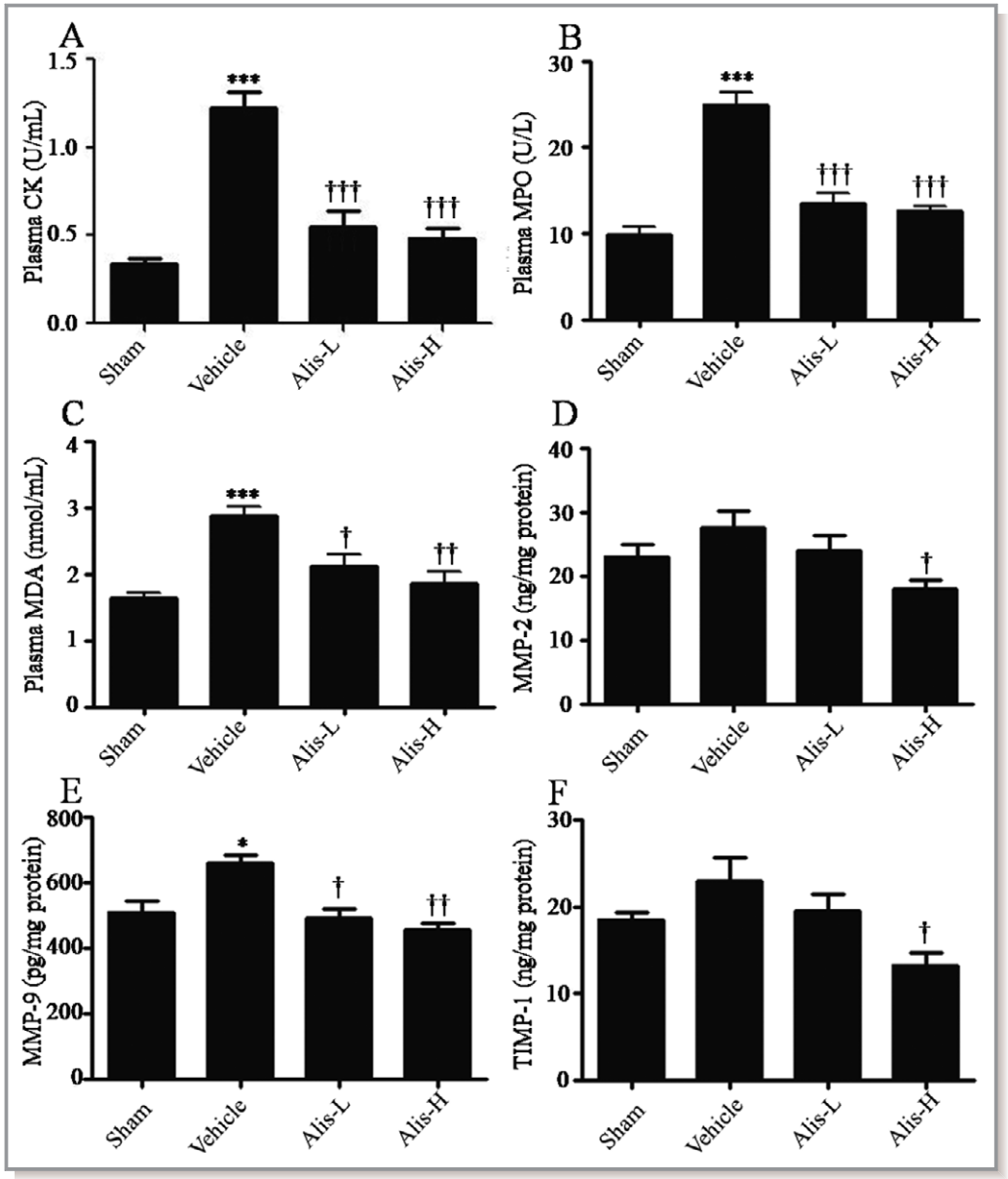


**Figure 3.** Effect of aliskiren on indices of oxidative stress, leukocyte activity, myocardial injury and matrix metalloproteinase (MMP) activity, post myocardial I/R in SHR. Aliskiren decreased plasma levels of (A) CK, (B) MPO, and (C) MDA (n=6), as well as myocardial expression of (D) MMP-2, (E) MMP-9, and (F) tissue inhibitor of metalloproteinase (TIMP)-1 (n=4 to 6). Sham, sham operation; Vehicle, I/R only; Alis-L, I/R and co-treatment with aliskiren 30 mg $kg^{-1}$ $day^{-1}$; Alis-H, I/R and co-treatment with aliskiren 60 mg $kg^{-1}$ $day^{-1}$. *$P$<0.05, ***$P$<0.001 vs Sham. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 vs Vehicle. Alis-H indicates high-dose aliskiren; Alis-L, low-dose aliskiren; CK, creatine kinase; I/R, ischemia/reperfusion; MDA, malondialdehyde; MPO, myeloperoxidase; SHR, spontaneously hypertensive rats.

prevented by both Alis-L and Alis-H (Figure 3B). Plasma MDA is a sensitive indicator of whole body oxidant stress. We found an increase in plasma MDA in the vehicle-treated group, as a result of myocardial I/R, as compared with the sham-operated group; and this increase was abolished by both Alis-L and Alis-H (Figure 3C).

Elevated activities of matrix metalloproteinases (MMPs) following acute myocardial infarction have been shown to mediate ventricular remodeling.[13,14] Compared with sham-operated SHR, myocardial MMP-9 expression increased after I/R and this rise was abolished by both doses of aliskiren (Figure 3E). Both myocardial MMP-2 and tissue inhibitor of metalloproeinases (TIMP)-1 expression were decreased by high-dose aliskiren, whereas the effect of low-dose aliskiren on both of these was nonsignificant (Figure 3D and 3F).

## The Cardioprotective Effect of Aliskiren Following Myocardial I/R is Mediated Through PI3K/Akt/NO Signaling

The PI3K-Akt-eNOS pathway has been reported to play a protective role in myocardial I/R.[10] Although aliskiren increases levels of vascular eNOS, phospho-eNOS and phospho-Akt,[15] it is not clear whether it does the same following cardiac I/R, and whether any resultant increase in NO biosynthesis may contribute to its cardioprotective effects. We found that 30 minutes of ischemia followed by 24 hours of reperfusion decreased expression of the p85α subunit of PI3K, phospho-Akt and phospho-eNOS (with no change in total Akt or eNOS), as compared with sham-operated animals; both Alis-L and Alis-H abrogated all of these changes (Figure 4A, 4C, and 4D). By contrast, I/R increased iNOS expression, and this increase was abolished by both Alis-L and Alis-H (Figure 4B).

To elucidate whether these effects on PI3K/Akt/NO signaling might contribute to the observed cardioprotective effect of aliskiren following I/R, we used a Langendorff perfusion assay. Hearts were perfused with the NOS inhibitor L-NAME, the PI3K inhibitor wortmannin, or corresponding vehicle, with or without aliskiren, then subjected to ischemia for 30 minutes and reperfusion for 60 minutes. L-NAME or wortmannin alone had no effect on infarct size, coronary flow,

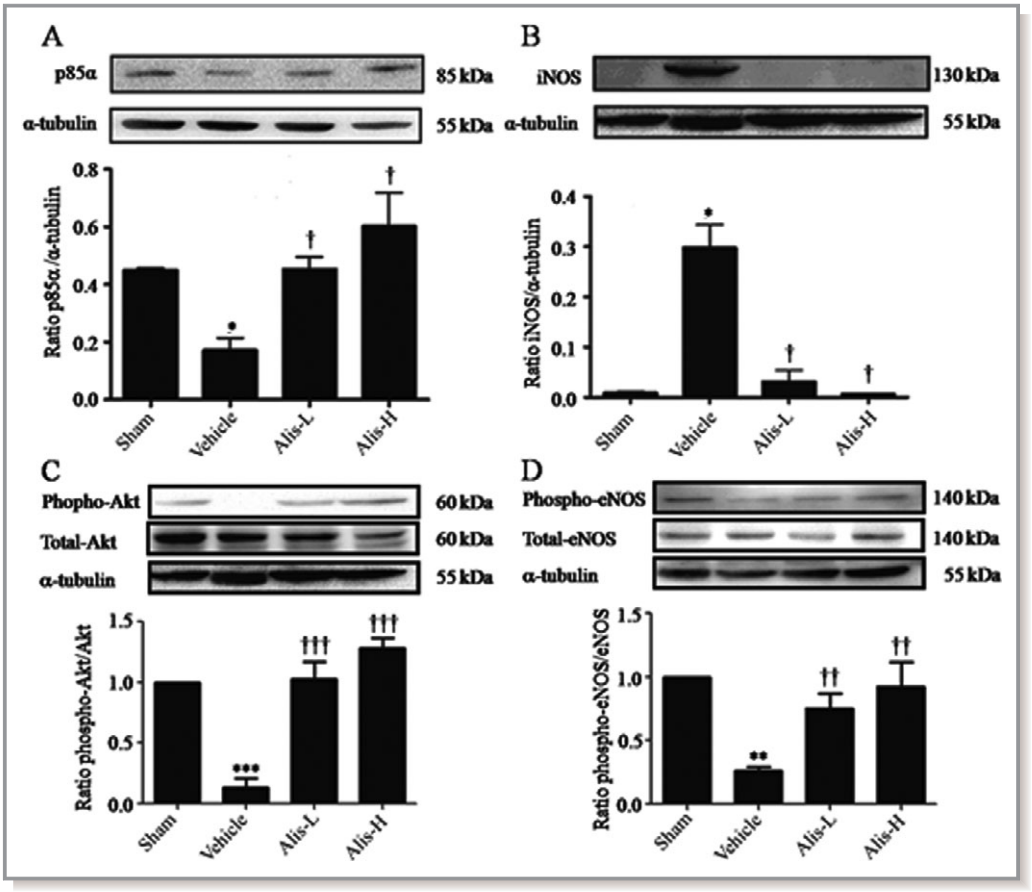


**Figure 4.** Effect of aliskiren on myocardial PI3K-Akt-eNOS signaling and iNOS expression, post I/R in SHR. Shown are myocardial western blot analyses of (A) the p85α subunit of PI3K (n=4), (B) iNOS (n=3), (C) phospho-Akt (n=5), and (D) phospho-eNOS (n=5). PI3K, phospho-Akt and phospho-eNOS decreased (with no corresponding change in total Akt or total eNOS expression), and iNOS increased, in response to I/R, and all of these changes were abrogated by aliskiren. p85α and iNOS are expressed as the densitometric ratio to α-tubulin, phospho-Akt as the densitometric ratio to total Akt, and phospho-eNOS as the densitometric ratio to total eNOS. Sham, sham operation; Vehicle, I/R only; Alis-L, I/R and co-treatment with aliskiren 30 mg $kg^{-1}$ $day^{-1}$; Alis-H, I/R and co-treatment with aliskiren 60 mg $kg^{-1}$ $day^{-1}$. *$P$<0.05, **$P$<0.01, ***$P$<0.001 vs Sham. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01, $^{\dagger\dagger\dagger}P$<0.001 vs Vehicle. Alis-H indicates high-dose aliskiren; Alis-L, low-dose aliskiren; eNOS, endothelial nitric oxide synthase; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; SHR, spontaneously hypertensive rats.

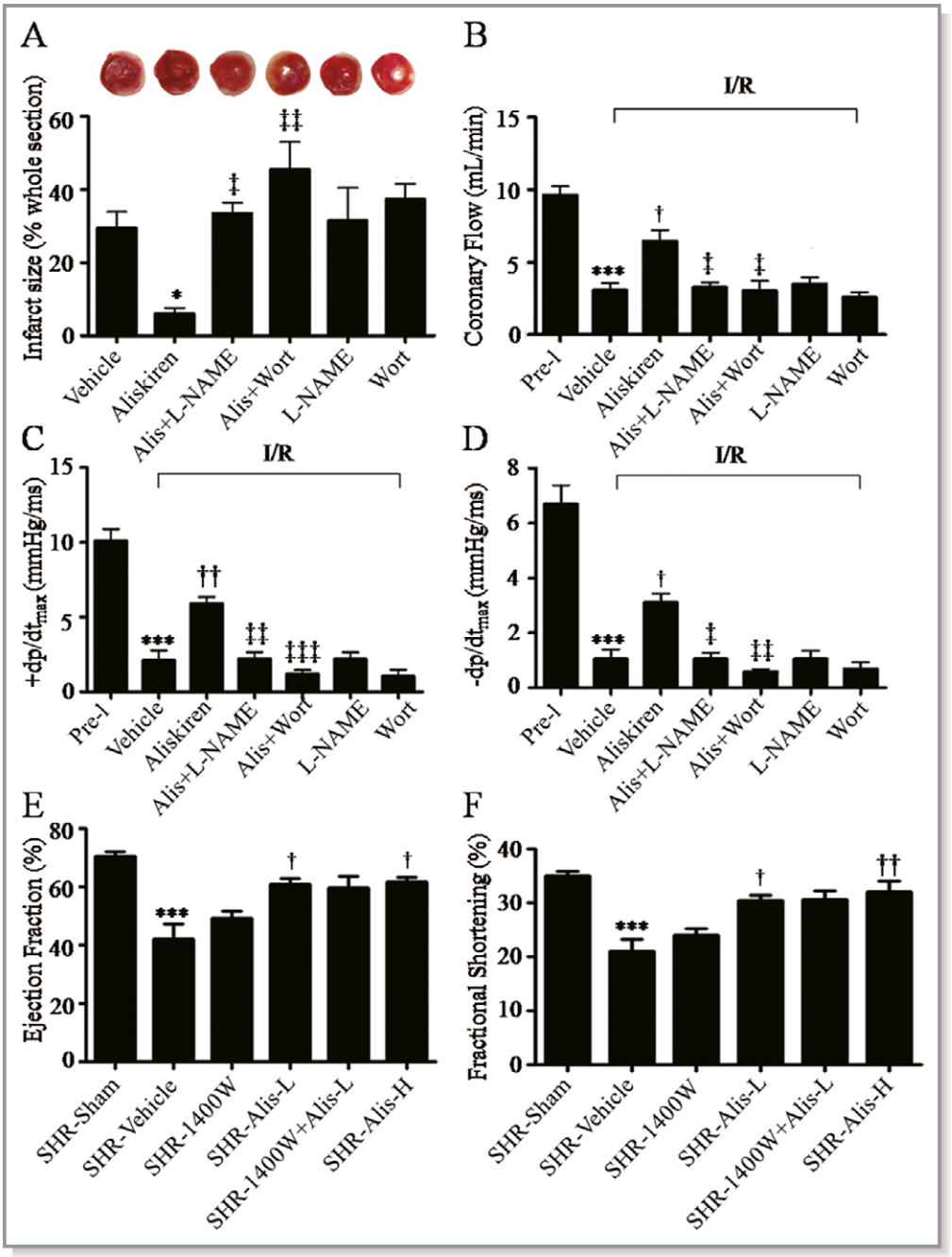


**Figure 5.** The role of PI3K-Akt-eNOS signaling and of iNOS in mediating the protective effect of aliskiren (Alis) on cardiac structure and function following I/R. In a Langendorff perfusion model of I/R, aliskiren reduced infarct size (A, n=5 to 9), increased coronary flow (B, n=6 to 13), and increased both $+dp/dt_{max}$ (C, n=3 to 9), and $-dp/dt_{max}$ (D, n=3 to 9); all of these effects of aliskiren were abrogated by NOS inhibition with L-NAME or by PI3K inhibition with wortmannin, while L-NAME and wortmannin alone had no effect on these parameters in the absence of aliskiren. Pre-I, pre-ischemia. In SHR in vivo, while aliskiren reversed the impairment in cardiac function caused by I/R (Vehicle), as assessed by ejection fraction (E, n=6) and fractional shortening (F, n=6), specific iNOS inhibition using 1400W either alone or in combination with aliskiren had no effect. Sham, sham operation; Vehicle, I/R only; Alis-L, I/R and co-treatment with aliskiren 30 mg $kg^{-1}$ $day^{-1}$; Alis-H, I/R and co-treatment with aliskiren 60 mg $kg^{-1}$ $day^{-1}$. For A, *$P$<0.05 vs vehicle. For B through D, ***$P$<0.001 vs pre-I. For E and F, ***$P$<0.001 vs SHR-Sham. For B through F, $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 vs vehicle. For B through D, $^{\ddagger}P$<0.05, $^{\ddagger\ddagger}P$<0.01, $^{\ddagger\ddagger\ddagger}P$<0.001 vs aliskiren. Alis-H indicates high-dose aliskiren; Alis-L, low-dose aliskiren; eNOS, endothelial nitric oxide synthase; I/R, ischemia/reperfusion; iNOS, inducible nitric oxide synthase; L-NAME, $N^{G}$-nitro-L-arginine methylester; PI3K, phosphatidylinositol 3-kinase; SHR, spontaneously hypertensive rats.

$+dp/dt_{max}$, or $-dp/dt_{max}$; by contrast, aliskiren markedly reduced infarct size, increased coronary flow and augmented both $+dp/dt_{max}$ or $-dp/dt_{max}$. All of these effects of aliskiren were abolished by combining it with L-NAME or with wortmannin (Figure 5A through 5D).

To further explore the possible role of iNOS during myocardial I/R, we employed a selective inhibitor of iNOS, 1400W, which was injected intraperitoneally 30 minutes prior to ischemia followed by 6 or 24 hours of reperfusion. SHR subjected to I/R and treated with 1400W exhibited no improvement in myocardial function compared with vehicle-treated animals, and 1400W had no discernible effect on the improvement in function elicited by aliskiren at either dose (Figure 5E and 5F), despite its marked effect on both systemic oxidant stress and myocardial superoxide anion generation (see below).

## Aliskiren Increases Myocardial cGMP Following Myocardial I/R

NO exerts the majority of its biological actions in mammalian tissues by binding to soluble guanylyl cyclase in target tissues, thereby catalyzing cGMP formation and subsequent activation of cGMP-dependent protein kinase (PKG). NO/cGMP/PKG signaling promotes vascular smooth muscle relaxation and platelet disaggregation.[16] cGMP is also a sensitive index of biological activity of NO. We therefore measured the effect of I/R, as well as that of concomitant aliskiren treatment, on myocardial cGMP levels in SHR. We found that myocardial cGMP was decreased by I/R, and that this decrease was abrogated by both Alis-L and Alis-H (Figure 6A).

## Aliskiren Abolishes the Increase in Cardiac Superoxide Anion Generation in Response to Myocardial I/R

Reactive oxygen species are believed to play a critical role in I/R injury; furthermore, as stated above, we found that myocardial I/R increases systemic markers of oxidative stress, and that these are abrogated by aliskiren. In cardiac tissue, I/R increased superoxide anion production, and this increase was abolished by treatment with either Alis-L or Alis-H (Figure 6B and 6C). Similarly, pretreatment of rats with 1400W prior to myocardial ischemia reduced the increase of both plasma MDA and cardiac superoxide anion production, as compared with vehicle-treated animals (Figure 6D and 6E); furthermore, it did so to a similar degree as aliskiren, despite its lack of effect (unlike aliskiren) on cardiac function.

All together, we here report that renin inhibition with aliskiren attenuates myocardial infarct size and protects cardiac function following acute coronary occlusion and subsequent reperfusion, and that these effects of aliskiren can be attributed to activation of NO signaling through stimulation of the PI3K/Akt/eNOS pathway.

# Discussion

The present study demonstrates that direct renin inhibition with aliskiren confers cardioprotection in response to cardiac I/R in SHR. We have shown that aliskiren prevents much of the myocardial damage of I/R; that these beneficial actions are associated with a decrease in oxidative stress, a reduction in leukocyte accumulation and an increase in NO bioavailability as well as an augmentation of PI3K/Akt/eNOS signaling; and, in a Langendorff model, that the beneficial effects of aliskiren on cardiac damage and function following I/R are entirely abolished by inhibition of NO biosynthesis with L-NAME or of upstream PI3K signaling with wortmannin, but not by selective inhibition of iNOS with 1400W.

In a previous study, Wood et al showed that aliskiren 30 mg $kg^{-1}$ $day^{-1}$ does not change mean arterial pressure in SHR.[17] In our study, we showed a slight (but nonsignificant) decrease in SBP with this dose of aliskiren whereas, at higher dose (60 mg $kg^{-1}$ $day^{-1}$), it effectively and significantly reduced SBP. That the lower dose of aliskiren affects SBP less may be due to its relative species specificity. Human renin inhibitors are much less potent toward rat and mouse renin.[18] Both angiotensin-converting enzyme inhibitors and angiotensin receptor blockers stimulate renal renin production by disrupting the inhibitory feedback of Ang II on juxtaglomerular cells; the resulting increase in renin eventually restores Ang II levels.[19] Aliskiren is the only orally active direct renin inhibitor currently available for clinical use. It blocks the generation of Ang I from angiotensinogen, the initial and rate-limiting step of the RAS, by inhibiting the active enzymatic site of renin. In our study, the cardioprotective actions of aliskiren were evident and indeed equal at both the 30 and 60 mg $kg^{-1}$ $day^{-1}$ doses, despite differential effects of the 2 doses on blood pressure, suggesting that the cardioprotective effects observed of direct renin inhibition were not simply consequent on blood pressure lowering.

Myocardial I/R injury gives rise to left ventricular dysfunction and heart failure. The present study demonstrates that cardiac function is markedly improved, and infarct size greatly reduced, in aliskiren-treated SHR after I/R. Aliskiren has been previously suggested to exert a protective role in cardiovascular disease. It has been found to augment both basal and acetylcholine-stimulated NO production, and to improve endothelium-dependent vasorelaxation, in thoracic aortic segments from Watanabe heritable hyperlipidemic rabbits.[8] Several studies also show that aliskiren reduces atherosclerosis in animal models, an effect at least partially independent of changes in blood pressure.[9,20] Aliskiren also prevents

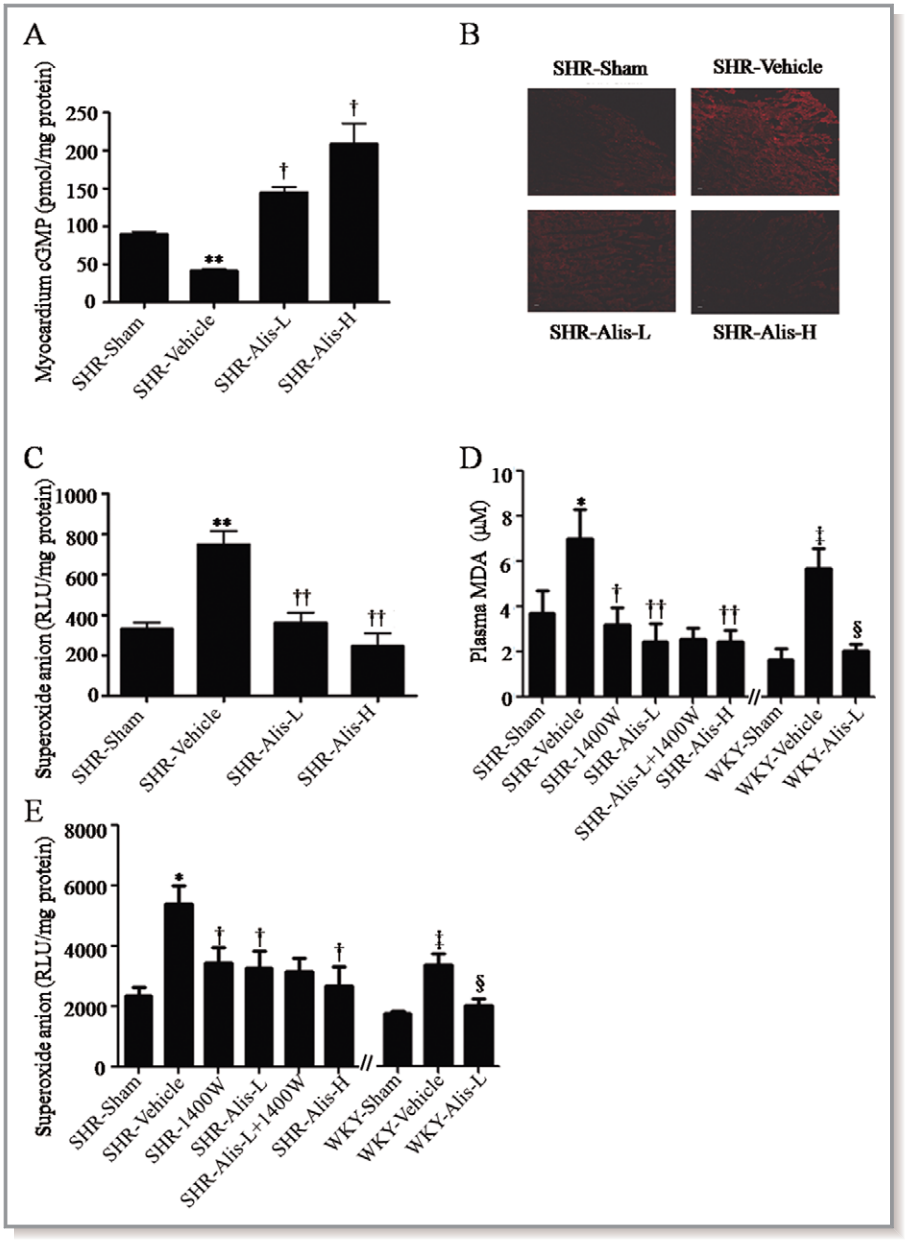


**Figure 6.** The effect of aliskiren on both systemic oxidative stress and myocardial superoxide anion generation, in SHR following I/R. A, Myocardial cGMP, an index of bioactive NO, was reduced by I/R in SHR, and this effect was abrogated by aliskiren (n=4 to 6). B, Representative photomicrographs of dihydroethidium-stained cardiac sections showing an increase in staining following I/R in SHR, which is abrogated by aliskiren. C, Lucigenin chemiluminescence confirmed that I/R increases myocardial superoxide anion generation in SHR, and that this increase is abrogated by aliskiren (n=3). D, Systemic oxidative stress, as assessed by plasma MDA, is increased by I/R in both SHR and WKY, and this increase is abrogated by aliskiren. Also shown is the effect of 1400W in SHR, demonstrating a similar abrogation in the increase in plasma MDA, with no added effect over and above aliskiren alone (n=4 to 6). E, Myocardial superoxide anion generation, as assessed by lucigenin chemiluminescence, is increased by I/R in both SHR and WKY, and this increase is abrogated by aliskiren. Also shown is the effect of 1400W in SHR, demonstrating a similar abrogation in the increase in superoxide anion, with no added effect over and above aliskiren alone (n=4 to 6). Sham, sham operation; Vehicle, I/R only; Alis-L, I/R and co-treatment with aliskiren 30 mg $kg^{-1}$ $day^{-1}$; Alis-H, I/R and co-treatment with aliskiren 60 mg $kg^{-1}$ $day^{-1}$. *$P$<0.05, **$P$<0.01 vs SHR-sham. $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 vs SHR-vehicle. $^{\ddagger}P$<0.05 vs WKY-Sham. $^{\S}P$<0.05 vs WKY-Vehicle. Alis-H indicates high-dose aliskiren; Alis-L, low-dose aliskiren; cGMP, cyclic guanosine-3′, 5′-monophosphate; I/R, ischemia/reperfusion; MDA, malondialdehyde; NO, nitric oxide; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto rats.

cardiovascular complications and pancreatic injury in obese type 2 diabetic mice,[21] improves coronary endothelial function and decreases cardiac hypertrophy in SHR, and reduces PRA and angiotensin levels in these SHR after 1 week of treatment both in plasma and tissue.[22]

There is evidence that activation of Akt may exert antiatherogenic effects by phosphorylating eNOS on Ser1177.[23] PI3K, an upstream kinase of Akt, can activate this enzyme by phosphorylating it on Ser473. We investigated the role of the PI3K-Akt-eNOS pathway in the observed aliskiren-induced changes in hearts of SHR after I/R. The cardiac expression of PI3K, as assessed from its p85α subunit, was increased in aliskiren-treated groups. We also found that aliskiren restored the attenuation in phospho-/total Akt, and phospho-/total eNOS ratios in myocardium from SHR following I/R, with no change in total Akt or eNOS expression. Additionally, aliskiren treatment abrogated the decrease in myocardial cGMP level (an index of bioactive NO) after I/R.

In order to clarify whether the protective effect of aliskiren on myocardial damage and function following I/R injury depends on increased eNOS and NO signaling, through activation of the PI3K-Akt-eNOS pathway, we examined the effect of co-administration of the NOS inhibitor L-NAME or of the PI3K inhibitor wortmannin, in the isolated working heart using a Langendorff perfusion assay. We found that, indeed, both L-NAME and wortmannin abolished the effects of aliskiren on myocardial I/R injury, both in terms of structure (infarct size) and function (coronary flow, $+dp/dt_{max}$, $-dp/dt_{max}$). In a parallel study, we treated rats with 1400W, a selective inhibitor of iNOS, 30 minutes before I/R, to examine the role of iNOS in this phenomenon. We found that iNOS inhibition did not affect myocardial function following I/R, either in the absence or presence of aliskiren, suggesting that iNOS-derived NO does not play an important role in modulating cardiac function in this situation.

NO scavenges superoxide anion and protects against impairment of vascular endothelial function.[24,25] There is accumulating evidence that oxidative stress induces I/R injury, and reactive oxygen species and free radicals have long been recognized as major mediators of I/R injury.[26] Therefore, we examined the potential contribution of oxidative stress to the protective effect of aliskiren against I/R injury. Previous studies have demonstrated that activated neutrophils are a major source of oxygen-derived free radicals during reperfusion after prolonged myocardial ischemia in vivo,[27] and MPO is a marker of activated neutrophils that is released into the bloodstream.[28] We confirmed that, indeed, MPO levels were higher after I/R; however, aliskiren treatment restored MPO levels to normal. MDA, one of the end products in the lipid peroxidation process, has also been used to assess oxygen-derived free radical-mediated injury.[29] In our study, plasma MDA level in both aliskiren-treated groups was lower than in the vehicle-treated group. Moreover, myocardial superoxide anion generation in response to I/R was attenuated by aliskiren treatment. Therefore, aliskiren may attenuate oxidative stress at least in part through reducing neutrophil accumulation with resultant superoxide anion generation. However, because 1400W reduced oxidative stress and myocardial superoxide anion production to a similar degree as aliskiren, but did not favorably affect the I/R-induced changes in myocardial structure or function, our data suggest that the beneficial effect of direct renin inhibition observed on these parameters was not due to its attenuating oxidative stress but rather to a direct action of NO generated through eNOS.

Changes in the MMP/TIMP system and development of left ventricular hypertrophy are associated with the transition from compensated to decompensated heart failure.[30,31] MMP-9 in particular is an important biomarker for post-infarct dilatation,[32] and contributes importantly to the risk of left ventricular rupture, so that its genetic deletion attenuates post-infarct mortality.[33] We found an increase in MMP-9 expression in the infarcted area of the left ventricle, and both MMP-2 and MMP-9 expression were inhibited by aliskiren. We also found that TIMP-1 expression is downregulated by aliskiren. Taken together, these results suggest that the overall effect of aliskiren is to attenuate the activation/deactivation cycle of matrix degradation in the context of I/R.

A limitation of the tail cuff method for measuring blood pressure, as used in this study, is its relative insensitivity as compared with telemetry. Nonetheless, despite any lack of sensitivity of the method, we observed a clear difference in that higher-dose aliskiren gave rise to a reduction in blood pressure which was clearly greater—and more easily detectable—than did lower-dose aliskiren; and indeed the reduction with the former, unlike with the latter, was significant. This supports the argument that the apparently equal effects of lower- and higher-dose aliskiren on other parameters measured in our study were not purely related to blood pressure reduction.

In conclusion, our data indicate that in SHR, aliskiren is cardioprotective in the context of myocardial I/R, predominantly through activation of the PI3K-Akt-eNOS pathway. This is the first demonstration to show that direct renin inhibition activates this pathway, giving rise to consequent functional effects, and has important potential implications as regards novel strategies to prevent and treat myocardial I/R injury.

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## Disclosures

None.

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# Direct Renin Inhibition With Aliskiren Protects Against Myocardial Ischemia/Reperfusion Injury by Activating Nitric Oxide Synthase Signaling in Spontaneously Hypertensive Rats

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