Smoking, Endothelial Function, and Rho-Kinase in Humans

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Objective—Smoking is associated with endothelial dysfunction and activated Rho-kinase in vascular smooth muscle cells (VSMCs) in humans. The purpose of this study was to elucidate the relationship between endothelial function and Rho-kinase activity in forearm VSMCs in healthy young men.

Methods and Results—We evaluated the forearm blood flow (FBF) responses to acetylcholine (ACh), fasudil, a Rho-kinase inhibitor, and sodium nitroprusside (SNP) in male smokers (n=10) and nonsmokers (n=14). FBF was measured by using a strain-gauge plethysmography. The vasodilatory effect of ACh was significantly smaller in smokers than that in nonsmokers. The vasodilatory effect of fasudil was significantly greater in smokers than that in nonsmokers. The vasodilatory effects of SNP in the 2 groups were similar. There was a significant correlation between the maximal FBF response to fasudil and that to ACh (r=-0.67; P<0.01). There was no significant correlation between the maximal FBF response to fasudil and that to SNP. The intra-arterial coinfusion of fasudil significantly increased the FBF response to ACh in smokers but not in nonsmokers. There were no significant differences between FBF response to fasudil alone and that in combination with N⁶-monomethyl-L-arginine in smokers and in nonsmokers. The intra-arterial coinfusion of ascorbic acid did not alter the FBF response to fasudil in both groups.

Conclusions—These findings suggest that smoking is involved in not only endothelial dysfunction but also activation of Rho-kinase in VSMCs in forearm circulation, and that there is a significant correlation between endothelial function and Rho-kinase activity in VSMCs. (Arterioscler Thromb Vasc Biol. 2005;25:2630-2635.)

Key Words: smoking ■ Rho-kinase ■ endothelial function ■ vascular smooth muscle cell ■ healthy young man

Cigarette smoking is a major risk factor for the development of atherosclerosis. Although several lines of evidence have indicated the mechanisms for endothelial dysfunction by smoking, the underlying mechanisms are not completely understood. Smoking causes endothelial dysfunction in smokers and passive smokers, leading to cardiovascular and cerebrovascular complications.

Recent in vitro and in vivo studies suggested that the Rho-associated kinase (Rho-kinase/ROK/ROCK) family, one of several putative small GTPase Rho effectors, plays major roles in actin cytoskeleton, organization, smooth muscle contraction, and gene expression, all of which may be involved in the pathogenesis of atherosclerosis. Results of previous studies have shown that Rho-kinase plays a key role in the contraction of vascular smooth muscle cells (VSMCs). Rho-kinase activates myosin light chain (MLC) kinase (MLCK) by phosphorylation of the myosin-binding subunit (MBS) in MLC phosphatase (MLCPH), leading to contraction of VSMCs. Smooth muscle dysfunction has been found in subjects with atherosclerosis. VSMC dysfunction may be partly attributable to the activation of Rho-kinase in VSMCs. It is thought that Rho-kinase activity also interacts endothelial function in humans. However, there is no information on the relationship between endothelial function and Rho-kinase activity in humans.

To evaluate the effects of smoking on endothelial function and Rho-kinase activity, and to determine the relationship between endothelial function and Rho-kinase activity in humans, we measured vascular responses to acetylcholine (ACh), fasudil, a specific inhibitor of Rho-kinase, and sodium nitroprusside (SNP), a direct vasodilator of VSMCs, in healthy young men.

Methods

Subjects

The subjects were 10 healthy young male smokers (mean age 24.9±5.3 years) and 14 healthy age-matched young male nonsmokers (mean age 25.1±4.6 years). All of the subjects were recruited from healthy volunteers. Normal blood pressure was defined as systolic blood pressure of <130 mm Hg and diastolic blood pressure of <80 mm Hg. The results of physical and routine laboratory examinations in all subjects were normal. None of the subjects had a family history of premature cardiovascular disease, and none of the...
subjects were taking oral contraceptives, antioxidant vitamins, or cardiovascular drugs. The study protocol was approved by the ethical committee of Hiroshima University Graduate School of Biomedical Sciences. Informed consent for participation in the study was obtained from all subjects. The definition of smokers was those who fulfilled the prespecified entry criteria: regular smoking history >5 pack-years. One pack-year was equivalent to 20 cigarettes smoked per day for 1 year. All of the smokers (11.4±13.2 pack years) had a smoking history of >5 years and abstained from smoking for ≥3 hours before the forearm blood flow (FBF) measurements. We defined nonsmokers as those who had never smoked.

Measurements of FBF
FBF was measured with a mercury-filled Silastic strain-gauge plethysmography (EC-5R; D.E. Hokanson, Inc.), as described previously.\textsuperscript{14,15}

Procedures
The forearm vascular responses to ACh (Daichi Pharmaceutical Co) were evaluated in 10 smokers and 14 nonsmokers, and fasudil (Asahi Chemical Industries) and SNP (Malushi Pharmaceutical Co) were evaluated in all subjects. The infusions of ACh, fasudil, and SNP were performed in a randomized fashion. The study began at 8:30 AM with the subjects in the fasting condition. A 23-gauge polyethylene catheter (Hokkow Co) was inserted into the left brachial artery for the infusion of ACh, fasudil, and SNP for the recording of arterial pressure with an AP-641G pressure transducer (Nihon Kohden Co) under local anesthesia (1% lidocaine). Another catheter was inserted into the left deep antecubital vein to obtain blood samples.

After 30 minutes in the supine position, we measured basal FBF and arterial blood pressure. Then, forearm vascular response to ACh, endothelium dependent vasodilator, fasudil, a specific Rho-kinase inhibitor, and SNP, a direct vasodilator of smooth muscle cells, on forearm hemodynamics were measured. ACh (3.75 and 7.5 μg/min), fasudil (3, 10, 30, and 100 μg/min), and SNP (0.75, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 minutes at each dose. Each study proceeded after the FBF returned to baseline.

To determine the coinfusion effect of fasudil on ACh-induced vasodilation, the forearm vascular response to ACh (3.75 and 7.5 μg/min) in combination with fasudil (10 μg/min) was evaluated in 6 smokers and 8 nonsmokers. Furthermore, after a 30-minute rest period, N\textsubscript{2}-monomethyl-L-arginine (L-NMMA), an NO synthase inhibitor, was infused intra-arterially at a dose of 8 μmol/min for 5 minutes while the basal FBF and arterial blood pressure were recorded and fasudil (3, 10, 30, and 100 μg/min) was administered.

On another day, to determine the effect of fasudil after inhibition of reactive oxygen species (ROS), the forearm vascular responses to fasudil (3, 10, 30, and 100 μg/min) alone and in combination with ascorbic acid (24 mg/min) were evaluated in 7 smokers and 7 nonsmokers.

Analytical Methods
Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein cholesterol, and triglycerides. Serum concentrations of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were determined using Friedewald’s methods. The concentration of angiotensin II was assayed by radioimmunnoassay. The plasma concentrations of norepinephrine were measured by high-performance liquid chromatography.

Statistical Analysis
Results are presented as the means±SD. Values of P<0.05 were considered to indicate statistical significance. The Mann–Whitney U test was used to evaluate differences between current smokers and nonsmokers concerning parameters at baseline. Comparisons between the 2 groups with respect to changes in parameters were performed with adjusted means on an ANCOVA, with baseline data used as the covariates. Comparisons of dose-response curves of parameters during infusion of the drug were analyzed by repeated-measures ANOVA. For the analysis of FBF response to ACh in combination with fasudil and that to fasudil in combination with L-NMMA, the absolute FBF changes from baseline values were used to compare the dose-response curves. Each FBF response to the vasoactive drugs was compared with that in the other group by Bonferroni correction. Spearman’s rank correlation was used to compare the maximal FBF response to ACh with that to fasudil and that to SNP. The data were analyzed using the software package StatView V (SAS Institute Inc.) and Super ANOVA (Abacus Concepts).

Results
Baseline Clinical Characteristics
The clinical characteristics of the 10 smokers and 14 nonsmokers are summarized in the Table. All of the parameters, including plasma insulin, plasma angiotensin II, norepinephrine, and lipid profiles, were similar in smokers and nonsmokers. Systemic and forearm hemodynamics such as basal FBF were also similar in the 2 groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Smoker (n=10)</th>
<th>Nonsmoker (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>24.9±5.3</td>
<td>25.1±4.6</td>
</tr>
<tr>
<td>Body mass index, kg/m\textsuperscript{2}</td>
<td>22.7±2.5</td>
<td>24.6±3.0</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>118.0±9.3</td>
<td>123.0±6.4</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>61.8±6.8</td>
<td>64.6±6.6</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>80.5±7.0</td>
<td>84.1±5.2</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>65.5±8.1</td>
<td>64.1±8.5</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.30±0.80</td>
<td>4.42±0.71</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.06±0.34</td>
<td>1.07±0.37</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.37±0.36</td>
<td>1.31±0.38</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.45±0.83</td>
<td>2.62±0.55</td>
</tr>
<tr>
<td>Mean fasting plasma glucose, mmol/L</td>
<td>5.0±0.4</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>Plasma NE, ng/mL</td>
<td>0.17±0.10</td>
<td>0.20±0.13</td>
</tr>
<tr>
<td>Plasma Ang II, pg/mL</td>
<td>7.0±2.31</td>
<td>6.00±3.57</td>
</tr>
<tr>
<td>FBF, mL/min per 100 mL tissue</td>
<td>6.9±1.9</td>
<td>7.1±2.8</td>
</tr>
</tbody>
</table>

Ang II indicates angiotensin II; FBF, forearm blood flow; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NE, norepinephrine. All results are presented as mean±SD.

FBF Responses to ACh in Smokers and Nonsmokers
The intra-arterial infusion of ACh significantly increased FBF in a dose-dependent manner in smokers and nonsmokers. The FBF response to ACh was significantly smaller in smokers than in nonsmokers (maximal FBF 12.3±3.3 versus 21.4±26.8 mL/min per 100 mL tissue; P<0.01; Figure 1, top). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of ACh in either.

FBF Responses to SNP in Smokers and Nonsmokers
The intra-arterial infusion of SNP significantly increased FBF in a dose-dependent manner in smokers and nonsmokers. There was no significant difference between FBF responses to SNP in the 2 groups (Figure 1, bottom). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of SNP in either.
FBF Responses to Fasudil in Smokers and Nonsmokers

The FBF response to fasudil was significantly greater in smokers than in nonsmokers (maximal FBF 23.4 ± 6.1 vs 14.6 ± 5.1 mL/min per 100 mL tissue; \( P < 0.01 \); Figure 2). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil in either.

There was a significant relationship between the maximal FBF response to ACh and that to fasudil (\( r = 0.67; P < 0.01 \)).

No significant relationship was found between the maximal FBF response to ACh and that to SNP (\( r = 0.08; P = \text{NS} \)), or between the maximal FBF response to fasudil and that to SNP (\( r = 0.28; P = \text{NS} \)).

FBF Responses to Fasudil Alone and in Combination With L-NMMA in Smokers and Nonsmokers

The intra-arterial infusion of L-NMMA significantly decreased basal FBF from 7.3 ± 2.8 to 5.1 ± 2.1 mL/min per 100 mL tissue (\( P < 0.05 \)) in smokers and from 8.4 ± 3.0 to 5.1 ± 2.1 mL/min per 100 mL tissue (\( P < 0.05 \)) in nonsmokers. Changes in basal forearm vascular responses to L-NMMA infusion were similar in the 2 groups. There were no significant differences between FBF response to fasudil alone and that in combination with L-NMMA in smokers and in nonsmokers (Figure 4). There was a significant difference between the changes in FBF response to fasudil after coinfusion of L-NMMA in the 2 groups (\( P < 0.01 \)). No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil alone and in combination with L-NMMA in either.

FBF Responses to Fasudil Alone and in Combination With Ascorbic Acid in Smokers and Nonsmokers

Ascorbic acid did not alter the FBF response to fasudil in smokers and in nonsmokers (Figure 5). No significant change

However, there was no significant relationship between the maximal FBF response to ACh and that to SNP (\( r = 0.08; P = \text{NS} \)) or between the maximal FBF response to fasudil and that to SNP (\( r = 0.28; P = \text{NS} \)).
was found in arterial blood pressure or heart rate with intra-arterial infusion of fasudil alone and in combination with ascorbic acid in either.

Discussion

In the present study, we demonstrated that not only endothelial dysfunction but also activated Rho-kinase in VSMCs were found in healthy young male smokers compared with nonsmokers. The results of the present study also showed for the first time that there is a significant correlation between endothelial function and Rho-kinase activity in forearm resistance arteries.

Endothelium-dependent vasodilation was impaired even in healthy young smokers compared with nonsmokers. Our findings are supported by results of previous studies showing that smoking is significantly associated with endothelial dysfunction and cardiovascular disease. Structural damage, a direct toxic effect, a decreased production or bioavailability of endothelial NO, and a superoxide anion by containing of a large number of free radicals and pro-oxidants in cigarette smoke have been proposed as mechanisms of smoking-induced vascular damage.

The main findings of the present study are that the forearm vasodilatory effect evoked by fasudil was greater in smokers than in nonsmokers, whereas SNP-induced vasodilation was similar in the 2 groups, and that there was a significant correlation between the forearm vasodilatory effect evoked by ACh and that by fasudil. Although the precise mechanism of the interaction between endothelial function and Rho-kinase activity remains to be cleared, our results suggest that smoking may contribute to the activation of Rho-kinase in VSMCs as well as endothelial dysfunction. Several lines of evidence have demonstrated that eNOS expression is upregulated by inhibition of Rho-kinase via increase of eNOS mRNA stability and eNOS phosphorylation. Hernandez-Perera et al reported that Rho is required for the basal expression of preproendothelin-1 in vascular endothelial cells, which gives rise to endothelin-1. In addition, several investigators demonstrated an interaction between NO and Rho/Rho-kinase in VSMCs. Several investigators demonstrated an interaction between NO and Rho/Rho-kinase in VSMCs. Sauzeau et al have shown that exogenous NO attenuates RhoA-dependent Ca²⁺ sensitization of blood vessel contraction by inhibiting RhoA translocation from the cytosol to membrane in VSMCs through activation of the cyclic GMP-dependent kinase pathway. These findings suggest that endothelial dysfunction may result in Rho-kinase activation in VSMCs through a decrease in NO production from the endothelium, and that activated Rho-kinase may inhibit eNOS expression in the endothelium. Consequently, endothelial dysfunction and activation of Rho-kinase in VSMCs may be evoked by smoking.

Recent studies have shown that Rho-kinase plays important roles in various cellular functions, including vascular smooth muscle contraction. Uehata et al reported that systemic administration of a Rho-kinase inhibitor, Y-27632, induced significant and persistent decreases in blood pressure in hypertensive rat models. In clinical studies, several investigators reported that hypertension, stable angina pectoris, and coronary vasospasm are associated with activation of Rho-kinase. These findings suggest that activation of Rho-kinase in VSMCs is involved in the development and progression of the atherosclerotic process. Furthermore, recent studies demonstrated the partial contribution of Rho-kinase to VSMC contraction. VSMC contraction is modulated in a dual manner by MLCK and MLCPh, so that the phosphorylation of MBS on MLCPh by Rho-kinase results in the phosphorylation of MLC and subsequent contraction of VSMCs. Moreover, MLC diphosphorylation as well as MLC monophosphorylation were found in impaired VSMCs. It is postulated that smoking is associated with Rho-kinase activity.

In the present study, we evaluated endothelial function by using ACh, which is well established as an endothelial dependent vasodilator, and we evaluated Rho-kinase activity in VSMCs by using fasudil, a Rho-kinase inhibitor.
Fasudil, which is currently used for prevention and treatment of cerebral vasospasm after subarachnoid hemorrhage, has been shown recently to be a potent and specific inhibitor of Rho-kinase.\textsuperscript{33,34} In addition, fasudil is used for assessment of Rho-kinase activity in humans.\textsuperscript{28–30} However, we cannot deny the possibility that fasudil, especially at high doses, has nonspecific effects on vasculature.

In the present study, L-NMMA did not alter the FBF response to fasudil in smokers or nonsmokers. Interestingly, confusion of fasudil significantly augmented the FBF response to ACh in combination with fasudil in smokers and that in nonsmokers in the present study. This may be explained by a decrease in Ca\textsuperscript{2+} sensitivity by inhibition of Rho-kinase in VSMCs in smokers. These findings support our hypothesis that Rho-kinase in VSMCs is activated in smokers compared with nonsmokers, although it remains to be clarified whether endogenous NO inhibits Rho-kinase activity in humans. Of additional interest, there was no significant difference between FBF response to ACh and fosfodiesterase inhibitor in healthy young male nonsmokers. We cannot deny the possibility that fasudil improves endothelial function via upregulation of eNOS expression in smokers.

Recently, Higashi et al\textsuperscript{36} demonstrated that Rho-kinase is substantially involved in production of ROS through the phosphatidylinositol 3-kinase/Akt pathway, resulting in increased NO production and subsequent cardiovascular protection. On the other hand, several investigators have shown that inhibition of Rho-kinase upregulates eNOS expression through increase in eNOS mRNA stability and eNOS phosphorylation.\textsuperscript{23,35} We cannot deny the possibility that fasudil improves endothelial function via upregulation of eNOS expression in smokers.

Several methods have been used to assess endothelial function in humans. Recently, several investigators, including us, evaluated the effects of intra-arterial infusion of NO agonists, such as ACh, methacholine, and bradykinin, and the effects of intra-arterial infusion of NO antagonists on FBF. The responses to intra-arterial infusion of vasoactive agents should be considered the gold standard for assessing endothelial function because the use of agonists to stimulate NO release and the use of antagonists of NO allow us to draw more specific conclusions concerning the role of basal and stimulated NO release. Measurement of flow-mediated vasodilation (FMD) in the brachial artery using ultrasound also reflects NO production well. It is accepted that measurement of FBF responses to vasoactive agents is an index of resistance artery endothelial function and that measurement of FMD is an index of conduit artery endothelial function. Both measurements of FBF responses to vasoactive agents and FMD would enable more specific conclusions concerning the relationship between Rho-kinase activity and endothelial function to be drawn. Unfortunately, we were not able to perform measurement of FMD as an index of conduit artery endothelial function in the present study.

Endothelial dysfunction and activation of Rho-kinase may play a critical role in the pathogenesis of atherosclerosis in smokers, leading to cardiovascular and cerebrovascular complications. Further studies on the mechanisms underlying the interaction between endothelial function and Rho-kinase, not only in smokers but also in other subjects who have cardiovascular risk factors such as hypercholesterolemia and diabetes mellitus, are awaited for future therapeutic benefits.

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References

Noma et al. Endothelial Function and Rho-Kinase Activity


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