Aliskiren Reduces Myocardial Ischemia–Reperfusion Injury by a Bradykinin B₂ Receptor– and Angiotensin AT₂ Receptor–Mediated Mechanism

Suang Suang Koid, James Ziogas, Duncan John Campbell

Abstract—Angiotensin-converting enzyme inhibitors and angiotensin AT₁ receptor blockers reduce myocardial ischemia–reperfusion injury via bradykinin B₂ receptor– and angiotensin AT₂ receptor–mediated mechanisms. The renin inhibitor aliskiren increases cardiac tissue kallikrein and bradykinin levels. In the present study, we investigated the effect of aliskiren on myocardial ischemia–reperfusion injury and the roles of B₂ and AT₂ receptors in this effect. Female Sprague-Dawley rats were treated with aliskiren (10 mg/kg per day) and valsartan (30 mg/kg per day), alone or in combination, together with the B₂ receptor antagonist icatibant (0.5 mg/kg per day) or the AT₂ receptor antagonist PD123319 (30 mg/kg per day), for 4 weeks before myocardial ischemia–reperfusion injury. Aliskiren increased cardiac bradykinin levels and attenuated valsartan-induced increases in plasma angiotensin II levels. In vehicle-treated rats, myocardial infarct size (% area at risk, mean±SEM, n=7–13) was 43±3%. This was reduced to a similar extent by aliskiren, valsartan, and their combination to 24±3%, 25±3%, and 22±2%, respectively. Icatibant reversed the cardioprotective effects of aliskiren and the combination of aliskiren plus valsartan, but not valsartan alone, indicating that valsartan-induced cardioprotection was not mediated by the B₂ receptor. PD123319 reversed the cardioprotective effects of aliskiren, valsartan, and the combination of aliskiren plus valsartan. Aliskiren protects the heart from myocardial ischemia–reperfusion injury via a B₂ receptor– and AT₂ receptor–mediated mechanism, whereas cardioprotection by valsartan is mediated via the AT₂ receptor. In addition, aliskiren attenuates valsartan-induced increases in angiotensin II levels, thus preventing AT₂ receptor–mediated cardioprotection by valsartan. (Hypertension. 2014;63:768-773.) • Online Data Supplement

Key Words: aliskiren • angiotensins • bradykinin • myocardial infarction

Inhibition of the renin–angiotensin system with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor type 1 (AT₁) receptor blockers (ARBs) lowers the risk of total mortality, cardiovascular mortality, nonfatal myocardial infarction, stroke, and other clinical outcomes in patients at increased cardiovascular risk or with stable ischemic heart disease.¹-³ Despite advances in the treatment of ischemic heart disease, myocardial infarction remains a major cause of death and hospitalization in developed countries. It has been argued that neither ACE inhibitors nor ARBs completely block the renin–angiotensin system cascade.⁴ Thus, combination treatment with a renin inhibitor may provide protection against myocardial infarction additional to that achieved using ACE inhibitors or ARBs alone.

Aliskiren is the first orally effective inhibitor of renin. At present, aliskiren is clinically approved for the treatment of hypertension. In animal models, aliskiren reduces myocardial infarct size and left ventricular remodeling after myocardial infarction,⁵-⁸ increases nitric oxide bioavailability,⁶ and inhibits atherosclerosis.⁹-¹¹ Recently, we reported that aliskiren increases tissue kallikrein and bradykinin levels in the rat heart at plasma levels within the therapeutic range in patients.¹² Both the dose-response and time course of the aliskiren-induced increases in cardiac bradykinin levels suggested that these actions of aliskiren were independent of renin inhibition.¹²

Bradykinin has well-established cardioprotective activity¹³-²² and is implicated in the reduction of myocardial ischemia–reperfusion (I/R) injury by both ACE inhibitors and ARBs. The bradykinin receptor type 2 (B₂) receptor antagonist icatibant prevents the reduction in I/R injury produced by ACE inhibitors,²³-²⁶ whereas both icatibant and the angiotensin II receptor type 2 (AT₂) receptor antagonist PD123319 attenuate cardioprotection by ARBs.²⁵-²⁸-²⁹ Our finding that aliskiren increases cardiac bradykinin levels¹² raised the possibility of cardioprotection, independent of any effect of aliskiren on renin.

The aims of the present study were to compare the effects of aliskiren, the AT₁ receptor antagonist valsartan, and their combination on myocardial I/R injury in rats and to investigate the role of the bradykinin B₂ and angiotensin AT₂ receptors in the mechanism of action of aliskiren. We show that aliskiren...
reduces myocardial I/R injury and that the cardioprotective effect of aliskiren is mediated via a B₂ receptor– and AT₂ receptor-mediated mechanism.

Methods
Detailed methods are provided in the online-only Data Supplement.

Results
Effects of Drug Treatment on Systolic Blood Pressure, Body Weight, Heart Weight/Body Weight Ratio, and Mean Arterial Blood Pressure During I/R Injury
Systolic blood pressure (SBP) of the 12 groups of rats during the 4-week treatment period before I/R injury is shown in the Table. In the present study, we used doses of aliskiren (10 mg/kg per day SC) and valsartan (30 mg/kg per day PO) that have little effect on blood pressure in normotensive Sprague-Dawley rats. Neither aliskiren nor valsartan caused any change in SBP during the 4 weeks (Table). However, SBP in rats receiving the combination of aliskiren plus valsartan was less than that in vehicle-treated rats. In contrast, SBP in rats receiving the B₂ receptor antagonist icatibant (0.5 mg/kg per day SC) alone was higher than that of vehicle-treated rats. Rats treated with aliskiren, valsartan, and the combination of aliskiren plus valsartan had lower SBP during combined treatment with icatibant than that of rats treated with icatibant alone. Treatment with the AT₂ receptor antagonist PD123319 (30 mg/kg per day SC) alone did not affect blood pressure. During combined treatment with PD123319, aliskiren did not lower SBP. However, PD123319 in combination with valsartan, and with aliskiren plus valsartan, lowered SBP relative to that of rats treated with PD123319 alone. Nevertheless, none of the changes in SBP during the course of the 4-week treatment period translated to differences in mean arterial blood pressure during cardiac I/R injury (Table S1 in the online-only Data Supplement). None of the treatments influenced body weight during the 4-week treatment period (Table S2). Neither aliskiren, valsartan, nor their combination influenced heart weight or heart weight/body weight ratio, except in rats administered PD123319 (Table S3). The heart weight/body weight ratio was lower in rats administered valsartan plus PD123319 or the combination of aliskiren and valsartan plus PD123319 compared with vehicle plus PD123319 alone.

Aliskiren Reduces Cardiac I/R Injury
Because aliskiren increases bradykinin levels in the heart¹² and bradykinin has well-established cardioprotective activity,¹³–²² we compared the effects of aliskiren with valsartan and their combination on cardiac I/R injury. Myocardial infarct size relative to the area at risk was 43% in the no inhibitor vehicle-treated group (Figure 1). Infarct size was reduced to 22% to 25% by aliskiren, valsartan, and their combination, representing ≈45% protection against I/R injury. Thus, in the Sprague-Dawley rat, aliskiren is cardioprotective to a similar extent as valsartan, and the combination of the 2 drugs did not result in an additive benefit.

Aliskiren Reduces Myocardial Infarct Size via a B₂ Receptor–Mediated Mechanism
The role of the B₂ receptor in aliskiren-induced cardioprotection was investigated by the coadministration of the B₂ receptor antagonist icatibant. Icatibant had no effect on myocardial infarct size in vehicle-treated rats but reversed the cardioprotective effects of aliskiren (Figure 1). However, icatibant had no effect on valsartan-induced cardioprotection. These results demonstrate that aliskiren reduces myocardial infarct size.

Table. Systolic Blood Pressure During the 4-Week Treatment Period

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Wk 0</th>
<th>Wk 1</th>
<th>Wk 2</th>
<th>Wk 3</th>
<th>Wk 4</th>
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</thead>
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<tr>
<td>Vehicle</td>
<td>121±3</td>
<td>117±3</td>
<td>123±3</td>
<td>124±3</td>
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<td>112±3</td>
<td>115±3</td>
<td>121±3</td>
<td>118±3</td>
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<tr>
<td>Valsartan</td>
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<td>113±4</td>
<td>115±4</td>
<td>117±3</td>
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<tr>
<td>Aliskiren+valsartan*</td>
<td>126±2</td>
<td>112±4</td>
<td>114±3</td>
<td>117±2</td>
<td>111±4</td>
</tr>
<tr>
<td>Vehicle+icatibant*</td>
<td>127±3</td>
<td>126±2</td>
<td>125±2</td>
<td>131±3</td>
<td>130±2</td>
</tr>
<tr>
<td>Aliskiren+icatibant†</td>
<td>123±2</td>
<td>116±2</td>
<td>118±2</td>
<td>118±2</td>
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</tr>
<tr>
<td>Valsartan+icatibant†</td>
<td>123±2</td>
<td>119±4</td>
<td>119±2</td>
<td>122±2</td>
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<tr>
<td>Aliskiren+valsartan+icatibant†</td>
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<td>116±3</td>
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<tr>
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<td>128±4</td>
<td>107±6</td>
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<td>108±5</td>
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</tbody>
</table>

Female Sprague-Dawley rats were administered vehicle, aliskiren (10 mg/kg per day), or valsartan (30 mg/kg per day), alone or in combination, for 4 wk in (1) the absence of an inhibitor; (2) the presence of the B₂ receptor antagonist icatibant (0.5 mg/kg per day); or (3) the presence of the AT₂ receptor antagonist PD123319 (30 mg/kg per day). Data represent means±SEM (n=7–13).

*P<0.05 compared with vehicle.
†P<0.05 compared with vehicle+icatibant.
‡P<0.05 compared with vehicle+PD123319 (wk 1–4, 2-way repeated-measures ANOVA).
via a B₂ receptor–mediated mechanism, whereas valsartan reduces myocardial infarct size via a mechanism that does not involve B₂ receptors. Interestingly, icatibant also prevented the cardioprotection induced by the combination of aliskiren plus valsartan, indicating that the non–B₂ receptor–mediated cardioprotection afforded by valsartan was attenuated by cotreatment with aliskiren (Figure 1).

**Aliskiren Reduces Myocardial Infarct Size via an AT₂ Receptor–Mediated Mechanism**

Because the angiotensin AT₂ receptor is implicated in the cardiovascular benefits of the ARB valsartan, we investigated the effect of coadministration of the AT₂ receptor antagonist PD123319 in the cardioprotective actions of aliskiren, valsartan, and their combination. PD123319 had no effect on myocardial infarct size in vehicle-treated rats but prevented the reduction in myocardial infarct size by all 3 drug treatments (Figure 1).

**Aliskiren Increases Cardiac Bradykinin Peptide Levels**

To explore the differences in the mechanisms of action of aliskiren and valsartan, we measured cardiac and blood bradykinin and angiotensin peptide levels in rats treated with aliskiren, valsartan, or their combination. Both active bradykinin [BK-(1–9)] and inactive metabolite of bradykinin [BK-(1–7)] peptide levels in the heart were higher in aliskiren- than in vehicle-treated rats (Figure 2A and 2B). The combination of aliskiren and valsartan caused a similar doubling of BK-(1–9) and BK-(1–7) levels in the heart compared with levels in the vehicle-treated group. In contrast, valsartan alone had no effect on cardiac BK-(1–9) or BK-(1–7) levels. These findings are consistent with the prevention by icatibant of aliskiren-induced, but not valsartan-induced, reductions in myocardial infarct size. Aliskiren alone also increased BK-(1–9) and BK-(1–7) levels in the blood, but neither valsartan nor the combination of aliskiren plus valsartan affected blood bradykinin peptide levels (Figure S1). Furthermore, in a separate series of rats we showed that coadministration of aliskiren and PD123319 did not prevent the aliskiren-induced increases in cardiac BK-(1–9) and BK-(1–7) levels (Figure S2).

**Aliskiren Attenuates Valsartan-Induced Increases in Angiotensin Peptide Levels**

Aliskiren had no effect on blood angiotensin I or angiotensin II levels (Figure 3). In contrast, valsartan caused 11- and 26-fold increases in blood angiotensin I and angiotensin II levels, respectively. Interestingly, in rats treated with the combination of aliskiren and valsartan, blood angiotensin I and angiotensin II levels were only 4- and 5-fold higher, respectively, than concentrations in vehicle-treated rats. Thus, aliskiren attenuated valsartan-induced increases in angiotensin I and angiotensin II levels by ≈60% and ≈80%, respectively (Figure 3).

Similar patterns to those seen for blood were observed for cardiac angiotensin I levels. Aliskiren alone had no affect on angiotensin I levels in the heart, valsartan increased angiotensin I levels 7-fold, and the valsartan-induced increase in angiotensin I levels was attenuated by cotreatment with aliskiren (Figure S3). None of the treatments influenced cardiac angiotensin II levels.

**Short-Term Aliskiren Administration Does Not Reduce Myocardial Infarct Size, Despite Reduction in Blood Angiotensin Peptide Levels**

To determine whether cardioprotection by aliskiren is related to the renin-inhibitory actions of aliskiren, we administered aliskiren intravenously 5 minutes before occlusion and throughout cardiac I/R injury. Treatment with aliskiren caused a 68% and 48% reduction in blood angiotensin I and

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Effect of aliskiren (10 mg/kg per day SC), valsartan (30 mg/kg per day PO), and the combination of aliskiren plus valsartan on cardiac ischemia–reperfusion injury, expressed as the ratio of infarct size to area at risk. Female Sprague-Dawley rats were treated with vehicle, aliskiren, or valsartan, alone or in combination, for 4 weeks before ischemia–reperfusion injury. In addition, rats received no inhibitor, icatibant (B₂ receptor antagonist; 0.5 mg/kg per day SC), or PD123319 (AT₂ receptor antagonist; 30 mg/kg per day SC). Data represent means±SEM (n=7–13). **P <0.01 compared with no inhibitor vehicle-treated rats (1-way ANOVA, Dunnett test); †P<0.05, ††P<0.01, comparisons as indicated (t tests).

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Cardiac bradykinin-(1–9) [BK-(1–9)] (A) and bradykinin-(1–7) [BK-(1–7)] (B) peptide levels in female Sprague-Dawley rats treated with vehicle, aliskiren, or valsartan, alone or in combination, for 4 weeks. Data represent means±SEM (n=8–10). **P<0.01, ***P<0.001 compared with vehicle-treated rats (1-way ANOVA, Dunnett test).
Angiotensin II levels, respectively (Figure S4). Myocardial infarct size was 45% in the vehicle-treated group (Figure S4). However, short-term aliskiren administration did not reduce myocardial infarct size.

**Discussion**

In the present study, we demonstrated that aliskiren protects the heart from myocardial I/R injury via a B₂ receptor– and AT₂ receptor–mediated mechanism. These effects of aliskiren were observed independent of blood pressure control and provide a novel insight into the interaction between inhibitors of the renin–angiotensin system. Although the cardioprotective effect of valsartan was mediated by the AT₂ receptor, the combination of aliskiren and valsartan did not provide additional cardioprotection. Rather, by attenuating the valsartan-induced increases in angiotensin II levels, aliskiren prevented the AT₂ receptor–mediated cardioprotection by valsartan.

Aliskiren increases tissue kallikrein that in turn increases bradykinin levels in the heart.12 Our finding that icatibant prevented the cardioprotective effect of aliskiren is consistent with the involvement of B₂ receptors, although unlike other studies we did not detect an effect of icatibant on valsartan-induced cardioprotection.25,27-29 Many previous studies have shown interaction between the B₂ and AT₂ receptors.33-38 Siragy et al35 showed that PD123319 suppressed basal bradykinin levels and prevented valsartan-induced increase in bradykinin levels in renal interstitial fluid. Our study is the first to examine this question in the heart. In contrast to the findings for the kidney by Siragy et al,35 we found that PD123319 did not modify basal cardiac bradykinin levels nor prevent the aliskiren-induced increase in bradykinin levels. Our study demonstrates that B₂ receptor–mediated cardioprotection is dependent on a functional AT₂ receptor, although the exact nature of the interaction between the B₂ and AT₂ receptors remains to be determined (Figure 4).

In contrast to the reduction in infarct size produced by short-term ACE inhibitor and ARB administration,23-29 we and others39 showed that short-term aliskiren administration did not produce cardioprotection, despite reduction in angiotensin peptide levels. Rather, a longer period of aliskiren administration, sufficient to increase tissue kallikrein protein and mRNA and bradykinin levels,12 was required to produce cardioprotection. These studies suggest that cardioprotection by aliskiren is not caused by a direct effect on renin and angiotensin peptide levels but rather that it is mediated through an increase in tissue kallikrein gene expression.12

Clinical data show that the antihypertensive effects of aliskiren are comparable with those of other antihypertensive agents, with an adverse event profile similar to that of placebo.40 However, therapeutic combination of aliskiren with either an ACE inhibitor or an ARB results in a greater frequency of adverse events, fails to improve left ventricular remodeling after myocardial infarction, fails to reduce cardiovascular and renal events in high-risk patients with type 2 diabetes mellitus, and fails to reduce postdischarge mortality and heart failure readmissions in hospitalized patients with heart failure.41-43 Similar increases in adverse effects were observed with combinations of ACE inhibitors and ARBs.44,45 The adverse effects associated with combinations of renin–angiotensin system inhibitors are likely due to excessive blockade of AT₁ receptor–mediated effects. Other mechanisms that contribute to the therapeutic effects of ACE inhibitors and ARBs include increased bradykinin, angiotensin II, and angiotensin-(1–7) levels and increased activation of B₂, AT₂, and Mas receptors.46-49 However, when given in combination with ACE inhibitors and ARBs, the renin inhibitor aliskiren may attenuate angiotensin II and angiotensin-(1–7) levels and thus

![Figure 3. Blood angiotensin I (Ang I) (A) and Ang II (B) peptide levels in female Sprague-Dawley rats treated with vehicle, aliskiren, or valsartan, alone or in combination, for 4 weeks. Data represent means±SEM (n=8–10). **P<0.01, ***P<0.001 compared with vehicle-treated rats or as indicated (1-way ANOVA, Tukey test).](http://hyper.ahajournals.org/)

![Figure 4. Interactions between the renin–angiotensin and kallikrein–kinin systems. Angiotensin II (Ang II) acts on AT₁ receptors, and bradykinin acts on B₁ and B₂ receptors. Valsartan blocks the AT₁ receptor, PD123319 blocks the AT₂ receptor, and icatibant blocks the B₂ receptor. By blocking the tonic Ang II–mediated inhibition of renin secretion, valsartan increases renin, Ang I, and Ang II levels. Increased Ang II levels stimulate the AT₂ receptor, thus mediating cardioprotection. Aliskiren inhibits renin, thereby preventing the valsartan-induced increase in Ang II levels and AT₂ receptor–mediated cardioprotection. Aliskiren also increases tissue kallikrein and bradykinin levels. The increased bradykinin levels act on the B₂ receptor to produce cardioprotection, which was prevented by icatibant.](http://hyper.ahajournals.org/)
prevent the therapeutic benefits resulting from the action of these peptides on the AT1 and Mas receptors (Figure 4).

Aliskiren is the only compound thus far shown to increase tissue kallikrein expression in the heart. This finding offers a new approach to cardioprotection. However, it may be necessary to separate the renin-inhibitory actions from the tissue kallikrein-stimulating actions of aliskiren for tissue kallikrein-mediated cardioprotection to be achieved in combination with the Mas receptor– and AT1, receptor–mediated benefits of ACE inhibitors and ARBs. Although additional studies are required to confirm whether this mechanism operates in the human heart, such a strategy offers the possibility of greater cardioprotection while avoiding the adverse effects of excessive AT1 receptor blockade.

Perspectives

The leading treatments for ischemic heart disease, including ACE inhibitors and ARBs, involve activation of bradykinin B1 receptor– and angiotensin AT1 receptor–mediated mechanisms. The renin inhibitor aliskiren increases cardiac bradykinin and tissue kallikrein levels, thus offering a new approach to cardioprotection. In the present study, aliskiren reduced myocardial infarct size by a B1 receptor– and AT1 receptor–mediated mechanism. In addition, aliskiren attenuated AT1 receptor–mediated cardioprotection by valsartan. These effects of aliskiren were observed independent of blood pressure control and provide a possible explanation for why combination of aliskiren with ARB therapies does not provide additional clinical benefit. Aliskiren is the only compound thus far shown to increase tissue kallikrein expression in the heart. Future therapies that increase tissue kallikrein levels without renin inhibition offer the possibility of greater benefit in combination with ACE inhibitors and ARBs.

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Disclosures

None.

References

Novelty and Significance

Summary

Aliskiren protects the heart from myocardial ischemia–reperfusion injury via a B, receptor– and AT1 receptor–mediated mechanism, whereas cardioprotection by valsartan is mediated by the AT1 receptor. Aliskiren prevents AT1 receptor–mediated cardioprotection by valsartan, and thus the combination of aliskiren and valsartan does not provide greater cardioprotection than either drug alone. These effects of aliskiren provide a novel insight into the mechanism of cardioprotection by aliskiren and the interaction between inhibitors of the renin–angiotensin system.

What Is New?
- Both the bradykinin B1 receptor antagonist icatibant and the angiotensin AT1 receptor antagonist PD123319 prevent aliskiren–mediated reduction of myocardial ischemia–reperfusion injury.
- When combined with the angiotensin AT1 receptor blocker valsartan, aliskiren attenuates the valsartan–induced increase in angiotensin II levels, thereby preventing AT1 receptor–mediated cardioprotection by valsartan.

What Is Relevant?
- This study provides a novel insight into interactions between inhibitors of the renin–angiotensin system, offering an explanation for why the combination of aliskiren with angiotensin AT1 receptor blocker therapies does not provide additional clinical benefit.

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SUPPLEMENTAL MATERIAL for

Aliskiren reduces myocardial ischemia-reperfusion injury by a bradykinin B2 receptor-and angiotensin AT2 receptor-mediated mechanism

Suang S Koid, James Ziogas, Duncan J Campbell

From St Vincent’s Institute of Medical Research and Department of Medicine (S.S.K., D.J.C.), and Department of Pharmacology and Therapeutics (S.S.K., J.Z.), University of Melbourne, Victoria, Australia.

Address for correspondence:  Duncan J Campbell
St Vincent’s Institute of Medical Research
41 Victoria Parade
Fitzroy
VIC 3065 Australia
Telephone number: + 61 3 9288 2501
Fax number: + 61 3 9416 2676
E-mail: dcampbell@svi.edu.au
SUPPLEMENTAL METHODS

Animals
Rats were housed in a room maintained at 22 ± 1°C on a 12-h light/dark cycle. Rats received normal chow (Certified Rodent Diet #5002; LabDiet, St. Louis, MO, USA) and drinking water ad libitum. All experimental procedures were approved by the St. Vincent’s Hospital Animal Research Ethics Committee in accordance with National Health and Medical Research Council of Australia’s Code for the Care and Use of Animals for Scientific Purposes.

Experimental procedures
For the long-term aliskiren administration experiment, 8-week old female Sprague-Dawley rats were randomly assigned to one of 12 treatment groups for 4 weeks: (1) vehicle; (2) aliskiren; (3) valsartan; (4) combination of aliskiren and valsartan; (5) icatibant; (6) aliskiren and icatibant; (7) valsartan and icatibant; (8) combination of aliskiren, valsartan, and icatibant; (9) PD123319; (10) aliskiren and PD123319; (11) valsartan and PD123319; (12) combination of aliskiren, valsartan, and PD123319. Aliskiren (10 mg/kg/day), icatibant (0.5 mg/kg/day), PD123319 (30 mg/kg/day) and vehicle (0.9% saline) were administered via subcutaneous minipumps implanted in the back of rats during brief anesthesia with alfaxalone (1.5 mL/kg, i.v.). Valsartan (30 mg/kg/day) or an equal volume of vehicle (0.25% methylcellulose) was administered by daily gavage. Vehicle-treated rats received both 0.9% saline and 0.25% methylcellulose. Aliskiren, valsartan, and PD123319 were gifts from Novartis Pharma (Basel, Switzerland). Icatibant was purchased from Auspep Pty Ltd (Tullamarine, Australia).

Prior to and at weekly intervals during the treatment period, body weight and systolic blood pressure were measured. After 4 weeks treatment, the myocardial responses to ischemia-reperfusion (I/R) injury and angiotensin and bradykinin peptides were measured.

In the short-term aliskiren administration experiment, 10-week old female Sprague-Dawley rats were randomly assigned to receive either vehicle or aliskiren (1 mg/kg, i.v.), administered 5 min before occlusion of the left anterior descending (LAD) coronary artery followed by an i.v. infusion of 10 µg/kg/min during myocardial ischemia and reperfusion. After 2 h reperfusion, blood samples (2 mL) were collected from the carotid artery into 10 mL of 4 mol/L guanidine thiocyanate for measurement of angiotensin peptides.

Blood pressure measurement
Systolic blood pressure was determined in pre-warmed conscious rats using a non-invasive tail-cuff and blood pressure monitor connected to a Powerlab system (Chart 5, v5.5.6; ADInstruments, Bella Vista, NSW, Australia).

Cardiac I/R injury
We used established methods to assess I/R injury. Rats were anesthetized with pentobarbitone sodium (60 mg/kg; i.p.), followed by administration of atropine (1 mg/kg; s.c.) and lignocaine (5 mg/kg; s.c.). A tracheotomy was performed and rats were ventilated with room air on a rodent ventilator (Model 7025; Ugo Basile, Comerio, VA, Italy). Stroke volume was set at 11 mL/kg and stroke rate at 40 breaths/min. Following cannulation of the right jugular vein, the left carotid artery was cannulated for continuous monitoring of heart rate, and phasic and mean arterial pressure via a Millar pressure catheter (SPR-407; Millar Instruments, Houston, TX, USA) connected to a PowerLab data acquisition system (Chart 5, v5.5.6; ADInstruments). Rats were subjected to thoracotomy at the 4th intercostal space to expose the heart. The LAD coronary artery was snared and blood flow occluded. After 30
min of ischemia, the ligature was released and blood flow restored. After 2 h reperfusion, the LAD coronary artery was re-occluded and 1 mL of 5% Evans blue dye injected intravenously to stain the perfused myocardium blue. When Evans blue staining of tissues was complete, the heart was removed and rinsed of excess dye. The left ventricle (LV) was then sliced transversely into 2-mm slices, photographed, and stained with 1% 2,3,5-triphenyltetrazolium chloride at 37°C for 15 min. The LV slices were then fixed in 10% formalin solution at room temperature overnight before repeat photography. Areas at risk (AAR) and infarct sizes were measured by planimetry (ImageJ, NIH, USA) for both sides of each slice. Animals with a mean arterial pressure <50 mmHg or those that exhibited prolonged arrhythmia during I/R injury were excluded from the study.

**Measurement of AAR and infarct size**
Each of the LV slices was weighed. The endpoint of cardioprotection was measured by infarct size as a ratio to AAR. The infarct size was calculated as described elsewhere. Briefly, the total area, AAR and infarcted area for both sides of each slice were averaged, and the weights of the AAR and the infarcted area for each slice were calculated from these averaged values. The ratio of infarct size to AAR was calculated from the sum of these weights and expressed as a percentage of AAR.

**Measurement of angiotensin and bradykinin peptides in heart and blood**
Angiotensin and bradykinin peptides were measured in heart and blood of female Sprague-Dawley rats treated with vehicle, aliskiren (10 mg/kg/day; s.c. minipump), valsartan (30 mg/kg/day; p.o.), or the combination of aliskiren and valsartan. After 4 weeks treatment, the 12-week old rats were anesthetized with a mixture of ketamine (75 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.). The carotid artery was cannulated and blood samples (2 mL) were collected into 10 mL of 4 mol/L guanidine thiocyanate for measurement of angiotensin and bradykinin peptides. The cardiac ventricles were excised, rapidly rinsed free of blood with 0.9% saline and homogenized immediately in 10 mL of 4 mol/L guanidine thiocyanate. Blood and tissue homogenates were adjusted to 0.1% trifluoroacetic acid, centrifuged at 5020 g at room temperature for 15 min, and then processed as described previously. Concentrations of angiotensin (Ang) I, AngII, bradykinin (BK)-(1-9) and its metabolite BK-(1-7) were measured by N-terminal-directed radioimmunoassays. Peptide data were corrected for recovery as reported previously.

**Statistical analysis**
Data are expressed as means ± SEM. The significance of differences in systolic blood pressure (SBP), body weight and mean arterial blood pressure (MAP) was analyzed by two-way repeated-measures analysis of variance (ANOVA). The effects of aliskiren, valsartan and their combination on myocardial infarct size were analyzed by one-way ANOVA, and comparisons of the effects of treatment with vehicle were evaluated using Dunnett’s test. The effects of icatibant and PD123319 were analyzed separately for each treatment group using Student’s t-test. For the purpose of statistical calculations, peptide levels below the minimum detectable level were taken as half the minimum detectable amount. All peptide concentrations were logarithmically transformed prior to statistical analysis (ANOVA, post-hoc Dunnett’s test) because of a skewed distribution. All statistical analyses were performed using IBM SPSS Statistics (Version 21.0. Armonk, NY) and two-tailed $P <0.05$ was considered significant.
SUPPLEMENTAL REFERENCES


### Table S1. Mean arterial blood pressure of anesthetized rats during cardiac I/R injury

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean arterial pressure (mmHg)</th>
<th>During ischemia</th>
<th>30 min reperfusion</th>
<th>1 h reperfusion</th>
<th>1.5 h reperfusion</th>
<th>2 h reperfusion</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td>80 ± 6</td>
<td>81 ± 6</td>
<td>80 ± 6</td>
<td>78 ± 5</td>
<td>75 ± 5</td>
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<tr>
<td>Aliskiren</td>
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<td>84 ± 5</td>
<td>85 ± 5</td>
<td>75 ± 4</td>
<td>78 ± 4</td>
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<td>79 ± 4</td>
<td>79 ± 4</td>
<td>74 ± 4</td>
<td>71 ± 4</td>
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<td>76 ± 5</td>
<td>76 ± 5</td>
<td>70 ± 5</td>
<td>67 ± 4</td>
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<tr>
<td>Vehicle + icatibant</td>
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<td>76 ± 5</td>
<td>71 ± 3</td>
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<td>71 ± 4</td>
<td>67 ± 4</td>
</tr>
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<td>61 ± 2</td>
<td>59 ± 2</td>
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<td>64 ± 3</td>
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</table>

Prior to I/R, female Sprague-Dawley rats were administered vehicle, aliskiren (10 mg/kg/day) or valsartan (30 mg/kg/day), alone or in combination, for 4 weeks in: (i) the absence of an inhibitor; (ii) the presence of the B2 receptor antagonist icatibant (0.5 mg/kg/day); or (iii) the presence of the AT2 receptor antagonist PD123319 (30 mg/kg/day). Data represent means ± SEM (n = 7-13). No significant differences were detected between any of the treatment groups (two-way repeated-measures ANOVA, comparisons against respective vehicle, vehicle + icatibant or vehicle + PD123319 groups).
Table S2. Body weight over the 4-week treatment period

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Week 0</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>197 ± 5</td>
<td>222 ± 5</td>
<td>235 ± 5</td>
<td>252 ± 5</td>
<td>263 ± 4</td>
</tr>
<tr>
<td>Aliskiren</td>
<td>196 ± 3</td>
<td>223 ± 4</td>
<td>246 ± 5</td>
<td>255 ± 4</td>
<td>268 ± 4</td>
</tr>
<tr>
<td>Valsartan</td>
<td>200 ± 4</td>
<td>229 ± 5</td>
<td>243 ± 5</td>
<td>256 ± 5</td>
<td>266 ± 5</td>
</tr>
<tr>
<td>Aliskiren + valsartan</td>
<td>194 ± 6</td>
<td>217 ± 7</td>
<td>240 ± 6</td>
<td>251 ± 6</td>
<td>256 ± 6</td>
</tr>
<tr>
<td>Vehicle + icatibant</td>
<td>198 ± 6</td>
<td>219 ± 5</td>
<td>238 ± 7</td>
<td>253 ± 6</td>
<td>267 ± 7</td>
</tr>
<tr>
<td>Aliskiren + icatibant</td>
<td>189 ± 3</td>
<td>214 ± 2</td>
<td>233 ± 3</td>
<td>250 ± 3</td>
<td>261 ± 3</td>
</tr>
<tr>
<td>Valsartan + icatibant</td>
<td>196 ± 6</td>
<td>219 ± 4</td>
<td>229 ± 5</td>
<td>242 ± 4</td>
<td>256 ± 4</td>
</tr>
<tr>
<td>Aliskiren + valsartan + icatibant</td>
<td>197 ± 6</td>
<td>217 ± 4</td>
<td>233 ± 5</td>
<td>245 ± 4</td>
<td>258 ± 4</td>
</tr>
<tr>
<td>Vehicle + PD123319</td>
<td>195 ± 5</td>
<td>225 ± 4</td>
<td>251 ± 5</td>
<td>264 ± 5</td>
<td>272 ± 5</td>
</tr>
<tr>
<td>Aliskiren + PD123319</td>
<td>197 ± 5</td>
<td>225 ± 5</td>
<td>248 ± 6</td>
<td>260 ± 7</td>
<td>269 ± 5</td>
</tr>
<tr>
<td>Valsartan + PD123319</td>
<td>202 ± 5</td>
<td>230 ± 6</td>
<td>256 ± 6</td>
<td>265 ± 5</td>
<td>272 ± 6</td>
</tr>
<tr>
<td>Aliskiren + valsartan + PD123319</td>
<td>199 ± 4</td>
<td>232 ± 6</td>
<td>251 ± 6</td>
<td>257 ± 5</td>
<td>269 ± 3</td>
</tr>
</tbody>
</table>

Female Sprague-Dawley rats were administered vehicle, aliskiren (10 mg/kg/day) or valsartan (30 mg/kg/day), alone or in combination, for 4 weeks in: (i) the absence of an inhibitor; (ii) the presence of the B₂ receptor antagonist icatibant (0.5 mg/kg/day); or (iii) the presence of the AT₂ receptor antagonist PD123319 (30 mg/kg/day). Data represent means ± SEM (n = 7-13). Body weight (adjusted for minipump weights) increased over Weeks 1-4, with no effect of treatment (two-way repeated-measures ANOVA, comparisons against respective respective vehicle, vehicle + icatibant or vehicle + PD123319 groups).
**Table S3.** Heart weight and heart weight/body weight ratio of Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Heart weight (g)</th>
<th>Heart weight/Body weight ratio (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.61 ± 0.01</td>
<td>2.24 ± 0.04</td>
</tr>
<tr>
<td>Aliskiren</td>
<td>0.60 ± 0.01</td>
<td>2.18 ± 0.06</td>
</tr>
<tr>
<td>Valsartan</td>
<td>0.57 ± 0.01</td>
<td>2.05 ± 0.05</td>
</tr>
<tr>
<td>Aliskiren + valsartan</td>
<td>0.58 ± 0.01</td>
<td>2.15 ± 0.05</td>
</tr>
<tr>
<td>Vehicle + icatibant</td>
<td>0.61 ± 0.01</td>
<td>2.20 ± 0.05</td>
</tr>
<tr>
<td>Aliskiren + icatibant</td>
<td>0.61 ± 0.01</td>
<td>2.25 ± 0.05</td>
</tr>
<tr>
<td>Valsartan + icatibant</td>
<td>0.58 ± 0.02</td>
<td>2.16 ± 0.08</td>
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<tr>
<td>Aliskiren + valsartan + icatibant</td>
<td>0.55 ± 0.01</td>
<td>2.05 ± 0.04</td>
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<tr>
<td>Vehicle + PD123319</td>
<td>0.63 ± 0.02</td>
<td>2.25 ± 0.07</td>
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<tr>
<td>Aliskiren + PD123319</td>
<td>0.58 ± 0.02</td>
<td>2.09 ± 0.04</td>
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<tr>
<td>Valsartan + PD123319</td>
<td>0.56 ± 0.01</td>
<td>2.04 ± 0.05†</td>
</tr>
<tr>
<td>Aliskiren + valsartan + PD123319</td>
<td>0.55 ± 0.01</td>
<td>2.02 ± 0.03‡</td>
</tr>
</tbody>
</table>

Female Sprague-Dawley rats were administered vehicle, aliskiren (10 mg/kg/day) or valsartan (30 mg/kg/day), alone or in combination, for 4 weeks in: (i) the absence of an inhibitor; (ii) the presence of the B₂ receptor antagonist icatibant (0.5 mg/kg/day); or (iii) the presence of the AT₂ receptor antagonist PD123319 (30 mg/kg/day). Data represent means ± SEM (n = 7-13). †P<0.05, ‡P<0.01 compared with vehicle + PD123319 (one-way ANOVA).
Figure S1. Blood bradykinin (BK)-(1-9) (A) and BK-(1-7) (B) peptide levels in female Sprague-Dawley rats treated with vehicle, aliskiren (10 mg/kg/day) or valsartan (30 mg/kg/day), alone or in combination, for 4 weeks. Data represent means ± SEM (n = 8-10). **P<0.01 compared with vehicle (one-way ANOVA, Dunnett’s test).
Figure S2. Cardiac bradykinin (BK)-(1-9) (A), and BK-(1-7) (B) peptide levels in female Sprague-Dawley rats treated with vehicle or aliskiren (10 mg/kg/day) in the absence and presence of PD123319 (30 mg/kg/day), for 4 weeks. Data represent means ± SEM (n = 9). **P<0.01, ***P<0.001 compared with vehicle (Student’s t-test).
Figure S3. Cardiac angiotensin (Ang) I (A), and AngII (B) peptide levels in female Sprague-Dawley rats treated with vehicle, aliskiren (10 mg/kg/day) or valsartan (30 mg/kg/day), alone or in combination, for 4 weeks. Data represent means ± SEM (n = 8-10). **P<0.01 compared with vehicle (one-way ANOVA, Dunnett’s test).
Figure S4. Effect of short-term administration of aliskiren on cardiac ischemia-reperfusion injury, expressed as the ratio of infarct size to area at risk (A); blood AngI (B), and AngII (C) peptide levels in female Sprague-Dawley rats administered 1 mg/kg i.v. bolus 5 min before occlusion followed by an infusion 10 µg/kg/min i.v. during ischemia and reperfusion. Data represent means ± SEM (n = 9-11). ***P<0.001 compared with vehicle (Student’s t-test).