Primary Aldosteronism

Effect of Aldosterone-Producing Adenoma on Endothelial Function and Rho-Associated Kinase Activity in Patients With Primary Aldosteronism

Takeshi Matsumoto, Kenji Oki, Masato Kajikawa, Ayumu Nakashima, Tatsuya Maruhashi, Yumiko Iwamoto, Akimichi Iwamoto, Nozomu Oda, Takayuki Hidaka, Yasuki Kihara, Nobuoki Kohno, Kazuaki Chayama, Chikara Goto, Yoshiki Aibara, Kensuke Noma, James K. Liao, Yukihito Higashi

Abstract—The purpose of this study was to evaluate vascular function and activity of Rho-associated kinases (ROCKs) in patients with primary aldosteronism. Vascular function, including flow-mediated vasodilation (FMD) and nitroglycerine-induced vasodilation, and ROCK activity in peripheral leukocytes were evaluated in 21 patients with aldosterone-producing adenoma (APA), 23 patients with idiopathic hyperaldosteronism (IHA), and 40 age-, sex-, and blood pressure–matched patients with essential hypertension (EHT). FMD was significantly lower in the APA group than in the IHA and EHT groups (3.2±2.0% versus 4.6±2.3% and 4.4±2.2%; P<0.05, respectively), whereas there was no significant difference in FMD between the IHA and EHT groups. There was no significant difference in nitroglycerine-induced vasodilation in the 3 groups. ROCK activity was higher in the APA group than in the IHA and EHT groups (1.29±0.57 versus 1.00±0.46 and 0.81±0.36l; P<0.05, respectively), whereas there was no significant difference in ROCK activity between the IHA and EHT groups. FMD correlated with age (r=−0.31; P<0.01), plasma aldosterone concentration (r=−0.35; P<0.01), and aldosterone:renin ratio (r=−0.34; P<0.01). ROCK activity correlated with age (r=−0.24; P=0.04), plasma aldosterone concentration (r=0.33; P<0.01), and aldosterone:renin ratio (r=0.46; P<0.01). After adrenalectomy, FMD and ROCK activity were restored in patients with APA. APA was associated with both endothelial dysfunction and increased ROCK activity compared with those in IHA and EHT. APA may have a higher risk of future cardiovascular events. (Hypertension. 2015;65:841-848. DOI: 10.1161/HYPERTENSIONAHA.114.05001.) • Online Data Supplement

Key Words: aldosterone ■ hyperaldosteronism ■ hypertension ■ Rho-associated kinases

Primary aldosteronism (PA) is one of the most common causes of secondary hypertension. The prevalence of cardiovascular events is higher in patients with PA than in patients with essential hypertension (EHT).1-3 Endothelial dysfunction is established in the initial step of atherosclerosis, leading to the development of atherosclerosis.3 In addition, it is well known that endothelial dysfunction is an independent predictor of cardiovascular events.4 Hypertension is associated with endothelial dysfunction and that circulating aldosterone levels significantly correlated with endothelial function.5,6 However, there is little information on the relationship between subtype of PA and grade of vascular function. In addition, the prevalence of cardiovascular events in patients with aldosterone-producing adenoma (APA) and patients with idiopathic hyperaldosteronism (IHA) remains unclear.

Rho-associated kinases (ROCKs), one of the first downstream targets of the small GTP-binding protein RhoA,
mediate various cellular physiological functions. Elevated ROCK activity would play an important pathophysiological role in the development and maintenance of hypertension. It has been reported that an increase in ROCK activity is associated with cardiovascular diseases, including hypertension. In addition, we have shown that leukocyte ROCK activity may be a new biomarker of cardiovascular events. Interestingly, it has been shown that activation of the RhoA/ROCK pathway impairs NO bioavailability through inhibition of endothelial NO synthase (eNOS) mRNA stability and eNOS protein phosphorylation at Ser 1177 via the Akt/PI3K pathway, suggesting the existence of an interaction between the eNOS/NO pathway and ROCK activity.

The purpose of this study was to evaluate vascular function, endothelium-dependent vasodilation induced by flow-mediated vasodilation (FMD) and endothelium-independent vasodilation induced by sublingual administration of nitroglycerin, and leukocyte ROCK activity before and after adrenalectomy in patients with APA compared with that in patients with IHA and patients with EHT.

Methods

Study Protocol 1: Vascular Function and ROCK Activity in Patients With EHT and Patients With PA
We studied 21 patients with APA (9 men and 12 women; mean age: 51±14 years), 23 patients with IHA (12 men and 11 women; mean age: 56±10 years), and 40 age-, sex-, and blood pressure–matched patients with EHT (24 men and 16 women; mean age: 53±11 years). Subjects were enrolled from the Hiroshima University Hypertension Database. The ethical committees of our institutions approved the study protocol. Written informed consent for participation in the study was obtained from all of the subjects.

Additional details on EHT are available in the online-only Data Supplement. PA, including the classification of PA, was defined according to the report of the guidelines for diagnosis and treatment of PA: the Japan Endocrine Society 2009 (additional details are available in the online-only Data Supplement). Additional details on study protocol are available in the online-only Data Supplement.

Study Protocol 2: Effect of Adrenalectomy on Vascular Function and ROCK Activity in Patients With APA
FMD, nitroglycerin-induced vasodilation, and ROCK activity were evaluated in the same manner as that in study 1 before adrenalectomy and at 12 weeks after this procedure in 12 of the 21 patients with APA (3 men and 9 women; mean age: 41±10 years). The surgical approach for adrenalectomy was laparoscopic adrenalectomy in all patients.

Measurement of FMD and Nitroglycerin-Induced Vasodilation
FMD and nitroglycerin-induced vasodilation were measured by ultrasonography with an automated edge tracking system (UNEXEF18G, UNEX) as previously described (additional details are available in the online-only Data Supplement).

Measurement of ROCK Activity
ROCK activity was assayed in peripheral blood leukocytes as the amount of phospho-Thr853 in the myosin-binding subunit of myosin light-chain phosphatase as previously described (additional details are available in the online-only Data Supplement).

Statistical Analysis
Results are presented as means±SD. All reported probability values were 2-sided, and a probability value of <0.05 was considered statistically significant. Categorical variables were compared by means of χ² test. Continuous variables were compared by using ANOVA for multiple groups. Relationships between variables were determined by Spearman correlation coefficients analysis. Multivariate regression analyses were performed to identify factors associated with FMD and ROCK activity in risk factors and laboratory data. The data were processed using the software package Stata version 9 (Stata Co., College Station, TX).

Results

Study Protocol 1: Vascular Function and ROCK Activity in Patients With EHT and Patients With PA
The baseline clinical characteristics of the 40 patients with EHT, 21 patients with APA, and 23 patients with IHA are summarized in Table 1. Plasma aldosterone concentration (PAC) was significantly higher in patients with APA than in patients with IHA or EHT. There was no significant difference in PAC between patients with IHA and patients with EHT. Plasma renin activity was significantly lower in patients with APA or IHA than in patients with EHT. There was no significant difference in plasma renin activity between patients with APA and patients with IHA. Aldosterone:renin ratio (ARR) was significantly higher in patients with APA or IHA than in patients with EHT and was higher in patients with APA than in patients with IHA. Serum potassium concentration was significantly lower in patients with APA than in patients with IHA or EHT. There was no significant difference in serum potassium concentration between patients with IHA and patients with EHT. The other parameters were similar in the groups.

FMD was significantly lower in the APA group than in the IHA group and EHT group (3.2±2.0% versus 4.6±2.3% and 4.4±2.2%; P<0.05, respectively), whereas there was no significant difference in FMD between the IHA group and EHT group (Figure 1A). There was no significant difference in nitroglycerin-induced vasodilation in the 3 groups (Figure 1B). FMD correlated with age (r=−0.29; P=0.007), PAC (r=−0.34; P=0.002; Figure S1A in the online-only Data Supplement), and ARR (r=−0.35; P=0.001; Figure S1B in the online-only Data Supplement). Nitroglycerin-induced vasodilation did not correlate with PAC (r=−0.19; P=0.11; Figure S1C in the online-only Data Supplement) or ARR (r=−0.13; P=0.24; Figure S1D in the online-only Data Supplement), or with any of the other parameters. Multivariate analysis revealed that age, PAC, and ARR were independent predictors of FMD (Table 2).

ROCK activity was significantly higher in the APA group than in the IHA group and EHT group (1.29±0.57 versus 1.00±0.46 and 0.81±0.36; P<0.001 and P=0.04, respectively), whereas there was no significant difference in ROCK activity between the IHA group and EHT group (Figure 2). ROCK activity correlated with PAC (r=0.33; P=0.004; Figure S2A in the online-only Data Supplement), ARR (r=0.46; P<0.001; Figure S2B in the online-only Data Supplement), and serum potassium concentration (r=−0.25; P=0.03). Multivariate analysis revealed that PAC and ARR were independent predictors of ROCK activity (Table 2).
Table 1. Clinical Characteristics of the Subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>EHT, n=40</th>
<th>IHA, n=23</th>
<th>APA, n=21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men, n (%)</td>
<td>24 (60.0)</td>
<td>12 (52.2)</td>
<td>9 (42.9)</td>
</tr>
<tr>
<td>Age, y</td>
<td>53±11</td>
<td>56±10</td>
<td>51±14</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.3±3.6</td>
<td>26.1±4.9</td>
<td>24.4±3.4</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>144.0±20.3</td>
<td>141.6±19.5</td>
<td>141.2±16.2</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>85.9±12.3</td>
<td>81.3±12.3</td>
<td>85.8±9.4</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>71.4±12.6</td>
<td>71.3±11.4</td>
<td>74.2±11.4</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>206.0±33.0</td>
<td>206.3±35.5</td>
<td>194.9±37.2</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>170.4±81.8</td>
<td>154.0±75.9</td>
<td>136.4±68.5</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mg/dL</td>
<td>57.9±13.8</td>
<td>58.9±13.6</td>
<td>51.9±11.6</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol, mg/dL</td>
<td>123.0±32.4</td>
<td>126.5±31.7</td>
<td>123.4±33.9</td>
</tr>
<tr>
<td>Serum potassium, mg/dL</td>
<td>4.1±0.4</td>
<td>3.9±0.3</td>
<td>3.4±0.7†</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>112.4±30.8</td>
<td>106.5±19.4</td>
<td>103.5±20.5</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.9±0.7</td>
<td>5.9±0.7</td>
<td>5.8±0.8</td>
</tr>
<tr>
<td>Plasma aldosterone concentration, ng/mL</td>
<td>13.7±5.4</td>
<td>15.6±5.8</td>
<td>35.3±28.3†</td>
</tr>
<tr>
<td>Plasma renin activity, ng/mL/h</td>
<td>2.0±1.8</td>
<td>0.5±0.2†</td>
<td>0.4±0.2‡</td>
</tr>
<tr>
<td>Aldosterone:renin ratio</td>
<td>10.6±5.9</td>
<td>36.1±8.4†</td>
<td>114.9±77.8‡</td>
</tr>
<tr>
<td>Medical history, n (%)</td>
<td>40 (100)</td>
<td>23 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>40 (100)</td>
<td>23 (100)</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>20 (50.0)</td>
<td>10 (43.5)</td>
<td>11 (52.4)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7 (17.5)</td>
<td>6 (26.1)</td>
<td>5 (23.8)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>18 (46.2)</td>
<td>10 (43.5)</td>
<td>7 (33.3)</td>
</tr>
<tr>
<td>Medications, n (%)</td>
<td>20 (50.0)</td>
<td>16 (70.0)</td>
<td>14 (66.7)</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>10 (25.0)</td>
<td>4 (17.4)</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Renin angiotensin system inhibitors</td>
<td>2 (5.0)</td>
<td>2 (8.7)</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>2 (5.0)</td>
<td>2 (8.7)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>Alpha blockers</td>
<td>2 (5.0)</td>
<td>2 (8.7)</td>
<td>2 (9.5)</td>
</tr>
<tr>
<td>Statins</td>
<td>7 (17.5)</td>
<td>3 (13.0)</td>
<td>3 (14.3)</td>
</tr>
<tr>
<td>Duration of hypertension, y</td>
<td>8.4±7.4</td>
<td>8.2±7.1</td>
<td>8.7±9.3</td>
</tr>
</tbody>
</table>

All results are presented as mean±SD. APA indicates aldosterone-producing adenoma; EHT essential hypertension; HbA1c, hemoglobin A1c; IHA, idiopathic hyperaldosteronism.

*P<0.01 vs IHA.
†P<0.01 vs EHT.
‡P<0.05 vs EHT.

Study Protocol 2: Effect of Adrenalectomy on Vascular Function and ROCK Activity in Patients With APA

The baseline clinical characteristics before and after adrenalectomy of the 12 patients with APA are summarized in Table 3. Adrenalectomy significantly decreased systolic blood pressure and PAC and significantly increased plasma renin activity, ARR and serum potassium concentration.

After adrenalectomy, FMD was enhanced from 3.6±2.0% to 5.0±2.5% (P=0.005; Figure 3A), whereas nitroglycerine-induced vasodilation was not significantly altered from 12.1±4.8% to 14.6±5.8% (P=0.15). The increase in FMD correlated significantly with the decrease in PAC (r=−0.42; P=0.02; Figure 4B). No significant correlation was found between the increase in FMD and changes in blood pressure or variables, such as lipid, glucose, and insulin concentrations, or between these variables and the increase in nitroglycerine-induced vasodilation.

Leukocyte ROCK activity was significantly lower after adrenalectomy than before adrenalectomy (1.09±0.41 versus 1.42±0.62; P=0.02; Figure 3B). The decrease in ROCK activity correlated significantly with the decrease in ARR (r=0.58; P=0.003; Figure 4D) but not with the decrease in PAC (r=0.34; P=0.10; Figure 4C). No significant correlation was found between the decrease in ROCK activity and changes in blood pressure or variables, such as lipid, glucose, insulin, and potassium concentrations.

Discussion

In this study, we demonstrated for the first time that (1) endothelial function was impaired in patients with APA compared with that in patients with IHA or EHT, whereas endothelial function was similar in patients with IHA and patients with EHT and it was impaired to a greater extent in relation to levels of PAC or ARR, (2) leukocyte ROCK activity was increased in patients with APA compared with that in patients with IHA or EHT, whereas leukocyte ROCK activity was similar in patients with IHA and patients with EHT and it was increased to a greater extent in relation to levels of PAC or ARR, and (3) surgical resection of APA improved endothelial function and inhibited leukocyte ROCK activity in patients with APA. There were significant correlations between improvement of FMD and ROCK activity and decrease in PAC or ARR.

Endothelial Function and Aldosterone

Patients with PA are ideal models for the study of how endothelial function is altered in the presence of excess vasoconstricting and proatherosclerotic factors. In this study, excess aldosterone blunted FMD as an index of endothelium-dependent vasodilation in patients with APA, whereas nitroglycerine-induced vasodilation was similar in patients with APA, IHA, and EHT, indicating that endothelial function, but not smooth muscle function, is selectively impaired and is restored after adrenalectomy in patients with APA.

Previous studies showing that PA is associated with endothelial dysfunction and that there is a significant relationship between PAC and endothelial function support our results. An imbalance between NO and aldosterone may directly result in conditions associated with endothelial dysfunction in humans. Aldosterone, as well as angiotensin II, plays an important role in the regulation of vascular function through the NO/eNOS pathway.

It has been reported that aldosterone attenuates eNOS activity through an increase in reactive oxygen species–induced eNOS uncoupling and protein phosphatase 2A–induced dephosphorylation of phospho-eNOS in a mineralocorticoid receptor–dependent manner in human umbilical vein endothelial cells.

The mineralocorticoid receptor blocker eplerenone improved endothelial function through enhancement of expression of the eNOS gene in Dahl salt–sensitive rats and...
2-kidney, 1 clip rats, suggesting that eplerenone directly enhances the NO/eNOS pathway. Inhibition of the aldosterone or mineralocorticoid receptor also may contribute to decrease in oxidative stress, resulting in the improvement of endothelial function through inhibition of NO inactivation. Eplerenone also improved FMD in patients with EHT. In addition, the nonselective mineralocorticoid receptor blocker spironolactone improved FMD and acetylcholine-induced vasodilation in patients with hyperaldosteronism and in patients with heart failure. In this study, there was a significant relationship between PAC and FMD in all patients, and there was a significant relationship between the decrease in PAC and improvement in FMD in patients with APA. These findings suggest that aldosterone per se or mineralocorticoid receptor may impair endothelial function through inactivation of the NO/eNOS pathway, including decrease in NO production and increase in NO inactivation.

Endothelial function becomes impaired as blood pressure increases, and the degree of dysfunction is related to the severity of hypertension. It is expected that endothelial dysfunction will be improved by antihypertensive therapy. However, several experimental and clinical studies have provided conflicting results about the relationship between reduction in blood pressure and improvement in endothelial function. Although adrenalectomy acutely decreased blood pressure in patients with APA in this study, changes in blood pressure did not correlate with improvement of FMD. In previous studies, we and other investigators have shown that although clinically effective antihypertensive therapy, such as antihypertensive drugs and aerobic exercise, restored resistance artery endothelial function of forearm circulation in patients with EHT, there is no significant correlation between the degree of reduction in blood pressure and the augmentation of endothelial function. Therefore, it is unlikely that a reduction in blood pressure per se is involved in the restoration of endothelial function in forearm circulation.

ROCK Activity and Aldosterone

Patients with PA are also ideal models for the study of how ROCK activity is altered in the presence of excess vasoconstricting and proatherosclerotic factors. In this study, leukocyte ROCK activity was increased in patients with APA compared with that in patients with IHA or EHT and was increased to a greater extent in relation to levels of PAC or ARR. The increase in ROCK activity was restored after adrenalectomy in patients with APA. These findings suggest that excess aldosterone plays a critical role in the increase in ROCK activity in humans.

ROCKs are one of the first downstream targets of the small GTP-binding protein RhoA. It is well known that angiotensin II is a potent stimulator of ROCK activity in endothelial cells or smooth muscle cells and subsequently modulates cell contraction, proliferation, apoptosis, and gene expression via several signaling pathways. ROCK activity is elevated in rats with aldosterone-induced hypertension, leading to vascular remodeling and tissue injury in the heart and kidney. In

### Table 2. Multivariate Analysis of Flow-Mediated Vasodilation and Rho-Associated Kinases Activity With Clinical Variables

<table>
<thead>
<tr>
<th>Variables</th>
<th>β</th>
<th>t Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow-mediated vasodilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>−0.26</td>
<td>−2.65</td>
<td>0.01</td>
</tr>
<tr>
<td>Men</td>
<td>0.01</td>
<td>0.09</td>
<td>0.93</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>0.05</td>
<td>0.43</td>
<td>0.68</td>
</tr>
<tr>
<td>Plasma aldosterone concentration, ng/dL</td>
<td>−0.33</td>
<td>−3.17</td>
<td>0.002</td>
</tr>
<tr>
<td>Flow-mediated vasodilation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>−0.32</td>
<td>−3.11</td>
<td>0.003</td>
</tr>
<tr>
<td>Men</td>
<td>−0.05</td>
<td>−0.51</td>
<td>0.61</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>0.05</td>
<td>0.45</td>
<td>0.66</td>
</tr>
<tr>
<td>Aldosterone:renin ratio</td>
<td>−0.38</td>
<td>−3.71</td>
<td>0.0004</td>
</tr>
<tr>
<td>Rho-associated kinase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>−0.14</td>
<td>−1.28</td>
<td>0.20</td>
</tr>
<tr>
<td>Men</td>
<td>−0.08</td>
<td>−0.73</td>
<td>0.47</td>
</tr>
<tr>
<td>Serum potassium, mEq/L</td>
<td>−0.14</td>
<td>−1.19</td>
<td>0.24</td>
</tr>
<tr>
<td>Plasma aldosterone concentration, ng/dL</td>
<td>0.30</td>
<td>2.65</td>
<td>0.01</td>
</tr>
<tr>
<td>Rho-associated kinase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td>−0.10</td>
<td>−0.98</td>
<td>0.33</td>
</tr>
<tr>
<td>Men</td>
<td>−0.04</td>
<td>−0.35</td>
<td>0.73</td>
</tr>
<tr>
<td>Serum potassium, mEq/L</td>
<td>−0.04</td>
<td>−0.35</td>
<td>0.73</td>
</tr>
<tr>
<td>Aldosterone:renin ratio</td>
<td>0.43</td>
<td>3.76</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Figure 1. Flow-mediated vasodilation (A) and nitroglycerine-induced vasodilation (B) in patients with essential hypertension (EHT), patients with aldosterone-producing adenoma (APA), and patients with idiopathic hyperaldosteronism (IHA).
addition, treatment with mineralocorticoid receptor blockers prevented cardiovascular injury through inhibition of ROCK activity in these animal models. We showed that in a previous blind, randomized, parallel group study, the mineralocorticoid receptor blocker eplerenone decreased leukocyte ROCK activity in patients with EHT. These findings suggest that aldosterone-induced activation of ROCK activity may be because of classical genomic actions that regulate gene transcription and protein synthesis through binding of aldosterone to the mineralocorticoid receptor. However, we cannot deny the possibility that nongenomic responses contribute to the aldosterone-induced activation of ROCK activity, especially under the condition of excess aldosterone. Future studies are needed to confirm the precise mechanisms by which the aldosterone/mineralocorticoid receptor is associated with activation of ROCK activity in vitro and in vivo and in a clinical setting.

Elevated ROCK activity would play an important pathophysiological role in the development and maintenance of hypertension. Hypertension is associated with activation of the Rho/ROCK pathway. Therefore, it is expected that increased ROCK activity will be improved by antihypertensive therapy. However, changes in blood pressure did not correlate with the decrease in ROCK activity in patients with APA. In previous studies, we showed that although clinically effective antihypertensive therapy using antihypertensive drugs, such as eplerenone and the calcium channel blocker nifedipine, decreased ROCK activity in patients with EHT, there was no significant correlation between the degree of reduction in blood pressure and decrease in ROCK activity. Therefore, it is unlikely that a reduction in blood pressure per se is involved in the restoration of leukocyte ROCK activity.

Some studies showed that either APA or IHA had a higher risk for target organ damage of the heart, brain, and kidneys than did EHT. However, unfortunately, there have been no large clinical trials in which differences in cardiovascular events between patients with APA and IHA were evaluated. Several lines of evidence have shown that endothelial function

### Table 3. Effect of 12 Weeks of Treatment With Adrenalectomy in Aldosterone-Producing Adenoma Group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Before Adrenalectomy, n=12</th>
<th>After Adrenalectomy, n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass index, kg/m²</td>
<td>23.9±3.1</td>
<td>23.5±2.9</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>140.8±17.4</td>
<td>127.6±8.1*</td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>88.4±8.5</td>
<td>82.6±9.0</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>75.7±13.3</td>
<td>75.4±10.6</td>
</tr>
<tr>
<td>High-density lipoprotein cholesterol, mg/dL</td>
<td>51.8±9.4</td>
<td>54.7±11.3</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol, mg/dL</td>
<td>127.1±34.9</td>
<td>110.9±22.6</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>107.8±20.7</td>
<td>117.4±36.3</td>
</tr>
<tr>
<td>Plasma aldosterone concentration, ng/dL</td>
<td>32.8±22.8</td>
<td>14.7±10.9*</td>
</tr>
<tr>
<td>Plasma renin activity, ng/mL/h</td>
<td>0.3±0.2</td>
<td>1.4±1.5*</td>
</tr>
<tr>
<td>Aldosterone:renin ratio</td>
<td>122.0±79.5</td>
<td>22.7±25.4†</td>
</tr>
<tr>
<td>Serum potassium, mEq/L</td>
<td>3.3±0.8</td>
<td>4.2±0.3†</td>
</tr>
</tbody>
</table>

All results are presented as mean±SD. *P<0.05 vs before. †P<0.01 vs before.
Hypertension

April 2015

is not only the initial step of atherosclerosis but also a predictor of cardiovascular events. In addition, we have recently shown that leukocyte ROCK activity is an independent predictor of cardiovascular events. In this study, patients with APA had vascular dysfunction and an increase in ROCK activity compared with those in patients with IHA, suggesting that the prevalence of future cardiovascular events may be higher in patients with APA than in patients with IHA.

Study Limitations
In this study, the number of patients with PA, especially patients with APA, was relatively small. Nonetheless, we observed a marked augmentation of FMD and reduction in ROCK activity after adrenalectomy in patients with APA and significant relationships between both increase in FMD and decrease in ROCK activity and decrease in PAC or ARR.

It is well known that various vasoconstricting factors other than aldosterone affect vascular function in humans. We confirmed in a preliminary study that circulating levels of endothelin-1 were normal and did not change after adrenalectomy (2.1±0.3 to 2.0±0.4 pg/mL) in 10 patients with APA (4 men and 6 women; mean age: 53±12 years). However, we cannot deny the possibility that other vasoconstrictors contribute to endothelial function and ROCK activity and were restored after adrenalectomy in patients with APA.

In a previous study, we confirmed that eplerenone improved endothelial function and decreased ROCK activity independently of blood pressure reduction in patients with EHT. Evaluation of whether there are benefits beyond blood pressure control for medical interventions using maximum tolerated doses of mineralocorticoid receptor blockers or adrenalectomy in patients with APA will enable more specific conclusions about the role of aldosterone in vascular function and ROCK activity to be drawn. These findings may help to know the indication for adrenalectomy in elderly patients with APA.

Perspectives
Endothelial function was impaired in patients with APA compared with that in patients with IHA or EHT, whereas endothelial function was similar in patients with IHA and patients with EHT and it was impaired to a greater extent in relation to levels of PAC or ARR. Leukocyte ROCK activity was increased in patients with APA compared with that in patients with IHA or EHT, whereas leukocyte ROCK activity was similar in patients with IHA and patients with EHT and it was increased to a greater extent in relation to levels of PAC or ARR. Surgical resection of APA improved endothelial function and inhibited leukocyte ROCK activity in patients with APA. There were significant correlations between improvement of FMD and ROCK activity and decrease in PAC or ARR. APA may have a higher risk of future cardiovascular events.

Acknowledgments
We thank Megumi Wakisaka, Ki-ichiro Kawano, and Satoko Michiyama for their excellent secretarial assistance.

Sources of Funding
This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (1859081500 and 21590898) and the National Institutes of Health (HL052233).
Disclosures

Dr K. Liao is a consultant for Asahi-Kasei Pharmaceutical, Inc. The other authors report no conflicts.

References


**Novelty and Significance**

**What Is New?**

- This study is the first study showing that increased secretion of aldosterone impairs vascular function and increases Rho-associated kinase activity, that patients with aldosterone-producing adenoma had impairment of endothelial function and increased Rho-associated kinase activity compared with those in patients with idiopathic hyperaldosteronism or essential hypertension, and that resection of an aldosterone-secreting tumor restores vascular function and Rho-associated kinase activity in patients with aldosterone-producing adenoma.

**What Is Relevant?**

- Aldosterone-producing adenoma may have a higher risk of future cardiovascular events.

**Summary**

The results of this study showed for the first time the relationships between plasma aldosterone concentration or aldosterone:renin ratio and vascular function and Rho-associated kinase activity in patients with essential hypertension, aldosterone-producing adenoma and idiopathic hyperaldosteronism.
Effect of Aldosterone-Producing Adenoma on Endothelial Function and Rho-Associated Kinase Activity in Patients With Primary Aldosteronism

Takeshi Matsumoto, Kenji Oki, Masato Kajikawa, Ayumu Nakashima, Tatsuya Maruhashi, Yumiko Iwamoto, Akimichi Iwamoto, Nozomu Oda, Takayuki Hidaka, Yasuki Kihara, Nobuoki Kohno, Kazuaki Chayama, Chikara Goto, Yoshiki Aibara, Kensuke Noma, James K. Liao and Yukihito Higashi

Hypertension. 2015;65:841-848; originally published online January 26, 2015; doi: 10.1161/HYPERTENSIONAHA.114.05001

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 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/65/4/841

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2015/01/26/HYPERTENSIONAHA.114.05001.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/
Online Supplement

Impact of Aldosterone-Producing Adenoma on Endothelial Function and Rho-Associated Kinase Activity in Patients With Primary Aldosteronism

Brief title: Aldosteronism and Vascular Function

Takeshi Matsumoto, MD;¹ Kenji Oki, MD, PhD;² Masato Kajikawa, MD;¹ Ayumu Nakashima, MD, PhD;³ Tatsuya Maruhashi, MD, PhD;¹ Yumiko Iwamoto, MD;¹ Akimichi Iwamoto, MD;¹ Nozomu Oda, MD;¹ Takayuki Hidaka, MD, PhD;¹ Yasuki Kihara, MD, PhD;¹ Nobuoki Kohno, MD, PhD;² Kazuaki Chayama, MD, PhD;² Chikara Goto, PhD;³ Yoshiki Aibara, MS;⁶ Kensuke Noma, MD, PhD;⁶ James K. Liao, MD;² Yukihito Higashi, MD, PhD, FAHA³,⁶

¹Department of Cardiovascular Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
²Department of Molecular and Internal Medicine, Graduate School of Biomedical Sciences, Hiroshima University
³Division of Regeneration and Medicine, Medical Center for Translational and Clinical Research, Hiroshima University Hospital, Hiroshima, Japan
⁴Department of Gastroenterology and Metabolism, Institute of Biomedical and Health Sciences, Graduate School of Biomedical and Health Sciences, Hiroshima University
⁵Hiroshima International University, Hiroshima, Japan
⁶Department of Cardiovascular Regeneration and Medicine, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
⁷Section of Cardiology, University of Chicago Medical Center, Chicago, IL, USA

Address for correspondence: Yukihito Higashi, MD, PhD, FAHA
Department of Cardiovascular Regeneration and Medicine, Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
Phone: +81-82-257-5831 Fax: +81-82-257-5831
E-mail: yhigashi@hiroshima-u.ac.jp
Supplementary Methods

Study protocol 1. Vascular function and ROCK activity in patients with EHT and patients with PA

**Patients with EHT**: Hypertension was defined as a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg measured in a sitting position on at least three different occasions in the outpatient clinic of Hiroshima University School of Medicine. Secondary hypertensive patients were excluded on the basis of a complete history and physical examination, radiologic and ultrasound examinations, and urinalysis. Plasma renin activity (PRA), plasma aldosterone concentration (PAC), and serum creatinine, potassium, calcium, and free thyroxine concentrations were determined; 24-hour urinary excretion of catecholamines, 17-hydroxycorticosteroids, 17-ketogenic steroids, and vanillylmandelic acid was also measured. Patients with a history of coronary heart disease, cerebrovascular disease, or peripheral arterial disease were excluded from this study.

**Patients with PA**: PA, including the classification of PA, was defined according to the report of the guidelines for diagnosis and treatment of primary aldosteronism: the Japan Endocrine Society 2009. Briefly, a diagnosis of PA was confirmed by the captopril-challenge test, upright furosemide-loading test, and saline-loading test after screening of PAC (>12ng/dL) and/or the aldosterone to renin ration (ARR; >20). Location of the tumor was identified by computed tomography, magnetic resonance imaging, and ¹³¹I-adosterone scintigraphy. Then, to identify the lateralization of aldosterone secretion, PAC and plasma cortisol concentration were measured in adrenal venous blood using an adrenal vein sampling technique under adrenocorticotropic hormone stimulation. No patient had multiple endocrine neoplasias. Familial hyperaldosteronism types I and II and aldosterone-producing carcinoma were also not included in this study. Patients with a history of coronary heart disease, cerebrovascular disease, or peripheral arterial disease were excluded from this study. No antihypertensive agents were taken by the patients for >2 weeks before the study.

**Study protocol**: We measured vascular responses to reactive hyperemia and sublingually administered nitroglycerine in the brachial artery in all subjects. Subjects fasted the previous night for at least 12 hours. The study began at 8:30 AM. The subjects were kept in the supine position in a quiet, dark, air-conditioned room (constant temperature of 22°C to 25°C) throughout the study. A 23-gauge polyethylene catheter was inserted into the left deep antecubital vein to obtain blood samples. Thirty minutes after maintaining the supine position, basal brachial artery diameter was measured. A high-resolution linear artery transducer was coupled
to computer-assisted analysis software (UNEXEF18G, UNEX Co, Nagoya, Japan) that used an automated edge detection system for measurement of brachial artery diameter. A blood pressure cuff was placed around the forearm. The brachial artery was scanned longitudinally 5 to 10 cm above the elbow. When the clearest B-mode image of the anterior and posterior intimal interfaces between the lumen and vessel wall was obtained, the transducer was held at the same point throughout the scan by a special probe holder (UNEX Co) to ensure consistency of the image. Depth and gain setting were set to optimize the images of the arterial lumen wall interface. When the tracking gate was placed on the intima, the artery diameter was automatically tracked, and the waveform of diameter changes over the cardiac cycle was displayed in real time using the FMD mode of the tracking system. This allowed the ultrasound images to be optimized at the start of the scan and the transducer position to be adjusted immediately for optimal tracking performance throughout the scan. Pulsed Doppler flow was assessed at baseline and during peak hyperemic flow, which was confirmed to occur within 15 s after cuff deflation. Blood flow velocity was calculated from the color Doppler data and was displayed as a waveform in real time. The baseline longitudinal image of the artery was acquired for 30 s, and then the blood pressure cuff was inflated to 50 mm Hg above systolic pressure for 5 min. The longitudinal image of the artery was recorded continuously until 5 min after cuff deflation. Pulsed Doppler velocity signals were obtained for 20 s at baseline and for 10 s immediately after cuff deflation. Changes in brachial artery diameter were immediately expressed as percentage change relative to the vessel diameter before cuff inflation. FMD was automatically calculated as the percentage change in peak vessel diameter from the baseline value. Percentage of FMD [(Peak diameter - Baseline diameter)/Baseline diameter] was used for analysis. Blood flow volume was calculated by multiplying the Doppler flow velocity (corrected for the angle) by heart rate and vessel cross-sectional area ($r^2$). Reactive hyperemia was calculated as the maximum percentage increase in flow after cuff deflation compared with baseline flow.

The response to nitroglycerine was used for assessment of endothelium-independent vasodilation. Nitroglycerine-induced vasodilation was measured as described previously. Briefly, after acquiring baseline rest images for 30 s, a sublingual tablet (75 μg nitroglycerine) was given, and images of the artery were recorded continuously until the dilation reached a plateau after administration of nitroglycerine. Subjects who had received nitrate treatment and subjects in whom the sublingually administered nitroglycerine tablet was not dissolved during the measurement were excluded from this study. Nitroglycerine-induced vasodilation was automatically calculated as a percent change in peak vessel diameter from the baseline value. Percentage of nitroglycerine-induced vasodilation [(Peak diameter - Baseline diameter)/Baseline diameter] was used for analysis.

The inter- and intra-coefficients of variation for the baseline diameter were 1.6% and 1.4%, respectively, in our laboratory.

The observers were blind to the form of examination.

**Measurement of ROCK Activity**
ROCK activity was assayed in peripheral blood leukocytes as the amount of phospho-Thr853 in the myosin-binding subunit of myosin light chain phosphatase. Blood was collected at room temperature in heparinized tubes (20 U/mL). After adding an equal volume of 2% dextran, each sample was kept at room temperature for
30 min. The supernatant was spun at 1450 rpm for 10 min. Red blood cells in the resulting cell pellet were lysed with the addition of water and spun at 1450 rpm for 10 min after the addition of Hank’s balanced salt solution (Hyclone, Logan, UT, USA). The resulting leukocyte pellet was resuspended in medium 199 (Sigma Chemical Co., Saint Louis, Missouri, USA) and the number of cells was counted using a hematocytometer. Cells were fixed in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane. After centrifugation, the cell pellets were stored at 80°C for Western blot analysis. Cells pellets were dissolved in 10 μL of 1 mol/L Tris base and then mixed with 100 μL of extraction buffer (8 mol/L urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol). Equal amounts of cell extracts were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analyses from several membranes. After serum starvation for 20 hours, confluent cells were stimulated with 10 μmol/L lysophosphatidic acid for 10 minutes and then subsequently fixed and harvested in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane. Following centrifugation at 1450 rpm for 10 minutes at 4°C, precipitates were dissolved in 10 μL of 1 mol/L Tris base and mixed with 100 μL of extraction buffer. An equal volume of positive control cell lysate was used for each gel. Membranes were incubated with rabbit anti–phospho-specific Thr853–myosin-binding subunit polyclonal antibody (Biosource Invitrogen, Carlsbad, California, USA), rabbit anti-myosin-binding subunit polyclonal antibody (Covance Laboratories, Evansville, Indiana, USA), or antiactin monoclonal antibody (Sigma). Bands were visualized using the ECL system (Amersham-Pharmacia Co., London, UK). Images were captured using Adobe Photoshop, and the band intensities were quantified using National Institutes of Health Image 1.61. ROCK activity was expressed as the ratio of phospho myosin-binding subunit in each sample to phospho myosin-binding subunit in each positive control divided by total myosin-binding subunit in each sample per total myosin-binding subunit in each positive control.

The observers were blind to the form of examination.
Supplementary references
Supplemental Figures

Figure S1

Figure S1. Relationship between flow-mediated vasodilation and plasma aldosterone concentration (A) and the aldosterone to renin ratio (B). Relationship between nitroglycerine-induced vasodilation and plasma aldosterone concentration (C) and the aldosterone to renin ratio (D). EHT indicates essential hypertension; APA, aldosterone-producing adenoma; IHA, idiopathic hyperaldosteronism.
Figure S2. Relationship between plasma aldosterone concentration and Rho-associated kinases (ROCK) activity (A). Relationship between the aldosterone to renin ratio and ROCK activity (B). EHT indicates essential hypertension; APA, aldosterone-producing adenoma; IHA, idiopathic hyperaldosteronism.