Smoking Activates Rho-Kinase in Smooth Muscle Cells of Forearm Vasculature in Humans

Kensuke Noma, Yukihi Higashi, Daisuke Jitsuiki, Keiko Hara, Masashi Kimura, Keigo Nakagawa, Chikara Goto, Tetsuya Oshima, Masao Yoshizumi, Kazuaki Chayama

Abstract—Previous studies have shown that smoking is strongly associated with atherosclerosis and coronary vascular disease. Rho-kinase plays an important role in various cellular functions associated with atherosclerosis and hypertension. However, there is no information on the relationship between smoking and Rho-kinase activity in humans. The purpose of this study was to determine the Rho-kinase activity in forearm vascular smooth muscle cells (VSMCs) in healthy young male smokers. We evaluated the forearm blood flow (FBF) responses to fasudil (3, 10, and 30 μg/min for 5 minutes), a Rho-kinase inhibitor, or sodium nitroprusside (0.75, 1.5, and 3.0 μg/min for 5 minutes) in current smokers (n=8) and nonsmokers (n=8). FBF was measured with a strain-gauge plethysmograph. The vasodilatory effect of fasudil was significantly greater in smokers than in nonsmokers (14.9±3.5 versus 10.5±3.6 mL/min per 100 mL tissue; P<0.01). The FBF responses to sodium nitroprusside were similar in the 2 groups (34.7±10.4 versus 33.2±10.2 mL/min per 100 mL tissue; P=0.78). These findings suggest that smoking activates Rho-kinase in forearm VSMCs but does not alter the vasodilatory effect induced by exogenous nitric oxide in forearm VSMCs in healthy young men. (Hypertension. 2003;41:1102-1105.)

Key Words: smoking ■ muscle, smooth, vascular ■ endothelium ■ kinase

It is well known that smoking is a major risk factor for the development of atherosclerosis, leading to cardiovascular and cerebrovascular complications.1,2 Although the underlying mechanisms are not completely understood, there is substantial evidence indicating that smoking causes injury to the vasculature by direct cytotoxicity.3,4 Recent studies in vitro and in vivo suggested that small GTPase Rho plays an important role in various cellular physiological functions, including actomyosin-based cellular processes such as cell adhesion, migration, motility, cytokinesis, and contraction,5,6 all of which may be involved in the pathogenesis of atherosclerosis. The Rho-associated kinase (Rho-kinase/ROK/ROCK) family, one of the several putative Rho effectors, plays major roles in actin cytoskeleton, organization,7,8 smooth muscle contraction,9,10 and gene expression.11 Although the precise mechanism remains unclear, there is growing evidence that phosphorylation of the myosin-binding subunit (MBS) of myosin light chain phosphatase (MLCPh) by Rho-kinase contributes to the contraction of vascular smooth muscle cells (VSMCs).12–15 It has been suggested that activation of Rho-kinase was at least partly responsible for the occurrence of the smooth muscle dysfunction in individuals with atherosclerosis.16 However, there is little information on the relationship between the activity of Rho-kinase in VSMCs and smoking, a major risk factor for atherosclerosis.

To evaluate the effect of smoking on Rho-kinase activity in VSMCs in humans, we measured vascular responses to fasudil, a specific inhibitor of Rho-kinase, and to sodium nitroprusside (SNP), a direct vasodilator of VSMCs, in healthy young men.

Methods

Subjects
The subjects were 8 healthy young male smokers (mean age, 23.6±5.1 years) and 8 healthy age-matched young male nonsmokers (mean age, 22.9±3.8 years). All of the subjects were recruited from healthy volunteers. The study protocol was approved by the Ethics Committee of Hiroshima University Faculty of Medicine. 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 of 1 to 3 pack-years. The degree of smoking was measured as pack-years. One pack-year was equivalent to 20 cigarettes smoked per day for 1 year. Twenty-five cigarettes smoked per day for 1 year would count as 1.25 pack-years. All of the smokers (1.3±0.7 pack-years) had a smoking history of more than 5 years and abstained from smoking for at least 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 plethysmograph (EC-5R, D.E. Hokanson, Inc), as previously described.\textsuperscript{17,18}

Procedures
After the patient had been in the supine position for 30 minutes, we measured basal FBF and arterial blood pressure. Forearm vascular responses to fasudil (Asahi Chemical Industries), a specific Rho-kinase inhibitor, and SNP (Mitsubishi Pharmaceutical Co), a direct vasodilator of SMCs, alone and after the infusion of N\textsuperscript{\textregistered}-monomethyl-L-arginine (L-NMMA, Sigma Chemical Co), were measured. Fasudil (3, 10, and 30 \textmu g/min) or SNP (0.75, 1.5, 3.0 \textmu g/min) were infused intra-arterially for 3 minutes at each dose. The clinical dosage of fasudil is \approx 50 \mu g/min. In a preliminary study, we confirmed that fasudil at 3 to 100 \mu g/min increased FBF without altering systemic hemodynamics. The FBF was measured during the last 2 minutes of infusion. The infusions of fasudil and SNP were carried out in a randomized fashion with a crossover design. Each study proceeded after the FBF returned to baseline.

After a 30-minute rest period, L-NMMA, an inhibitor of nitric oxide (NO) synthase, was infused intra-arterially at a dose of 8 \mu mol/min for 5 minutes, and fasudil was administered at 5 minutes after initiation of the L-NMMA.

Analytical Methods
Routine chemical methods were used to determine serum concentrations of total cholesterol, HDL cholesterol, and triglycerides. Serum concentrations of insulin were measured by using an automated radioimmunoassay technique. Serum concentrations of LDL were determined by means of Friedewald’s methods. The concentration of angiotensin II (Ang II) was assayed by radioimmunoassay.\textsuperscript{19} The plasma concentrations of norepinephrine were measured by high-performance liquid chromatography.\textsuperscript{20}

Statistical Analysis
Results are presented as mean\( \pm \)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 covariates. Comparisons of dose-response curves of parameters during the infusion of drug were analyzed by ANOVA for repeated measures with Bonferroni correction.

Results
Baseline Clinical Characteristics of Smokers and Nonsmokers
The clinical characteristics of the 8 smokers and 8 nonsmokers are summarized in the Table. All parameters, including plasma insulin, plasma Ang II, norepinephrine, and lipid profiles, were similar in smokers and nonsmokers. Systemic and forearm hemodynamics in the 2 groups were also similar.

FBF Responses to Fasudil in Smokers and Nonsmokers
The intra-arterial infusion of fasudil significantly increased FBF in smokers but not in nonsmokers. The FBF response to fasudil was significantly higher in smokers than in nonsmokers (maximal FBF, 14.9\( \pm \)3.5 versus 10.5\( \pm \)3.6 mL/min per 100 mL tissue; \( P<0.01 \), Figure 1). No significant change was observed in arterial blood pressure or heart rate with intra-arterial infusion of fasudil.

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

FBF Responses to Fasudil After L-NMMA in Smokers and Nonsmokers
The intra-arterial infusion of L-NMMA significantly decreased basal FBF in both smokers and nonsmokers (7.9\( \pm \)2.8 mL/min per 100 mL tissue) after the L-NMMA infusion. Fasudil was infused intra-arterially at a dose of 8 \mu mol/min for 5 minutes, and fasudil was administered at 5 minutes after initiation of the L-NMMA.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Smoker (n=8)</th>
<th>Nonsmoker (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>23.6( \pm )5.1</td>
<td>22.9( \pm )3.8</td>
</tr>
<tr>
<td>Body mass index, kg/m(^2)</td>
<td>21.8( \pm )2.2</td>
<td>24.8( \pm )2.7</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>116.2( \pm )8.6</td>
<td>118.1( \pm )4.1</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>59.1( \pm )4.4</td>
<td>63.3( \pm )6.7</td>
</tr>
<tr>
<td>Mean blood pressure, mm Hg</td>
<td>78.3( \pm )5.1</td>
<td>80.1( \pm )4.7</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>67.1( \pm )8.5</td>
<td>61.3( \pm )6.5</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.16( \pm )0.86</td>
<td>4.03( \pm )0.42</td>
</tr>
<tr>
<td>Triglyceride, mmol/L</td>
<td>1.08( \pm )0.52</td>
<td>0.95( \pm )0.40</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.31( \pm )0.20</td>
<td>1.26( \pm )0.30</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.36( \pm )0.82</td>
<td>2.33( \pm )0.39</td>
</tr>
<tr>
<td>Serum insulin, pmol/L</td>
<td>77.9( \pm )50.5</td>
<td>110.4( \pm )95.3</td>
</tr>
<tr>
<td>Plasma NE, ng/mL</td>
<td>0.20( \pm )0.10</td>
<td>0.22( \pm )0.13</td>
</tr>
<tr>
<td>Plasma Ang II, pg/mL</td>
<td>7.0( \pm )2.1</td>
<td>5.4( \pm )2.5</td>
</tr>
<tr>
<td>FBF, mL/min per 100 mL tissue</td>
<td>5.9( \pm )1.5</td>
<td>7.4( \pm )2.5</td>
</tr>
</tbody>
</table>

All results are presented as mean\( \pm \)SD. HDL indicates high density lipoprotein; LDL, low density lipoprotein; NE, norepinephrine; Ang II, angiotensin II; and FBF, forearm blood flow.

FBF Responses to Fasudil After L-NMMA in Smokers and Nonsmokers
The intra-arterial infusion of L-NMMA significantly decreased basal FBF in both smokers and nonsmokers (7.9\( \pm \)2.8 mL/min per 100 mL tissue) after the L-NMMA infusion. Fasudil was infused intra-arterially at a dose of 8 \mu mol/min for 5 minutes, and fasudil was administered at 5 minutes after initiation of the L-NMMA.

Figure 1. Effects of fasudil on FBF in smokers (●) and nonsmokers (○). Fasudil significantly increased FBF in smokers compared with that in nonsmokers. Results are presented as mean\( \pm \)SD. Probability value refers to comparison of time course curves by ANOVA for repeated measurements.
Effects of fasudil after L-NMMA on FBF in smokers

**Figure 2.** Effects of SNP on FBF in smokers (○) and nonsmokers (□). SNP increased FBF in a dose-dependent manner in both smokers and nonsmokers. FBF responses to SNP in the 2 groups were similar. Results are presented as mean±SD. Probability value refers to comparison of time course curves by ANOVA for repeated measurements.

Effects of SNP on FBF in smokers (○) and nonsmokers (□). SNP increased FBF in a dose-dependent manner in both smokers and nonsmokers. FBF responses to SNP in the 2 groups were similar. Results are presented as mean±SD. Probability value refers to comparison of time course curves by ANOVA for repeated measurements.

**Figure 3.** Effects of fasudil after L-NMMA on FBF in smokers (○) and nonsmokers (□). Fasudil significantly increased FBF in smokers compared with that in nonsmokers after L-NMMA. Results are presented as mean±SD. Probability value refers to comparison of time course curves by ANOVA for repeated measurements.

Discussion

In this study, we demonstrated for the first time that the vasodilator response to fasudil, a specific Rho-kinase inhibitor, was significantly greater in the forearm resistance arteries in healthy smokers than in those in nonsmokers, whereas the vasodilator responses to SNP, an exogenous NO, in the 2 groups were similar. There was a significant difference between FBF response to fasudil in smokers and nonsmokers after L-NMMA infusion.

In the present study, we used fasudil as a Rho-kinase inhibitor. Fasudil, currently used for prevention and treatment of cerebral vasospasm, which usually develops after subarachnoid hemorrhage, has recently been shown to be a potent and specific inhibitor of Rho-kinase. Nagumo et al²⁴ demonstrated that GTPγS stimulation of permeabilized SMCs caused a decrease in MLCPh activity with an increase in the extent of phosphorylation of the 130-kDa MBS on MLCPh in a Rho-dependent manner with the use of a fasudil. Shimokawa et al²⁵ recently demonstrated not only an inhibitory effect of fasudil on coronary vasospastic response in a swine model but also a remarkable specific inhibitory effect of fasudil on Rho-kinase compared with its effects on other protein kinases. These findings suggest that fasudil is a specific inhibitor of Rho-kinase. The results would be more convincing if a second inhibitor of Rho-kinase, for example, Y27632, were to show the same results.

The main finding of the current study is that the forearm vasodilatory effect evoked by fasudil was greater in smokers than in nonsmokers, whereas the effects evoked by SNP were similar in the 2 groups. These findings indicate that smoking may contribute to the activation of Rho-kinase in VSMCs in forearm circulation. Smoking, one of the major risk factors for atherosclerosis and cardiovascular disease, may be involved in the pathogenesis of atherosclerosis in VSMCs.

In this study, basal FBF in smokers and nonsmokers was similar. These data are consistent with the results of previous studies showing that there was no significant difference between basal FBF in normal control subjects and smokers or between basal levels in normal control subjects and patients with essential hypertension who have activated Rho-kinase on forearm VSMC.¹⁷,¹⁸,²⁶ Recently, Kobayashi et al²⁷ demonstrated that expression of the endothelial NO synthase gene was enhanced by inhibition of Rho-kinase. However, in the current study, fasudil increased FBF even after L-NMMA infusion in smokers. FBF response to fasudil after L-NMMA was significantly higher in smokers than in nonsmokers. These findings suggest that fasudil-induced vasodilation may not be due to eNOS gene expression-related NO release in the endothelium in smokers.

Recent studies have shown that Rho-kinase plays an important role in various cellular functions, including smooth muscle contraction.⁹,¹⁰,¹²–¹⁵,²⁵ Uehata et al²⁸ reported that systemic administration of a specific Rho-kinase inhibitor, Y27632, induced significant and persistent decreases in blood pressure in hypertensive rat models. Masumoto et al²⁹ provided evidence that Rho-kinase may be activated in forearm VSMCs in patients with essential hypertension. These findings suggest that activation of Rho-kinase in VSMCs affects the development and progression of hypertension. Although the mechanism by which Rho-kinase activation is involved in vasoconstriction remains to be clarified, recent evidence indicates that contraction of VSMC is partially dependent on Ca²⁺ sensitivity, which is modulated in a dual manner by myosin light chain kinase and MLCPh. Therefore, the phosphorylation of MBS on MLCPh by Rho-kinase results in the...
phosphorylation of myosin light chain (MLC) and subsequent contraction of VSMCs. Moreover, MLC diphosphorylation as well as MLC monophosphorylation was induced in impaired VSMC. Therefore, it is plausible that the vaso-dilatory response to a Rho-kinase inhibitor is greater in impaired VSMCs than in intact VSMCs. However, the precise mechanisms by which smoking activates Rho-kinase in forearm VSMCs, even in healthy young men, remain unclear.

Perspectives

Smoking activates Rho-kinase in forearm VSMCs in healthy young men. Rho-kinase activation may play a critical role in the pathogenesis of atherosclerosis in smokers, leading to cardiovascular and cerebrovascular complications. Further studies regarding the role of Rho-kinase in atherosclerosis, hypercholesterolemia, and diabetes mellitus are awaited with great interest. Elucidation of the role of Rho-kinase activity in atherosclerosis would be useful for the development of new treatments.

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References

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