Renin Inhibitor Aliskiren Improves Impaired Nitric Oxide Bioavailability and Protects Against Atherosclerotic Changes

Toshio Imanishi, Hiroto Tsujioka, Hideyuki Ikejima, Akio Kuroi, Shigeho Takarada, Hironori Kitabata, Takashi Tanimoto, Yasuteru Muragaki, Seiichi Mochizuki, Masami Goto, Kiyoshi Yoshida, Takashi Akasaka

Abstract—We investigated whether aliskiren, a direct renin inhibitor, improves NO bioavailability and protects against spontaneous atherosclerotic changes. We also examined the effects of cotreatment with aliskiren and valsartan, an angiotensin II receptor blocker, on the above-mentioned outcomes. Watanabe heritable hyperlipidemic rabbits were treated with vehicle (control), aliskiren, valsartan, or aliskiren plus valsartan for 8 weeks. Then, acetylcholine-induced NO production was measured as a surrogate index of endothelium protective function, and both superoxide and vascular peroxynitrite were measured. Tetrahydrobiopterin in aortic segments was assessed by high-performance liquid chromatography with fluorescence detection. Plaque area was quantified by histology. Increase in plasma NO concentration in response to intra-aortic acetylcholine infusion was significantly greater in all of the test groups than in controls. Aliskiren + valsartan cotreatment increased acetylcholine-induced NO by 6.2 nmol/L, which was significantly higher than that with either aliskiren or valsartan alone. Vascular superoxide and peroxynitrite levels were both significantly higher in controls and significantly lower in the aliskiren + valsartan group than in the aliskiren or valsartan group. The highest tetrahydrobiopterin levels were observed after aliskiren + valsartan cotreatment. Histology of the thoracic aorta revealed that the plaque area was significantly decreased with combination therapy compared with monotherapy. Treatment with a direct renin inhibitor has protective effects on endothelial function and atherosclerotic changes. Furthermore, cotreatment with a direct renin inhibitor and an angiotensin II receptor blocker has additive protective effects on both. (Hypertension. 2008;52:563-572.)

Key Words: NO ■ endothelial function ■ renin inhibitor ■ oxidative stress

The renin-angiotensin system (RAS) activity may be a key factor in the pathophysiology and development of hypertension, atherosclerosis, heart failure, and renal disease in a substantial number of patients.1,2 However, it remains unclear whether angiotensin-converting enzyme (ACE) inhibitors and angiotensin (Ang) II receptor blockers (ARBs) have fully delivered the expected reduction in cardiovascular risk. In fact, optimized RAS suppression is difficult to achieve with currently available antihypertensive agents, partly because ACE inhibitors and ARBs both activate compensatory feedback mechanisms that result in renin release and increase plasma renin activity (PRA).3-5 In contrast, renin inhibitors neutralize any compensatory increase in PRA and prevent the formation of both Ang I and Ang II.6

Aliskiren, the first in a new class of orally effective renin inhibitors for the treatment of hypertension, is a potent and specific inhibitor of human renin in vitro with an IC50 value in the low nanomolar range.7,8 Studies in healthy volunteers have shown that aliskiren treatment leads to dose-dependent reductions in PRA and Ang II levels.9 Early clinical trials in hypertensive patients showed that this drug provided antihypertensive efficacy comparable to those of the ARBs losartan and irbesartan.10,11

In the present study, we hypothesized that aliskiren improves the impaired NO bioavailability and suppresses atherosclerotic plaque formation. To test this hypothesis, we examined the effects of aliskiren on NO bioavailability measured with our sensor12-14 and atherosclerotic plaque formation, as well as on vascular superoxide and peroxynitrite levels in Watanabe heritable hyperlipidemic (WHHL) rabbits. We also examined the effects of aliskiren + valsartan cotreatment on the above-mentioned outcomes.

Materials and Methods
An expanded Methods section is available in an online data supplement at http://hyper.ahajournals.org.
Catheter-Type NO Sensor
The integrated architecture and performance of our catheter-type NO sensor have been described previously. In brief, the oxidative current of NO was measured using the NO sensor (amino-700 XL, Innovative Instruments, detection tip diameter 700 μm) and an NO monitor (model inNO-T, Innovative Instruments). The sensor was calibrated with NO-saturated water, and the baseline (0 level) was set arbitrarily using the amperometric method. The change in current from baseline was expressed as the change in NO concentration (nmol/L). The mean peak response with NO concentration was 327 ± 11 pA/nmol/L among the sensors in the present study, and this value was compatible with those with the original sensor.

Animal Preparation
Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol was approved by the institutional animal care and use committee of Wakayama Medical University. Forty-eight 3-month-old male WHHL rabbits were assigned randomly to 1 of 4 groups. The groups received vehicle (0.5% carboxymethylcellulose sodium), 40 mg/kg per day of aliskiren, 5 mg/kg per day of valsartan, or 40 mg/kg per day of aliskiren plus 5 mg/kg per day of valsartan daily by oral gavage for 8 weeks. Both aliskiren and valsartan were provided by Novartis Pharma AG.

Rabbits were anesthetized with xylazine (10 mg/kg IM), ketamine (50 mg/kg IM), and pentobarbital sodium (10 mg/kg IV), followed by administration of heparin (1000 U intravenously) as an anticoagulant. A catheter for acetylcholine (ACH) or N\textsuperscript{G}-methyl-L-arginine (L-NMMA; NO synthase inhibitor) infusion was introduced into the aortic arch via the external carotid artery, whereas the NO sensor was inserted into the abdominal aorta through the left femoral artery. Aortic blood pressure was monitored simultaneously through a stiff cannula with a strain gauge pressure transducer (Nihon Kohden).

Plasma Biochemistry and Inflammatory Mediator Analysis
Blood samples were drawn from the ear artery and collected in EDTA bulbs (1 mg/mL) before and 8 weeks after treatment. Plasma was separated, and the total cholesterol and triglyceride concentrations were determined using a commercially available kit (Wako Pure Chemical Industries). PRA was determined using a radioimmunoassay of generated Ang I (SRL renin kit, TFB Co). Intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 were measured by using ELISA kits (Cytimmune Co).

ACh-Induced Vasorelaxation in Isolated Thoracic Arteries
The thoracic aorta was dissected and adhering perivascular tissue was removed as we described previously (please see the online data supplement).

Measurement of NO Production
Basal NO production was measured after L-NMMA (5 mg/kg) was infused at 1 mL/min for 10 minutes. Endothelium-dependent NO production was measured after ACh was administered at 4 μg/kg per minute for 5 minutes. Plasma NO concentration in the abdominal aorta was monitored over the entire time course.

Vessel Preparation
The aorta was placed in chilled modified Krebs/HEPES buffer (buffer A composition [mmol/L]: NaCl, 99.01; KCl, 4.69; CaCl\textsubscript{2}, 1.87; MgSO\textsubscript{4}, 1.20; K\textsubscript{2}HPO\textsubscript{4}, 1.03; NaHCO\textsubscript{3}, 25.0; Na-Hepes, 20.0; and glucose, 11.1; pH 7.4), cleaned of excessive adventitial tissue, and cut into 5-mm ring segments.

Estimation of Aortic Superoxide Production
Superoxide anion production in the aortic segments was measured by lucigenin (5 μmol/L)-derived chemiluminescence, as described previously. Briefly, after preparation, the vessels were placed in modified Krebs/HEPES buffer and equilibrated for 30 minutes at 37°C. Scintillation vials containing 2 mL of Krebs/HEPES buffer with 5 μmol/L of lucigenin were placed in a scintillation counter operating in the out-of-coincidence mode. After dark adaptation, background counts were recorded, and a vascular segment was added to the vial. Scintillation counts were then recorded every 2 minutes for 15 minutes, and the respective background counts were subtracted. The vessels were then dried in an oven at 90°C for 24 hours to determine their dry weight. In addition, to address the influence of NO synthase (NOS)-mediated superoxide production, vessels were incubated with N\textsuperscript{G}-nitro-L-arginine (l-NNA; 1 mmol/L) for 30 minutes, as described previously.

Estimation of Reduced Nicotinamide-Adenine Dinucleotide Phosphate Oxidase Activity in Vessel Homogenates
Reduced nicotinamide-adenine dinucleotide phosphate oxidase activity was measured as described previously (please see the online data supplement).

Western Blotting
Aorta samples were homogenized in ice-cold RIPA buffer (150 mmol/L of NaCl, 50 mmol/L of Tris-Cl, 5 mmol/L of EDTA, 1% vol/vol of Nonidet P-40, 0.5% weight/vol of deoxycholate, 10 mmol/L of phenylmethylsulfonyl fluoride, 2 μg/mL of aprotonin, and 2 μg/mL of leupeptin). Aortic extracts (30 μg protein per lane) were mixed with a sample loading buffer and separated on 12% SDS-polyacrylamide gel. Proteins were electrotransferred on polyvinylidene fluoride membranes (0.2 μm: Immun-Blot, Bio-Rad). The membrane was blocked in 5% nonfat dry milk and immunoblotted for anti-endothelial NOS (eNOS; Signal Transduction Laboratories, Cell Signaling Technology), antiphospho-Akt (New England Biolabs), antiheat shock protein 90 (Cytimmune Co), anti-Akt (New England Biolabs), antiphospho-Akt (New England Biolabs), antiheat shock protein 90 (Cell Signaling Technology), anti-Akt (New England Biolabs), antiphospho-Akt (New England Biolabs), antiheat shock protein 90 (Cell Signaling Technology), anti-Akt (New England Biolabs), and antiphospho-Akt (New England Biolabs). The bands were detected using a chemiluminescence assay (ECL Plus, Amersham). The profile of each band was plotted using National Institutes of Health Image software, and the densitometric intensity corresponding with each band was normalized using α-tubulin expression.

Measurement of Vascular Nitrotyrosine
Peroxynitrite is a strong oxidant formed by the reaction of NO with superoxide under atherosclerotic stimuli: the subsequent reaction of peroxynitrite with proteins results in the formation of nitrotyrosine. As a stable end product of peroxynitrite-mediated oxidation/nitration, nitrotyrosine can be used as a surrogate index of in vivo uncoupled NOS-dependent damage. We measured vascular nitrotyrosine in the thoracic aortic segments of rabbits from the control, aliskiren, valsartan, and aliskiren+valsartan groups. Vascular nitrotyrosine in proteins was measured using the NWLSS nitrotyrosine ELISA kit (Northwest Life Science Specialties, LLC) according to the manufacturer’s protocol.

Measurement of Aortic Luminal Surface Area
Each aorta (n=6 in each group) was opened longitudinally and stained with oil red O solution (Ceristain, Merck). The percentage of oil red O–positive area in relation of the total vessel area was quantified using Adobe Photoshop and National Institutes of Health Image software.

Histological Examination of Atherosclerosis
For the histological examination, a small portion of thoracic aorta was fixed in 10% neutral buffered formalin for 48 hours. After fixation and standard paraffin embedding, serial cross-sections were
processed for general histological staining with hematoxylin/eosin. The method of determining the intimal/medial area ratio of the thoracic aorta, which is used as a measure of atherosclerotic burden, has been described previously. Percentage aortic wall thickness [(outer wall area−area of lumen)/(outer wall area)]×100% was determined from the hematoxylin/eosin sections of the thoracic aorta. These slides were scanned microscopically (Keyence, Inc) to enable computerized image analysis with Adobe Photoshop and National Institutes of Health Image software. The luminal, intimal, medial, and outer wall areas were traced manually and quantified, which allowed for the calculation of the intimal/medial area ratio. The ratio was determined as the average value of 6 sections from each rabbit aorta.

The cellular components of the atherosclerotic lesion in the thoracic aorta were examined as follows. Macrophages in the lesion were stained immunohistochemically with a Vectorstain Universal ABC kit (Vector Laboratories) using an antimacrophage antibody (RAM-11, Dako) as the primary antibody and a biotin-labeled ABC kit (Vector Laboratories) using an antimacrophage antibody which allowed for the calculation of the intimal:medial area ratio. The method of determining the intimal:medial area ratio of the aorta. These slides were scanned microscopically (Keyence, Inc) to enable computerized image analysis with Adobe Photoshop and National Institutes of Health Image software. The luminal, intimal, medial, and outer wall areas were traced manually and quantified, which allowed for the calculation of the intimal/medial area ratio. The ratio was determined as the average value of 6 sections from each rabbit aorta.

Measurement of Tetrahydrobiopterin in Aortic Segments

Measurement of tetrahydrobiopterin (BH₄) was performed by high-performance liquid chromatography analysis after iodine oxidation under acidic or alkaline conditions, as described previously. Briefly, aortic segments from rabbits treated with the vehicle (control), aliskiren, valsartan, or aliskiren+valsartan were harvested, snap-frozen in liquid nitrogen or dry ice, and stored at −80°C. The frozen segments were divided into 2 fractions of known weight, one of which was suspended in HCl (0.25 mL, 0.1 N) and the other of which was suspended in NaOH (0.3 mL, 0.1 N). A solution of 4% iodine/8% KI was added to each fraction, which was kept on ice and protected from light. Each fraction was sonicated twice in a water/ice bath for 1 minute at 25% sonicator full power to break the cells. After 90-minute incubation at room temperature, 50 μL of 50% ascorbate were added to remove the excess iodine, and the sample was centrifuged at 14 000 rpm for 10 minutes to remove tissue debris. After adjustment of the pH to 4.0 with HCl, the supernatants were injected onto a Kromasil C-18 column equilibrated with phosphate buffer (0.15 mmol/L, pH 6.4), with a mobile phase of 5% methanol/95% water as a solvent at a flow rate of 1.0 mL/min. The fluorescence detector was set at 350 nm for excitation and 450 nm for emission. The BH₄ levels were determined from the difference between the total (BH₄+dihydrobiopterin+biopterin) and alkaline-stable oxidized (dihydrobiopterin+biopterin) compounds.

Statistical Analysis

All of the data were expressed as means±SEMs based on 6 independent experiments. Differences between groups were analyzed by ANOVA, followed by the Scheffé test, and were considered to be significant when the P value was <0.05.

Results

Table 1. Final Hemodynamic Data by Group

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
<th>BW, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (control)</td>
<td>78.7±2.2</td>
<td>178±3</td>
<td>2.49±0.04</td>
</tr>
<tr>
<td>Aliskiren</td>
<td>73.8±1.3</td>
<td>173±2</td>
<td>2.47±0.03</td>
</tr>
<tr>
<td>Valsartan</td>
<td>74.2±1.1</td>
<td>178±3</td>
<td>2.50±0.03</td>
</tr>
<tr>
<td>Aliskiren+valsartan</td>
<td>72.1±1.5</td>
<td>175±2</td>
<td>2.49±0.03</td>
</tr>
</tbody>
</table>

Plasma Renin Activity

PRA was significantly lower in the aliskiren group than in the control group (Figure 1). On the other hand, PRA was significantly greater in the valsartan group than in the control group. Aliskiren+valsartan cotreatment neutralized PRA almost to the levels achieved in the controls (Figure 1).

Inflammatory Mediators

There were no significant differences in the mean values of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1 levels between any of the groups at baseline (Table 2). After the 8-week treatment, the levels of these 3 inflammatory mediators with each drug treatment, but not vehicle, had a significant decrease compared with basal (P<0.01; Table 2). Furthermore, at the 8-week treatment, circulating levels of these 3 inflammatory mediators were significantly lower with each drug treatment than with the control treatment (P<0.01; Table 2). Interestingly, the levels of 3 inflammatory mediators were significantly lower with the aliskiren+valsartan group than with aliskiren or valsartan alone (Table 2).

Vascular Responses

In all of the rabbits, ACh-induced vasorelaxation was observed (Figure S1). The vasorelaxation in the vehicle (control)–treated WHHL rabbits was significantly lower than that in the drug-treated rabbits (Figure S1). Of note, vasorelaxation in the aliskiren+valsartan–treated rabbit was significantly greater than that in the either aliskiren- or valsartan-treated rabbit (Figure S1). Additional observation demonstrated that pretreatment with the NO synthase inhibitor N'-nitro-L-arginine methyl ester significantly inhibited ACh-induced vasorelaxation in the aliskiren–treated rabbit (Figure S1). On the other hand, the vasodilator response to N-methyl-N'-nitro-N-nitrosoguanidine was similar in all of the groups, with the strongest response in the aliskiren+valsartan–treated rabbits (Figure S1).

ACh-Induced Increase in NO Synthesis

Endothelial function was determined based on ACh-induced NO synthesis (Figure 2A). Intra-aortic ACh infusion (4 μg/kg per minute for 5 minutes) increased the plasma NO concentration, as shown by the peak response (Figure 2B) and the integrated response over the entire time course (Figure 2C). Both types of responses were significantly greater with each drug treatment than with the control treatment (P<0.01). Furthermore, the increase in
the plasma NO concentration was significantly greater with aliskiren+valsartan than with aliskiren or valsartan alone (P<0.01; Figure 2B and 2C).

**Basal NO Synthesis**
The effect of treatment on local basal NO concentration was evaluated based on the decrease in NO concentration in the presence of 5 mg/kg of L-NMMA (Figure 3A). All of the drug treatments caused significant decreases in the basal NO concentrations when compared with those in the controls (P<0.01). The L-NMMA–induced decrease in the basal peak NO concentration was significantly greater with aliskiren+valsartan than with aliskiren or valsartan alone (P<0.01; Figure 3B). Similar results were obtained for the 1-NMMA–induced change in the basal-integrated plasma NO concentration (Figure 3C).

**Vascular eNOS, Akt, and Hsp90 Protein Expressions**
The test groups showed significantly higher vascular eNOS, phospho-eNOS, and phospho-Akt but not Akt and Hsp90 expressions than the control group (Figure 4). Aliskiren+valsartan cotreatment resulted in significantly higher vascular eNOS, phospho-eNOS, and phospho-Akt than either aliskiren or valsartan alone.

**Change in Vascular Nitrotyrosine**
Vascular nitrotyrosine was measured as a surrogate index of vascular peroxynitrite and was significantly lower in the test groups than in the control group (Figure 5). Vascular nitrotyrosine levels were significantly lower with the aliskiren+valsartan cotreatment than with aliskiren or valsartan alone (P<0.01). This demonstrates an additive effect of the combination treatment on decreasing peroxynitrite production by reducing the reaction between superoxide and NO, thereby increasing NO bioavailability.

**Vascular Reactive Oxygen Species**
Superoxide anion formation was significantly lower in the test groups than in the control group (Figure S2A). The aliskiren+valsartan cotreatment produced a significantly lower level of superoxide production than either aliskiren...
or valsartan alone. Incubation of vessels from WHHL with L-NNA decreased superoxide production, compatible with an uncoupling of NOS (Figure S2A). In contrast, L-NNA incubation of vessels from WHHL rabbits treated with aliskiren increased superoxide production, compatible with a prevention of NOS uncoupling (Figure S2A). The activities of reduced nicotinamide-adenine dinucleotide phosphate–dependent oxidases were significantly lower in the test groups than in the control group, and the aliskiren and valsartan cotreatment produced a significantly lower level of oxidase activity than either aliskiren or valsartan alone (Figure S2B). In addition, gp91phox protein expression, a component of reduced nicotinamide-adenine dinucleotide phosphate oxidase, was significantly lower in drug-treated animals than in the control (Figure 6). The aliskiren and valsartan cotreatment produced a significantly lower level of gp91phox protein than either aliskiren or valsartan alone (Figure 6).

Atherosclerotic Plaque Formation
We determined atherosclerotic plaque formation in the typical histological sections of the thoracic aorta of WHHL rabbits. En face oil red O staining revealed that the plaque area was significantly smaller in animals treated with aliskiren or valsartan alone than in the controls and was decreased further by the aliskiren+valsartan cotreatment (Figure 7A and 7B). Atherosclerotic changes were also quantified by calculating the intimal:medial area ratio in the sections (Figure 7C). The ratio was smaller in animals treated with aliskiren or valsartan alone than in the controls and was decreased further by the aliskiren+valsartan cotreatment (Figure 7C). Immunostaining of the sections with the monoclonal antibody RAM-11, a macrophage marker, revealed that the plaque was almost exclusively composed of macrophages, regardless of the type of treatment (data not shown).

Vascular BH4 Levels
BH4 is of fundamental importance for normal endothelial NO synthase activity, and all of the drug treatments significantly increased the BH4 levels (Figure 8) when compared with those in the controls. The BH4 levels in the thoracic aorta were significantly higher with aliskiren+valsartan than with aliskiren or valsartan alone (P<0.01; Figure 8).

Discussion
Using a catheter-type NO sensor, we demonstrated for the first time that aliskiren administration increases ACh-induced and basal plasma NO concentrations in WHHL rabbits.

Figure 2. ACh-induced plasma NO production in the aorta of WHHL rabbits. A, Typical changes in plasma NO concentration induced by 4 μg/kg per minute of ACh infusion (5 minutes) in the thoracic aorta of WHHL rabbits (n=6 in each group) treated with vehicle (control), aliskiren, valsartan, or aliskiren+valsartan combination for 8 weeks. ACh-induced increases in the NO concentration measured by the peak response (B) and the integrated response over the entire time course (C) are also shown (n=6 in each group). Bars represent mean±SEM values. *P<0.01 vs control.
Moreover, aliskiren+/valsartan cotreatment increased the ACh-induced and basal plasma NO concentrations to a significantly greater extent than either aliskiren or valsartan alone. In addition, in terms of endothelial-dependent vasodilation, aliskiren+/valsartan cotreatment significantly improved ACh-induced vasorelaxation more than either aliskiren or valsartan alone. Finally, we found that aliskiren+/valsartan cotreatment was more effective than either drug alone in protecting against atherosclerotic lesion formation.

Endothelial function was traditionally quantified by measuring secondary effects of NO, such as biologically inactive products of NO (nitrite and nitrate) or flow-dependent endothelium-mediated vasodilation. On the other hand, our catheter-type NO sensor can directly measure intra-arterial NO concentration in vivo. The sensitivity and specificity in the NO sensor was reported previously. When NO was reacted with the superoxide produced by xanthine and xanthine oxidase, the measured NO current decreased quickly, demonstrating that NO instantaneously reacted with the superoxide, and the sensor measured changes in NO concentration at high selectivity and temporal resolution.

The RAS has emerged as a key target for antihypertensive therapy, with major clinical outcome trials showing the effectiveness of ACE inhibitors and ARBs in reducing cardiovascular events in a broad range of patients. However, RAS blockade with ACE inhibitors or ARBs does not ensure appropriate control of cardiovascular events. We found that the addition of aliskiren, a direct renin inhibitor, to the ARB valsartan improves NO bioavailability and protects against spontaneous atherosclerotic change in the WHHL rabbit. This finding might support a possibility that an ARB alone does not effectively suppress the RAS. Both ACE inhibitors and ARBs attenuate the inhibition of renin release, which occurs via negative feedback by inhibiting the production or action of Ang II, resulting in increased plasma renin concentration and PRA. In fact, in the present study, valsartan treatment significantly increased PRA compared with that in controls, whereas aliskiren+/valsartan cotreatment neutralized it almost to the levels in the controls. These results are in agreement with those of a previous clinical study. Azizi et al have shown that aliskiren, in combination with valsartan, neutralized the compensatory rise in PRA and Ang II levels induced by ARBs, suggesting that a direct renin inhibitor might be able to enhance the efficacy of the existing RAS inhibitors.

The improvement in NO bioavailability achieved with aliskiren treatment could be explained by several potential

Figure 3. Basal plasma NO concentration. A, Typical changes in plasma NO concentration under L-NMMA infusion (10 minutes) in the thoracic aorta of WHHL rabbits treated with vehicle (control), aliskiren, valsartan, or aliskiren+/valsartan combination for 8 weeks. Basal plasma NO concentrations measured by the peak response (B) and the integrated response over the entire time course (C) are also shown (n=6 in each group). Bars represent mean±SEM values (n=6). *P<0.01 vs control.
mechanisms. First, aliskiren may modulate the influence of Ang II on superoxide-generating oxidases. The present study showed that aliskiren treatment decreased PRA, and this action was accompanied by a reduction in superoxide production and peroxynitrite levels. Both superoxide and peroxynitrite have been demonstrated to oxidize BH4, a critical eNOS cofactor, leading to eNOS uncoupling.27 Oelze et al18 demonstrated eNOS uncoupling in WHHL rabbits by conducting lucigenin (5 μmol/L)-derived chemiluminescence experiments with and without L-NNA. That is, they showed

Figure 4. eNOS and associated protein expressions in WHHL rabbits. Expressions of eNOS (A), phospho-eNOS (B), Akt (right) and phospho-Akt (left; C), and Hsp90 (D) in the thoracic aorta of WHHL rabbits after treatment with vehicle (control), aliskiren, valsartan, or aliskiren + valsartan combination (top). Densitometric intensity corresponding with each band was normalized using α-tubulin expression (A, B, C, and D). Data are expressed as a ratio of the test value to the control value. Bars represent mean±SEM values. *P<0.01 vs control.
that incubation of aortic tissue from WHHL with L-NNA decreased rather than increased superoxide signals identifying eNOS as a significant superoxide source. In contrast, incubation of vessels from ARB irbesartan-treated WHHL rabbits with L-NNA increased vascular superoxide rather than decreased it, which indicates that ARB may reduce oxidative stress at least in part by preventing eNOS uncoupling. This study showed that incubation of vessels from aliskiren-treated rabbits with L-NNA increased vascular superoxide rather than decreased it (compatible with changes observed in controls), which indicates for the first time that aliskiren, similar to treatment with ARB, was able to prevent eNOS uncoupling. Interestingly, Laursen et al28 have shown that peroxynitrite is much more potent than superoxide in oxidizing BH4. In the present study, we showed that aliskiren or valsartan increased vascular BH4 levels in WHHL rabbits. However, the increase in vascular BH4 levels was significantly greater with the aliskiren + valsartan cotreatment than with aliskiren or valsartan alone. In the state of eNOS uncoupling, electron flow through the enzyme results in reduction of molecular oxygen at the prosthetic heme site rather than NO formation.29 Taken together, our findings in WHHL rabbits suggest that the inhibitory effects of aliskiren on the peroxynitrite and superoxide production would, at least in part, result in potentiated NO bioavailability through the suppression of NO breakdown. Second, this study showed that aliskiren treatment significantly upregulated eNOS phosphorylation, which is crucial to eNOS activity, and Akt phosphorylation. We also showed that aliskiren + valsartan cotreatment resulted in significantly higher vascular eNOS phosphorylation and Akt phosphorylation than either aliskiren or valsartan alone. Increasingly, eNOS is being shown to be highly regulated by posttranscriptional modifications that involve Akt phosphorylation, as well as Hsp90, among others. In fact, the Hsp90-Akt-eNOS complex is believed to be integral to eNOS-dependent NO production. Finally, aliskiren might modulate the direct influence of renin or prorenin on NO bioavailability. Indeed, Nguyen et al34 have demonstrated the existence of a renin/prorenin receptor. The renin receptor can trigger intracellular signals by activating the extracellular signal–regulated kinase 1/2 pathway, and it also acts as a cofactor by increasing the efficiency of angiotensinogen cleavage by receptor-bound renin, thereby facilitating Ang II generation and action on the cell surface. However, we could not elucidate the extent to which renin receptor activation affects endothelial dysfunction. Additional studies are required to clarify this issue.

The present histological analysis has demonstrated that both aliskiren and valsartan reduced the plaque area. More importantly, aliskiren + valsartan cotreatment dramatically reduced the plaque area. eNOS dysfunction was shown to accelerate atherosclerotic lesion formation in mice, whereas eNOS overexpression in mice with hypercholesterolemia resulted in increased eNOS-derived superoxide production and promotion of atherogenesis. In this study, as mentioned above, aliskiren may reduce oxidative stress in part by preventing eNOS uncoupling. We also showed that both aliskiren and the combination aliskiren + valsartan significantly reduced inflammatory mediators, such as intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and monocyte chemoattractant protein-1, compared with those of controls. Interestingly, aliskiren + valsartan cotreatment resulted in significantly lower levels of these inflammatory mediators than either aliskiren or valsartan alone. Taken together, the inhibiting effects of both aliskiren and valsartan on oxidative stress, as well as inflammatory mediators, may result in the reduction of the plaque area in WHHL rabbits.

In conclusion, this study showed for the first time that aliskiren + valsartan cotreatment may have beneficial effects on NO bioavailability and may protect against spontaneous atherosclerotic changes in WHHL rabbits.
Perspectives
Although ACE inhibitors and ARBs have provided an excellent starting point for therapies targeting the RAS, clinical trial evidence indicates that there is a significant scope for testing whether increased and more comprehensive RAS suppression could yield additional benefits. The present results might provide an experimental rationale for the combined application of ARBs and renin inhibitors in the treatment of hypertension and related cardiovascular diseases. The catheter-type NO sensor is potentially useful as a tool for investigating the relationship between increased NO bioavailability and reduced atherosclerosis. Importantly, the NO sensor may be applied to clinically evaluate endothelial function, ie, reduced endothelium-derived NO bioavailability in patients with cardiovascular diseases. Future studies are needed to ascertain whether combined treatment with ARBs and renin inhibitors could improve NO bioavailability (tested with the NO sensor) and induce plaque regression and/or stabilization (tested using intravascular ultrasound and optical coherence tomography) more effectively than monotherapy.

Disclosures
None.

References


Renin Inhibitor Aliskiren Improves Impaired Nitric Oxide Bioavailability and Protects Against Atherosclerotic Changes
Toshio Imanishi, Hiroto Tsujioka, Hideyuki Ikejima, Akio Kuroi, Shigeo Takarada, Hironori Kitabata, Takashi Tanimoto, Yasuteru Muragaki, Seiichi Mochizuki, Masami Goto, Kiyoshi Yoshida and Takashi Akasaka

_Hypertension_. 2008;52:563-572; originally published online July 21, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.111120

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/52/3/563

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2008/07/21/HYPERTENSIONAHA.108.111120.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org//subscriptions/
The Renin Inhibitor Aliskiren Improves Impaired NO Bioavailability and Protects against Atherosclerotic Changes

Toshio Imanishi1*, Hiroto Tsujioka1 Hideyuki Ikejima1, Akio Kuroi1, Shigeho Takarada1, Hironori Kitabata1, Takashi Tanimoto1, Yasuteru Muragaki2, Seiichi Mochizuki3, Masami Goto3, Kiyoshi Yoshida4, and Takashi Akasaka1

1. Department of Cardiovascular Medicine, Wakayama Medical University, Wakayama, Japan
2. Department of Pathology, Wakayama Medical University, Wakayama, Japan
3. Department of Medical Engineering, Kawasaki Medical School, Okayama, Japan
4. Division of Cardiology, Kawasaki Medical School, Okayama, Japan

Running Title: Aliskiren and valsartan combination treatment

Tables: 2
Figures: 10 (including 2 supplement figures)
Total pages: 38
Word count: 5921

*Address correspondence to:
Toshio Imanishi, M.D., PhD
Department of Cardiovascular Medicine
Wakayama Medical University
811-1, Kimiidera, Wakayama City
Wakayama 641-8510
Japan
Telephone: +81-73-441-0621
Fax: +81-73-446-0631
E-mail: t-imani@wakayama-med.ac.jp
Arterial rings (5 mm long) were cut and suspended from strain gauges to measure the isometric circumferential force in an organ chamber (25 mL) filled with Tyrode’s solution consisting of 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl₂, 1.1 mmol/L MgCl₂, 0.42 mmol/L NaH₂PO₄, 12 mmol/L NaHCO₃, and 5.7 mmol/L glucose, pH 7.4. Aortic rings were precontracted with $1 \times 10^{-6}$ mol/L of phenylephrine 30 min after being incubated with or without N⁶-nitro-L-arginin methyl ester (L-NAME; 100 μmol/L). Once a stable contraction was obtained, ACh, an agent that induces vasorelaxation via stimulation of NO production from the endothelium, was added to the bath in cumulative concentrations of $10^{-9}$-$10^{-6}$ mol/L to determine endothelial function and agonist-stimulated NO production for the endothelium. Endothelium-independent relaxation by nitroglycerin (NTG) ($10^{-9}$-$10^{-6}$ mol/L) was also examined. Once 1 experiment was completed, more than a 1h interval was obtained before starting the next experiment. The maximal response attained at each concentration was used in the calculation. ACh-induced vasorelaxation in phenylephrine-precontracted thoracic arteries treated with vehicle (control) (open circle), aliskiren (40 mg/kg/day) (closed circle), valsartan (5 mg/kg/day) (open triangle), aliskiren and valsartan in combination (closed triangle) for 8 weeks. In addition, to investigate possible mechanisms responsible for aliskiren-induced vasorelaxation, the arterial rings were contracted 30 min being incubated with L-NAME, followed by ACh addition (aliskiren+L-NAME; open square). The results are given as the percentage of the maximal relaxation for ACh (left) or nitroglycerin (NTG) (right). *$P < 0.01$ vs. control. #$P < 0.01$ vs aliskiren only and valsartan only groups. **$P < 0.01$ vs aliskiren only group.
S2

O2\textsuperscript{−} production of control and aliskiren with or without L-NNA, valsartan alone, or aliskiren plus valsartan combination. Bars represent mean ± SEM values (n = 6 in each group). *P < 0.01 vs. control. 

NADPH oxidase activity in the different experimental groups. A 10% vessel homogenate was prepared in 50 nmol/L phosphate buffer by homogenizing the aortic segments in a glass-to-glass motorized homogenizer. The homogenizing buffer consisted of a 50-mM phosphate buffer containing 0.01 mM EDTA. The homogenate was subjected to low-speed centrifugation (1,000 g) for 10 min to remove unbroken cells and debris. Aliquots of the supernatant were then added to the scintillation vials containing lucigenin (5 μmol/L) in 2 mL buffer B. Over the ensuing 5 min, chemiluminescence occurring in response to the addition of nicotinamide adenine dinucleotide (NADPH) (both 100 μ mol/L) was recorded by a luminometer (LB9505; Berthold Technologies, Germany). Values were standardized to the amount of protein present, which was measured using a commercially available kit (DC Protein Assay; BioRad Laboratories). NADPH oxidase activities in aortic homogenates after treatment with vehicle (control), aliskiren, valsartan, or aliskiren plus valsartan combination for 8 weeks. Bars represent mean ± SEM values (n = 6 in each group). *P < 0.01 vs. control.