Autologous Bone-Marrow Mononuclear Cell Implantation Improves Endothelium-Dependent Vasodilation in Patients With Limb Ischemia

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Background—Patients with limb ischemia were associated with endothelial dysfunction. The purpose of this study was to determine whether autologous bone-marrow mononuclear cell (BM-MNC) implantation improves endothelial dysfunction in patients with limb ischemia.

Methods and Results—We evaluated the leg blood flow (LBF) response to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, before and after BM-MNC implantation in 7 patients with limb ischemia. LBF was measured with a mercury-filled Silastic strain-gauge plethysmograph. The number of BM-MNCs implanted into ischemic limbs was $1.6\times10^9\pm0.3\times10^9$. The number of $CD34^+$ cells included in the implanted BM-MNCs was $3.8\times10^7\pm1.6\times10^7$. BM-MNC implantation improved the ankle-brachial pressure index (0.33 ± 0.21 to 0.39 ± 0.17, $P=0.06$), transcutaneous oxygen pressure (28.4 ± 11.5 to 36.6 ± 5.2 mm Hg, $P=0.03$), and pain-free walking time (0.8 ± 0.6 to 2.9 ± 2.2 minutes, $P=0.02$). After BM-MNC implantation, LBF response to ACh was enhanced (19.3 ± 6.8 versus 29.6 ± 7.1 mL/min per 100 mL; $P=0.002$). The vasodilatory effect of SNP was similar before and after BM-MNC implantation.

Conclusions—These findings suggest that BM-MNC implantation augments endothelium-dependent vasodilation in patients with limb ischemia. ***(Circulation.* 2004;109:1215-1218.)**

Key Words: angiogenesis ■ cells ■ endothelium ■ ischemia

Recent studies have shown that bone-marrow mononuclear cell (BM-MNC) implantation increases collateral vessel formation in both ischemic limb models and patients with limb ischemia.[1,2] However, it is not clear whether these collateral arteries have normal vascular function, especially endothelial function. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis and plays an important role in development and maintenance of atherosclerosis.[3] Limb ischemia is generally associated with endothelial dysfunction.[4,5] Therefore, it is clinically important to evaluate the vascular function of collateral arteries induced by BM-MNC implantation. We hypothesized that BM-MNC implantation would improve impaired endothelial function in patients with limb ischemia.

To determine the effect of BM-MNC implantation on endothelial function in patients with limb ischemia, we evaluated endothelium-dependent vasodilation induced by acetylcholine (ACh) and endothelium-independent vasodilation induced by sodium nitroprusside (SNP) before and after BM-MNC implantation.

Methods

Subjects

Seven patients with peripheral arterial disease (6 men and 1 woman; mean age, 64 ± 9 years) who had rest pain and nonhealing ulcers and who were not candidates for angioplasty or surgical revascularization were enrolled in this study. The diagnosis of limb ischemia was confirmed by angiography. Patients with diabetes mellitus, coronary artery disease, and history of malignant disorders were excluded. Four of the 7 patients had smoking habits, and those 4 patients stopped smoking 2 months before BM-MNC implantation. The drugs used were not changed throughout the study. Lifestyle also was regulated throughout the study. The study protocol was approved by the Ethics Committee of the Hiroshima University Graduate School of Medicine. Written informed consent for participation was obtained from all subjects.

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Patient	Age, y	Sex	Disorders	Drugs	BM-MNC Count, No. of Cells	CD34 (+) Cells in BM-MNC	ABI	TcO_2, mm Hg	Pain-Free Walking, min	Basal LBF mL · min^{-1} · 100 mL^{-1}
Patient 1										
Before	74	Male	HL	CaB, statin, APA	1.8×10^9	2.5×10^7	0.35	36	0.91	1.3
After 4 wk							0.44	41	1.22	2.4
After 24 wk							0.41	40	1.35	2.3
Patient 2										
Before	62	Male	HL	Statin	1.4×10^9	3.3×10^7	0.60	42	2.14	3.5
After 4 wk							0.62	45	6.89	3.9
After 24 wk							...	...	...	...
Patient 3										
Before	73	Male	HT, HL	CaB, ACEI, APA	1.6×10^9	4.2×10^7	0.23	25	1.21	2.2
After 4 wk							0.21	31	3.35	2.1
After 24 wk							0.22	32	2.98	2.2
Patient 4										
Before	56	Male	None	APA	2.2×10^9	6.2×10^7	ND	9	0	0.2
After 4 wk							0.26	37	2.34	0.6
After 24 wk							0.28	33	2.99	0.6
Patient 5										
Before	73	Female	HT	CaB, APA	1.5×10^9	1.3×10^7	0.39	38	0.56	1.9
After 4 wk							0.40	37	1.63	3.8
After 24 wk							0.38	36	1.35	3.2
Patient 6										
Before	51	Male	HL	Statin, APA	1.4×10^9	3.9×10^7	0.19	20	0.33	0.3
After 4 wk							0.22	31	0.98	0.3
After 24 wk							0.21	29	1.01	0.3
Patient 7										
Before	59	Male	HT	CaB, APA	1.2×10^9	5.1×10^7	0.52	29	0.76	2.6
After 4 wk							0.61	34	4.75	3.3
After 24 wk							0.59	32	5.11	3.1

ABI indicates ankle-brachial pressure index; TcO_2, transcutaneous oxygen; HL, hyperlipidemia; HT, hypertension; CaB, calcium blocker; APA, antiplatelet agent; ACEI, angiotensin-converting enzyme inhibitor; and ND, not detected.

BM-MNC Implantation

BM-MNCs were sorted and implanted in patients with limb ischemia as previously described.[2]

Effect of BM-MNC Implantation on Endothelial Function in Patients With Limb Ischemia

Leg vascular responses to ACh (Daiichi Pharmaceutical Co) and SNP (Maluishi Pharmaceutical Co) were evaluated by use of a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc) before and at 4 weeks after BM-MNC implantation in all subjects and at 24 weeks after BM-MNC implantation in 6 of the 7 subjects. Subjects fasted for at least 12 hours before cell implantation. They were kept in the supine position in a quiet, dark, air-conditioned room (temperature, 22°C to 25°C) throughout the study. A 23-gauge polyethylene catheter was inserted into the BM-MNC–implanted femoral artery for the infusion of ACh and SNP under local anesthesia. After each patient had spent 30 minutes in the supine position, we measured leg blood flow (LBF) and arterial blood pressure. Then, the effects of the ACh and SNP infusion on leg hemodynamics were measured. ACh (7.5, 15, and 30 μg/min) and SNP (0.75, 1.5, and 3.0 μg/min) were infused intra-arterially for 5 minutes at each dose. The infusions of ACh and SNP were performed in random order. Each study proceeded after the LBF had returned to baseline.

To evaluate the drug-related effect on endothelium-dependent vasodilation, the infusion of ACh and SNP was performed using a protocol identical to that used for the study of limb ischemic patients with implanted MN-MNCs before and after 4 weeks of follow-up in 5 patients with limb ischemia (4 men and 1 woman; mean age, 65±7 years) as a control group. Five patients were taking ACE inhibitors and antiplatelet agents; 3 of those 5 patients were taking calcium antagonists, and 2 were taking statins. The patients were subjected to 4 weeks of follow-up without any drug treatment or lifestyle modification.

Measurement of LBF

The blood flow was measured using a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc) as previously described.[6,7]

Statistical Analysis

Results are presented as the mean±SD. All reported probability values were 2-tailed. Values of $P<0.05$ were considered significant. Comparisons of parameters before and after BM-MNC implantation were performed with adjusted means by ANCOVA using baseline data as covariates. Comparisons of time-course curves of parameters during the infusions of ACh and SNP were analyzed by 2-way ANOVA for repeated measures on 1 factor followed by the Bonferroni correction for multiple-paired comparisons.

Results

Clinical Characteristics

The baseline clinical characteristics before and at 4 weeks and 24 weeks after BM-MNC implantation of patients with limb ischemia are summarized in the Table. The number of BM-MNCs implanted into ischemic limbs was $1.6\times10^9\pm0.3\times10^9$. The number of $CD34^+$ cells included in the implanted BM-MNCs was $3.8\times10^7\pm1.6\times10^7$. BM-MNC implantation improved the ankle-brachial pressure index from 0.33 ± 0.21 to 0.39 ± 0.17 after 4 weeks ($P=0.06$) and to 0.35 ± 0.38 after 24 weeks ($P=0.16$), transcutaneous oxygen pressure from 28.4 ± 11.5 to 36.6 ± 5.2 mm Hg after 4 weeks ($P=0.03$) and to 33.7 ± 3.8 mm Hg after 24 weeks ($P=0.06$), pain-free walking time from 0.8 ± 0.6 to 2.9 ± 2.2 minutes after 4 weeks ($P=0.02$) and to 2.5 ± 1.6 minutes after 24 weeks ($P=0.03$), and basal LBF from 1.7 ± 1.2 to 2.4 ± 1.4 mL/min per 100 mL tissue after 4 weeks ($P=0.04$) and to 2.0 ± 1.2 mL/min per 100 mL tissue after 24 weeks ($P=0.05$).

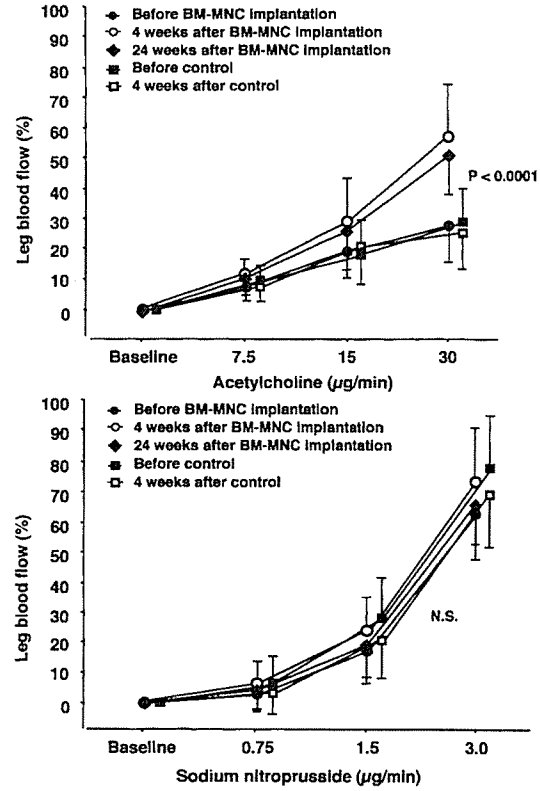
BM-NMC implantation did not alter blood pressures (mean blood pressure, from 86.2 ± 10.3 to 88.1 ± 11.2 mm Hg after 4 weeks and to 87.3 ± 12.1 mm Hg after 24 weeks) or serum concentrations of total cholesterol (from 5.28 ± 1.24 to 5.22 ± 1.06 mmol/L after 4 weeks and to 5.23 ± 1.18 mmol/L after 24 weeks), LDL cholesterol (from 3.88 ± 0.78 to 3.78 ± 0.72 mmol/L after 4 weeks and to 3.72 ± 0.81 mmol/L after 24 weeks), glucose (from 4.6 ± 0.4 to 4.5 ± 0.5 mmol/L after 4 weeks and to 4.6 ± 0.6 mmol/L after 24 weeks), and insulin (from 41.8 ± 9.8 to 42.3 ± 10.1 pmol/L after 4 weeks and to 43.6 ± 11.4 pmol/L after 24 weeks).

Effect of BM-MNC Implantation on Endothelial Function in Patients With Limb Ischemia

The intra-arterial infusion of ACh increased LBF in a dose-dependent manner. After BM-MNC implantation, LBF responses to ACh were enhanced in patients with limb ischemia (Figure, top). There was no significant difference in LBF response to ACh after 4 weeks and 24 weeks of follow-up (Figure, top). The intra-arterial infusion of SNP also increased LBF in a dose-dependent manner. The LBF response to SNP was unaffected by BM-MNC implantation (Figure, bottom). In the control group, there was no significant difference in LBF responses to ACh and SNP before and those after 4 weeks and 24 weeks of follow-up (Figure). No significant change was observed in arterial blood pressure or heart rate in response to intra-arterial infusion of either ACh or SNP before or after BM-MNC implantation and after 4 weeks of follow-up.

Discussion

In the present study, BM-MNC implantation improved not only limb ischemic symptoms and findings of angiography but also endothelium-dependent vasodilation in patients with limb ischemia. This beneficial effect of BM-MNC implantation on vascular function may be selective in endothelium-dependent vasodilation (endothelial cell function) but not in endothelium-independent vasodilation (smooth muscle cell function).



Comparison of LBF (as % change from basal flow) response to ACh administration (top) and SNP administration (bottom) before and after BM-NMC implantation of 4 weeks and 24 weeks of follow-up in patients with limb ischemia.

Our results showed that BM-MNC implantation increased the ankle-brachial pressure index, transcutaneous oxygen pressure, and basal LBF per se. Therefore, one possible mechanism by which BM-MNC implantation augments endothelium-dependent vasodilation is by increasing shear stress results from blood flow. Acute or chronic increases in shear stress stimulate the release of nitric oxide in isolated vessels and cultured cells through the enhanced expression of endothelial nitric oxide synthase gene.[8,9]

BM-MNCs ($CD34^+$ fraction) include endothelial progenitor cells and various angiogenetic growth factors, such as the vascular endothelial growth factor (VEGF) and angiopoietin families. Supplementation of the progenitor endothelial cells results in augmentation of neovascularization of ischemic tissue and repair of mature endothelial cells that release nitric oxide.[10] VEGF induces the formation of collateral vessels and increases collateral blood flow, leading to improvement in endothelium-dependent vasodilation.[11] In addition, VEGF directly upregulates endothelial nitric oxide synthase expression and increases subsequent nitric oxide release.[12] Rajagopalan et al[5] recently reported that gene therapy using an adenoviral vector encoding a 121-amino-acid isoform of VEGF augmented ACh-induced vasodilation in lower-leg circulation in patients with peripheral arterial disease. Although the mechanism by which BM-MNC implantation improves endothelial function in patients with limb ischemia is not clear, the multiplier effect of progenitor endothelial cells and VEGF may contribute to the angiogenesis-induced improvement in endothelium-dependent vasodilation.

We have recently shown that antihypertensive agents, such as ACE inhibitors, restore endothelial function in patients with mild to moderate hypertension but not in patients with severe hypertension.[13] It is clinically important that endothelial dysfunction is reversible by BM-MNC implantation in patients with severe atherosclerosis. BM-MNC implantation is expected to prevent the development of atherosclerosis through improvement in endothelial function.

Although a drastic change in endothelial function was observed after BM-MNC implantation, the number of subjects in this study was small, and the observation period is relatively short. In addition, this phase 1 clinical trial was not placebo-controlled. Controlled studies using a large population of patients and with long observation periods are needed to determine the role of BM-MNC implantation in endothelial function in patients with severe atherosclerosis.

Although the effectiveness of therapeutic angiogenesis with VEGF gene therapy in patients with peripheral arterial diseases has been established, BM-MNC implantation therapy may provide a new aspect of therapeutic angiogenesis in such patients.

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ORIGINAL ARTICLE

Strategy for treating elderly Japanese with hypercholesterolemia*

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Background: It has been widely accepted that control of serum cholesterol levels is effective for prevention of cardiovascular events. Recent data have suggested that this is also the case in the elderly.

Methods: A research group (chaired by T. Kita) was organized as part of the Comprehensive Research on Aging and Health conducted by the Japanese Ministry for Health, Labour, and Welfare in 1999–2002 to determine the best strategy for control of cholesterol levels in elderly Japanese with hypercholesterolemia. In order to do this a review of the literature was conducted.

Conclusion: The research group concluded: (i) Japanese patients aged 65–74 years with hypercholesterolemia should be treated by following the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases by the Japan Atherosclerosis Society (2002), as cholesterol-lowering therapy would bring a similar, or even larger, preventive effect to the elderly, whose absolute risk of cardiovascular events is higher than that in the younger population; (ii) target cholesterol levels in elderly Japanese aged ≥ 75 years with

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hypercholesterolemia should be determined individually according to their physical activities. It is noted that the elderly are more susceptible to drug-related adverse effects than the younger since renal and liver functions, required for metabolizing drugs, in the elderly are relatively weaker.

Keywords: cardiovascular event, elderly, hypercholesterolemia, Japanese, statin.

Introduction

It is well known that cardiovascular events occur in elderly people more frequently than in the younger population. It is also known that the incidence of these events increases as serum cholesterol levels are elevated. In Japan, populations of elderly people are rapidly increasing and serum cholesterol levels have been clearly rising in all ranges of ages probably due to westernization of our dietary habits.[1] Therefore, a rapid increase in atherosclerotic diseases is anticipated in Japan, especially in the elderly, without appropriate prevention.

Data obtained in many clinical studies performed in Western countries have demonstrated that cholesterol-lowering therapy with HMG-CoA reductase inhibitors, statins, reduces cardiovascular events by 26–37%.[2–4] Therefore, therapeutic intervention to control serum cholesterol levels is widely accepted. So far, guidelines for controlling cholesterol levels have been established in several countries, such as ATPIII (http://www.nhlbi.nih.gov/guidelines/cholesterol/atp_iii.htm) in the USA. Since the incidence of cardiovascular events in the Japanese population is clearly lower than that in Western countries, establishment of the Japanese guideline has been considered necessary. The first Japanese guideline was established by the Japanese Atherosclerosis Society in 1997 and it has been revised in 2002 (http://jas.umin.ac.jp). Since the subjects for the guideline are those aged ≤ 65 years, the guideline for elderly Japanese has been expected to be established.

In 1996–99, the research group for 'Establishing Japanese guidelines for treating atherosclerotic diseases in the elderly' was organized as part of the Comprehensive Research on Aging and Health conducted by the Japanese Ministry for Health, Labour and Welfare and the first guideline was proposed in 1999 (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). In this guideline, the target cholesterol levels for the elderly were recommended to be 20 mg/dL higher than those for the younger population, based on the comparison of relative risk increase in relation to serum cholesterol levels between younger people and the elderly (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). Since then, several important clinical datasets in Western countries and results of studies conducted in Japan,[2–4] such as the KLIS,[5,6] the J-LIT and PATE have been produced.[7–9] Therefore, the research group was again organized in 1999–2002 in order to conduct a research project entitled 'Long-term prognosis of the elderly with hyperlipidemia' (chaired by T. Kita) as a part of the Comprehensive Research on Aging and Health with a view to re-evaluating the proposed guideline (Kita & Hata *et al.* unpublished report to the Japanese Ministry of Health and Welfare 1999). The research group has concluded that serum cholesterol levels in Japanese aged 65–74 years are recommended to be controlled in the same way as for patients aged ≤ 65 years by following the Guideline for Diagnosis and Treatment of Atherosclerotic Cardiovascular Diseases (2002) by the Japan Atherosclerosis Society (http://jas.umin.ac.jp/), and that for those aged ≥ 75 years the control levels should be determined individually based on their physical activities (Kita & Matsuzawa *et al.* unpublished report to the Japanese Ministry of Health and Welfare 2002).

Clinical data in Western countries

Secondary prevention studies such as 4S and CARE have been analyzed with a focus on the elderly.[10,11] In both studies, treatment with simvastatin and pravastatin in the elderly patients was as safe and effective for reducing serum cholesterol levels as it was in younger patients.[10,11] In the 4S study, 4444 patients with established coronary heart diseases were divided into simvastatin and placebo groups, and followed for 5.4 years.[10] In this study, simvastatin treatment reduced total cholesterol levels by 26% in the elderly aged 65–70 years and by 25% in younger patients,[10] indicating that the cholesterol lowering effect of simvastatin in the elderly is similar to that in the younger. The relative risk reduction of major coronary events, including coronary artery death and non-fatal myocardial infarction, by simvastatin in the elderly patients was 34%, similar to that in younger patients aged < 65 years.[10] In the CARE study, 4159 patients were divided into pravastatin and placebo groups and followed for 5 years.[11] In this study, pravastatin treatment reduced total cholesterol levels by 19% in the elderly aged 65–75 years and by 20% in patients aged < 65 years,[11] indicating that the cholesterol lowering effect of simvastatin in the elderly is similar to that in younger patients. The relative risk reduction in the elderly group was 39% while that in the younger was 13%.[11] Because of the higher absolute risk and greater effect on risk reduction in the elderly group, the number

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 N^G-monomethyl-L-arginine in smokers and in nonsmokers. The intra-arterial coinfusion 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,[1,2] the underlying mechanisms are not completely understood. Smoking causes endothelial dysfunction in smokers and passive smokers,[3,4] leading to cardiovascular and cerebrovascular complications.[5]

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,[6,7] smooth muscle contraction,[8] and gene expression,[9] 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.[10–12] Smooth muscle dysfunction has been found in subjects with atherosclerosis.[13] 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

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subjects were taking oral contraceptives, antioxidant vitamins, or cardioactive 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.[14,15]

Procedures

The forearm vascular responses to ACh (Daiichi Pharmaceutical Co) were evaluated in 10 smokers and 14 nonsmokers, and fasudil (Asahi Chemical Industries) and SNP (Maluishi 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^G-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) were determined using Friedewald's methods. The concentration of angiotensin II was assayed by radioimmunoassay. 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).

Clinical Characteristics of Smokers and Nonsmokers

Variables	Smoker (n=10)	Nonsmoker (n=14)
Age, y	24.9±5.3	25.1±4.6
Body mass index, kg/m²	22.7±2.5	24.6±3.0
Systolic blood pressure, mm Hg	118.0±9.3	123.0±6.4
Diastolic blood pressure, mm Hg	61.8±6.8	64.6±6.6
Mean blood pressure, mm Hg	80.5±7.0	84.1±5.2
Heart rate, bpm	65.5±8.1	64.1±8.5
Total cholesterol, mmol/L	4.30±0.80	4.42±0.71
Triglyceride, mmol/L	1.06±0.34	1.07±0.37
HDL cholesterol, mmol/L	1.37±0.36	1.31±0.38
LDL cholesterol, mmol/L	2.45±0.83	2.62±0.55
Mean fasting plasma glucose, mmol/L	5.0±0.4	5.2±0.4
Plasma NE, ng/mL	0.17±0.10	0.20±0.13
Plasma Ang II, pg/mL	7.00±2.31	6.00±3.57
FBF, mL/min per 100 mL tissue	6.9±1.9	7.1±2.8

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.

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 baseline FBF were also similar in the 2 groups.

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±6.8 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.

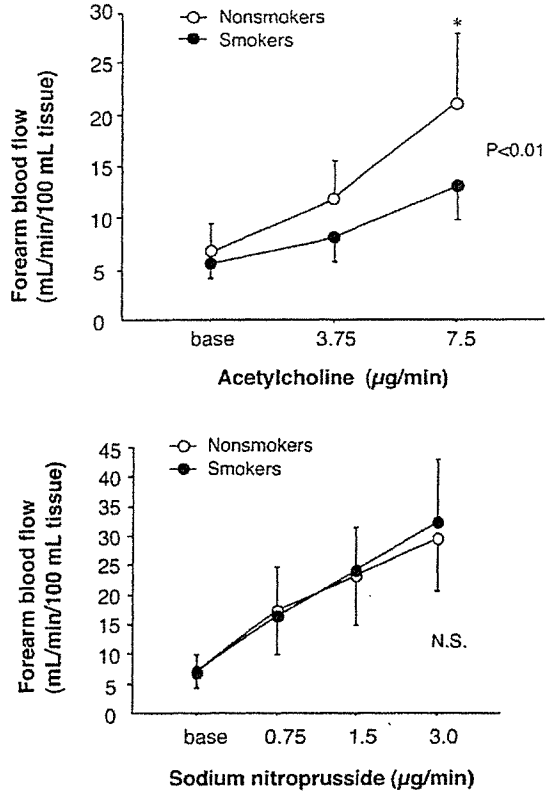

Figure 1. Effects of ACh on FBF in smokers (●) and nonsmokers (○; top). *$P<0.01$ vs smokers. Effects of SNP on FBF in smokers (●) and nonsmokers (○; bottom). Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

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 versus 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 ($r=-0.67$; $P<0.01$).

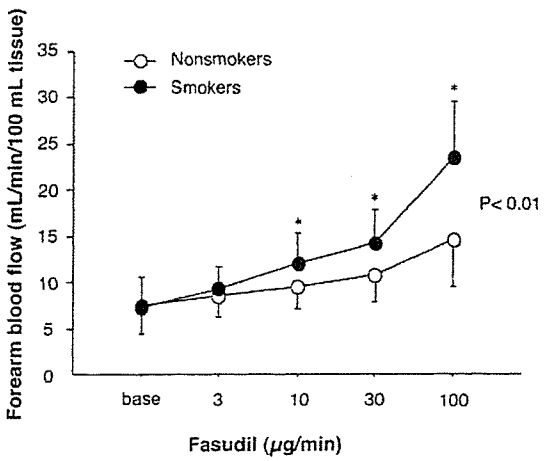

Figure 2. Effects of fasudil on FBF in smokers (●) and nonsmokers (○). *$P<0.05$ vs nonsmokers. Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

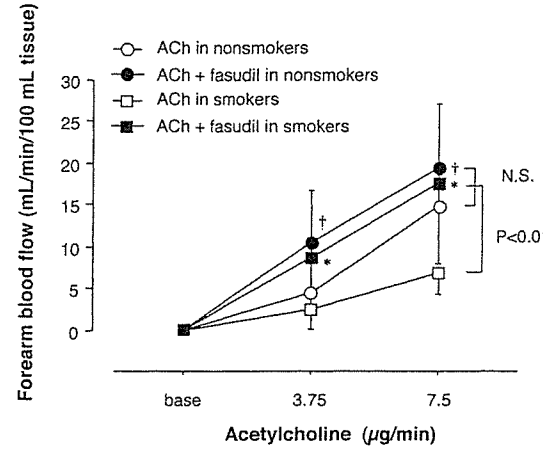

Figure 3. Effects of ACh alone (○) and in combination with fasudil (●) on FBF in smokers and nonsmokers. *$P<0.05$ vs before fasudil in smokers. †$P<0.05$ vs before fasudil in nonsmokers. Results are presented as mean±SD. The P value refers to a comparison of time course curves by ANOVA for repeated measurements.

However, there was no significant relationship between the maximal FBF response to ACh and that to SNP ($r=0.08$; P=NS) or between the maximal FBF response to fasudil and that to SNP ($r=0.28$; P=NS).

FBF Responses to ACh Alone and in Combination With Fasudil in Smokers and Nonsmokers

The intra-arterial coinfusion of fasudil significantly augmented FBF response to ACh in smokers ($P<0.01$; Figure 3) but not in nonsmokers (P=NS; Figure 3). During coinfusion of fasudil, there was no significant difference in ACh-induced vasodilation between the 2 groups. No significant change was found in arterial blood pressure or heart rate with intra-arterial infusion of ACh alone and in combination with fasudil in either.

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

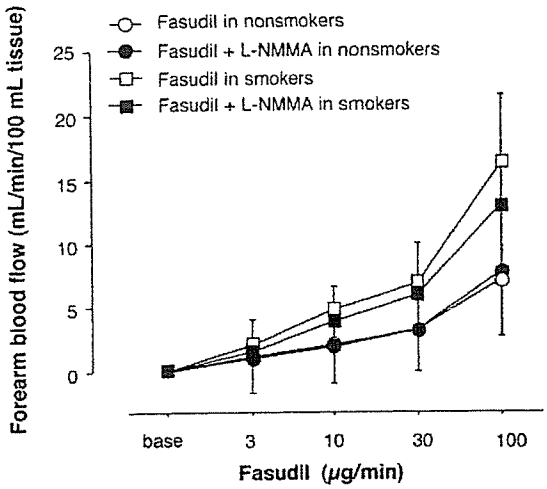

Figure 4. Effects of fasudil alone (□) and in combination with L-NMMA (■) on FBF in smokers and those of fasudil alone (○) and in combination with L-NMMA (●) on FBF in nonsmokers. Results are presented as mean±SD. The *P* value refers to a comparison of time course curves by ANOVA for repeated measurements.

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

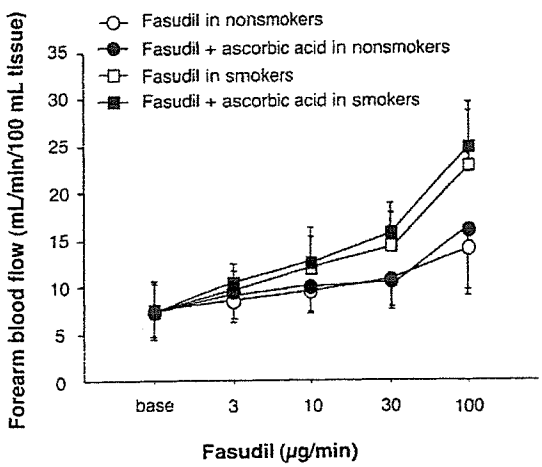

Figure 5. Effects of fasudil alone (□) and in combination with ascorbic acid (■) on FBF in smokers, and those of fasudil alone (○) and in combination with ascorbic acid (●) on FBF in nonsmokers. Results are presented as mean±SD. The *P* value refers to a comparison of time course curves by ANOVA for repeated measurements.

that smoking is significantly associated with endothelial dysfunction and cardiovascular disease.[3,5,16,17] Structural damage,[18] a direct toxic effect,[19] a decreased production or bioavailability of endothelial NO,[1,2] and a superoxide anion by containing of a large number of free radicals and prooxidants in cigarette smoke[20] 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.[21,22] Hernandez-Perera et al[23] 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.[24,25] Sauzeau et al[24] have shown that exogenous NO attenuates RhoA-dependent Ca^{2+} 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.[8,11,12,26] Uehata et al[27] 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.[28–30] 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.[31] Moreover, MLC diphosphorylation as well as MLC monophosphorylation were found in impaired VSMCs.[26,32] 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,[14] and we evaluated Rho-kinase activity in VSMCs by using fasudil, a Rho-kinase inhibitor.[15]

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.[33,34] In addition, fasudil is used for assessment of Rho-kinase activity in humans.[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, coinfusion of fasudil significantly augmented the FBF response to ACh in smokers but not in nonsmokers. These results may be attributable to decreased Ca^{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 in combination with fasudil in smokers and that in nonsmokers in the present study. This may be explained by a decrease in Ca^{2+} sensitivity attributable to inhibition of Rho-kinase in VSMCs and by an increase in phosphorylation of eNOS attributable to inhibition of Rho-kinase in endothelial cells in smokers.[22] Wolfrum et al[35] demonstrated in a rat model of myocardial infarction that acute administration of fasudil leads to rapid activation of eNOS 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.[23,35] We cannot deny the possibility that fasudil improves endothelial function via upregulation of eNOS expression in smokers.

Recently, Higashi et al[36] demonstrated that Rho-kinase is substantially involved in production of ROS through NAD(P)H oxidase upregulation. Moreover, several investigators have shown a possible interaction between Rho/Rho-kinase and ROS.[37,38] In the present study, coinfusion of antioxidant ascorbic acid had no effect on FBF response to fasudil in smokers or nonsmokers. It is unlikely that ROS has effects on Rho-kinase activity in healthy young male 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.

Acknowledgments

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Effects of Acute Administration of Caffeine on Vascular Function

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Caffeine is the most widely used pharmacologic substance in the world. It is found in common nonessential grocery items (e.g., coffee, tea, cocoa, and chocolate). The effects of caffeine on cardiovascular diseases, including hypertension, remain controversial, and there is little information on its direct effect on vascular function. The purpose of this study was to determine the effect of caffeine on endothelial function in humans. This study was a double-blind, randomized placebo and active drug study. Forearm blood flow (FBF) responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and to sodium nitroprusside, an endothelium-independent vasodilator, were evaluated in healthy young men before and after the oral administration of caffeine 300 mg (n = 10) or placebo (n = 10). FBF was measured by using a strain-gauge plethysmograph. Caffeine significantly increased systolic and diastolic blood pressures by 6.0 ± 6.0 and 2.6 ± 3.1 mm Hg ($p < 0.05$), respectively, but did not alter heart rate or baseline FBF. Caffeine augmented the FBF responses to ACh from 21.2 ± 7.1 to 26.6 ± 8.1 ml/min/100 ml tissue ($p < 0.05$), whereas sodium nitroprusside–stimulated vasodilation was not altered by caffeine administration. The intra-arterial infusion of N^G-monomethyl-L-arginine, a nitric oxide synthase inhibitor, abolished the caffeine-induced augmentation of FBF response to ACh. In the placebo group, the ACh- and sodium nitroprusside–stimulated vasodilation was similar before and after the follow-up period. In conclusion, these findings suggest that the acute administration of caffeine augments endothelium-dependent vasodilation in healthy young men through an increase in nitric oxide production. (Am J Cardiol 2006;98:1538–1541)

This study was designed to examine the effects of the acute administration of caffeine on systemic hemodynamics and endothelial function in humans by measuring forearm blood flow (FBF) responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and to sodium nitroprusside (SNP), an endothelium-independent vasodilator.

Methods and Results

The subjects were 20 young healthy men recruited from healthy volunteers. All were nonhabitual caffeine consumers who did not consume caffeine every day. This study was a double-blind, randomized placebo and active drug study. The 20 subjects were randomly assigned to receive caffeine (caffeine group; n = 10, mean age 26.8 ± 5.2 years) or placebo (control group; n = 10, mean age 26.1 ± 3.8 years). The study protocol was approved by the ethics committee of the Hiroshima University Graduate School of Biomedical Sciences. Informed consent was obtained from all subjects before participation.

FBF was measured with the use of a mercury-filled Silastic strain-gauge plethysmograph (EC-5R, D.E. Hokanson, Inc., Bellevue, Washington), as previously described.[1,2] Three plethysmographic measurements were averaged to determine FBF at baseline and during the administration of each drug. FBF is expressed in milliliters per minute per 100 ml of forearm tissue volume. FBF was calculated by 2 independent observers blinded to the study protocol from the linear portions of plethysmographic recordings. The intraobserver coefficient of variation was 3.0%. We confirmed the reproducibility of FBF responses to ACh and

Table I
Clinical characteristics of the control and caffeine groups

Variable	Control (n = 10)	Caffeine (n = 10)
Body mass index (kg/m^2)	22 ± 2	22 ± 1
Systolic blood pressure (mm Hg)	114 ± 7	117 ± 10
Diastolic blood pressure (mm Hg)	64 ± 8	61 ± 8
Heart rate (beats/min)	65 ± 7	61 ± 9
Total cholesterol (mmol/L)	4.12 ± 0.59	4.05 ± 0.65
Triglycerides (mmol/L)	1.27 ± 0.67	1.11 ± 0.55
Triglycerides (mg/dl)	112 ± 59	98 ± 49
Low-density lipoprotein cholesterol (mmol/L)	2.25 ± 0.54	2.12 ± 0.56
Low-density lipoprotein cholesterol (mg/dl)	87 ± 21	82 ± 22
Serum creatinine (μmol/L)	80 ± 9	79 ± 9
FBF (ml/min/100 ml tissue)	6.3 ± 2.8	6.1 ± 3.0

All results are expressed as mean ± SD.

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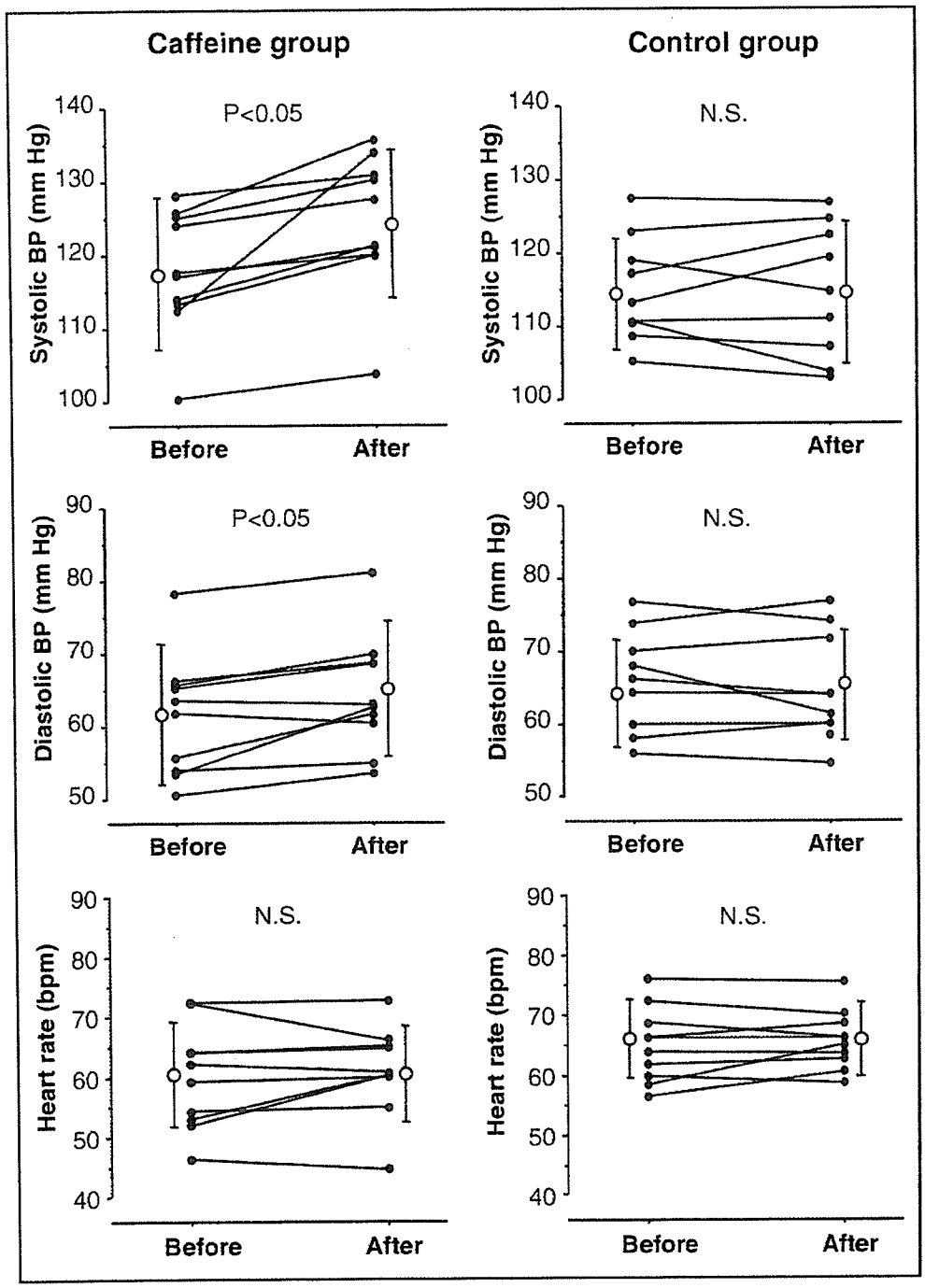

Figure 1. Effects of caffeine *(left)* and placebo *(right)* on systolic blood pressure (BP), diastolic BP, and heart rate.

SNP on 2 separate occasions in 10 healthy men (mean age 24 ± 4 years). The coefficients of variation were 6.2% and 4.6%, respectively.

All measurements were performed for subjects in the supine position in a temperature-controlled (22°C to 25°C), quiet, dark laboratory. All subjects abstained from caffeine, ethanol, and nicotine for ≥24 hours before the start of the study. After 30 minutes in the supine position, baseline FBF, heart rate, and arterial blood pressure were measured. Then the intra-arterial infusions of the endothelium-dependent vasodilator ACh (3.75, 7.5, and 15 μg/min) or the endothelium-independent vasodilator SNP (0.75, 1.5, and 3.0 μg/min) were performed randomly every 5 minutes, and FBF during the final 2 minutes of each infusion was measured.

After a 30-minute rest period, caffeine 300 mg or placebo was administered orally to each subject. Baseline FBF, heart rate, and arterial blood pressure were measured 1 hour after the oral administration of caffeine or placebo. The effects of ACh and SNP were determined again by the same

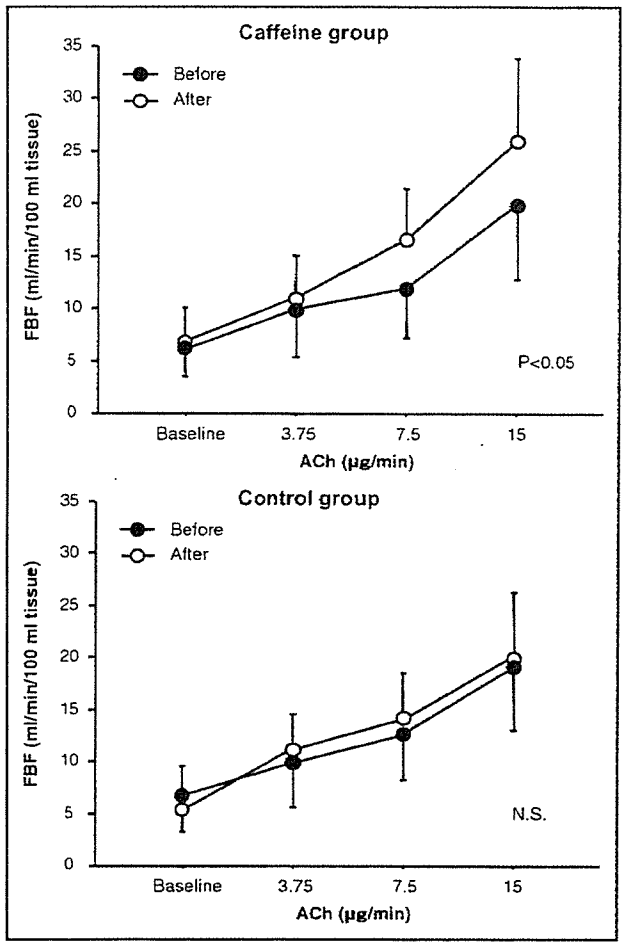

Figure 2. Effects of ACh on FBF before and after caffeine or placebo administration.

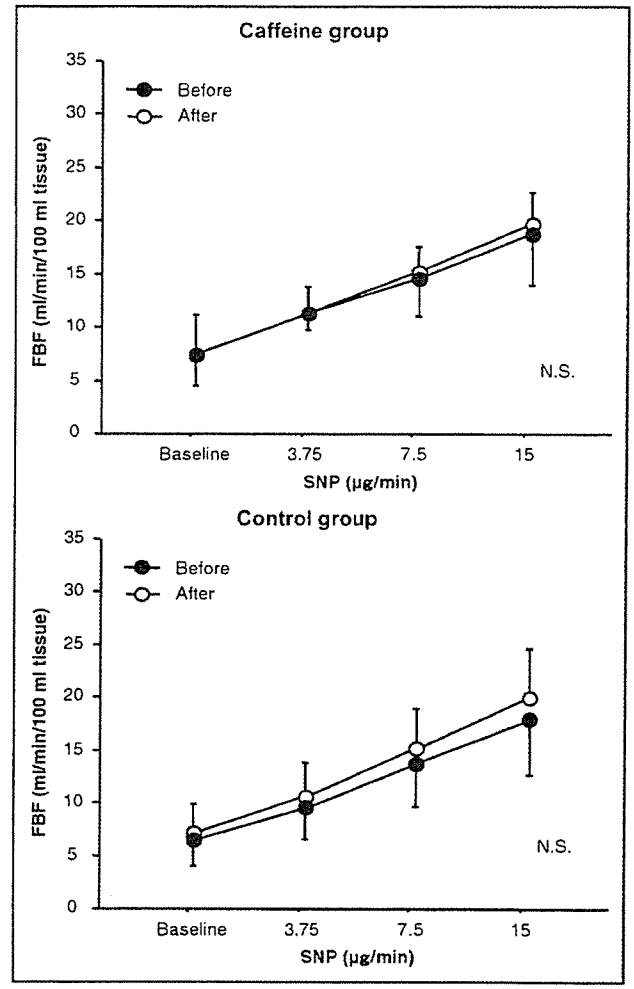

Figure 3. Effect of SNP on FBF before and after caffeine or placebo administration.

method as that used before caffeine and placebo administration.

After a 30-minute rest period, N^G-monomethyl-L-arginine (L-NMMA; Clinalfa Company, Läufelfiger, Switzerland), a nitric oxide synthase inhibitor, was infused intra-arterially at a dose of 8 μg/min for 5 minutes while baseline FBF and arterial blood pressure were recorded, and ACh (3.75, 7.5, and 15 μg/min) was administered.

The results are expressed as mean ± SD. Values of $p < 0.05$ were considered to indicate statistical significance. Baseline characteristics between 2 groups were compared using the Mann-Whitney U-statistic test. The effects of interventions on blood pressure, heart rate, and FBF were analyzed with the paired Student's *t* test. Comparisons of dose-response curves of parameters during the infusion of the drugs were analyzed with repeated-measures analysis of variance. The data were processed using the software package StatView V (SAS Institute Inc., Cary, North Carolina).

Baseline clinical characteristics in the caffeine group and control group are summarized in Table 1. There were no significant differences between the 2 groups in systolic blood pressure, diastolic blood pressure, heart rate, FBF, and other parameters.

Caffeine elevated systolic blood pressure from 117 ± 10 to 123 ± 9 mm Hg ($p < 0.05$) and elevated diastolic blood pressure from 61 ± 8 to 64 ± 8 mm Hg ($p < 0.05$) but did not alter heart rate (Figure 1). There were no significant differences in systolic and diastolic blood pressures or heart rate after placebo ingestion.

The intra-arterial infusion of ACh and SNP significantly increased FBF in a dose-dependent manner in the caffeine and control groups. FBF responses to ACh and SNP were similar in the 2 groups. Neither caffeine nor placebo altered baseline FBF. Caffeine significantly augmented FBF response to ACh ($p < 0.05$), whereas placebo did not alter FBF response to ACh (Figure 2). Neither caffeine nor placebo altered FBF response to SNP (Figure 3). No significant change was observed in arterial blood pressure or heart rate with the intra-arterial infusion of either ACh or SNP in any of the subjects.

The intra-arterial infusion of L-NMMA reduced baseline FBF and abolished the caffeine-induced augmentation of FBF response to ACh (Figure 4). No significant change was observed in arterial blood pressure or heart rate with the intra-arterial infusion of ACh in the presence of L-NMMA.

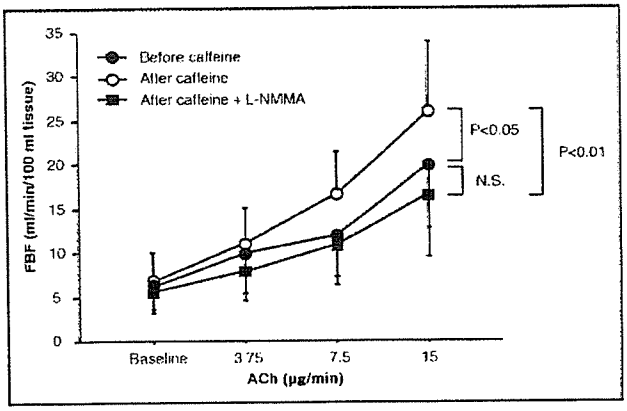

Figure 4. Effects of ACh on FBF in the presence of L-NMMA after caffeine administration.

Discussion

To our knowledge, this is the first study to demonstrate that the oral administration of caffeine increases FBF response to ACh, an endothelium-dependent vasodilator. In addition, L-NMMA completely abolished the caffeine-induced augmentation of FBF response to ACh. Caffeine elevated systolic and diastolic blood pressures but did not alter heart rate.

Some investigators have hypothesized that caffeine is a vasoconstrictive substance.[3–9] In the present study, systolic and diastolic blood pressures were elevated after caffeine ingestion, suggesting vasoconstrictive effects of caffeine.[5] Caffeine should be an antagonist of the adenosine receptor.[10,11] It is well known that adenosine induces vasodilation. Therefore, antagonization of the adenosine receptor could induce vasoconstriction. However, although oral caffeine ingestion did not change baseline FBF, FBF response to ACh was significantly increased in the caffeine group. Hatano et al[12] reported that caffeine promotes nitric oxide synthesis in the endothelium by the release of Ca^{2+} from the endoplasmic reticulum through activation of the ryanodine-sensitive Ca^{2+} channel and the suppression of cyclic guanosine monophosphate degradation in the isolated rat aorta, resulting in the caffeine-induced augmentation of endothelium-dependent vasodilatation. In the present study, L-NMMA, a nitric oxide synthase inhibitor, completely abolished the caffeine-induced augmentation of endothelium-dependent vasodilation. These findings suggest that caffeine augments endogenous nitric oxide production by agonist stimulation. A balance of the vasodilatory effect of caffeine as an endothelium-dependent vasodilator and the vasoconstrictive effect of caffeine as an adenosine receptor antagonist may regulate vascular function.

In the present study, caffeine ingestion elevated systolic and diastolic blood pressures in the brachial artery. Our results support those of previous studies showing that the acute administration of caffeine elevates peripheral blood pressure.[6–9] Karatzis et al[13] demonstrated the augmentation of central blood pressure after the acute administration of caffeine, but peripheral systolic blood pressure did not significantly change. It has been reported that various factors, such as hypertension, exercise stress, and age, influence blood pressure response to caffeine.[14] These observations suggest that the confounding factors should be kept fairly constant for the assessment of changes in blood pressure during caffeine administration.

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Roles of Rho-Associated Kinase and Oxidative Stress in the Pathogenesis of Aortic Stiffness

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Objectives The purpose of this study was to determine the relationship between Rho-associated kinase (ROCK) activity and aortic stiffness in humans.

Background Epidemiologic studies have shown that there is a relationship between aortic stiffness and cardiovascular complications. Recent evidence suggests that ROCK plays an important role in the process of atherosclerosis.

Methods We evaluated the forearm blood flow (FBF) response to sodium nitroprusside (SNP), a nitric oxide donor, acetylcholine (ACh), an endothelium-dependent vasodilator, and fasudil, a specific ROCK inhibitor, in 51 healthy male subjects (mean age 45.6 ± 3.0 years). The FBF was measured by using a strain-gauge plethysmography. Carotid-femoral pulse wave velocity (cf-PWV) was measured to assess the aortic stiffness using a pulse wave velocimeter.

Results Intra-arterial infusion of SNP alone, ACh alone, or fasudil alone and after co-infusion of N^G-monomethyl-L-arginine (L-NMMA), a nitric-oxide synthase inhibitor, significantly increased FBF in a dose-dependent manner ($p < 0.01$). Multivariate analysis showed that age and number of pack-years smoked were independent predictors of ROCK activity before or after co-infusion of L-NMMA ($p < 0.01$) and that age and ROCK activity before or after co-infusion of L-NMMA were independent predictors of cf-PWV ($p < 0.01$). The concentration of serum malondialdehyde-modified low-density lipoprotein, an index of oxidative stress, was significantly correlated with ROCK activity before and after co-infusion of L-NMMA and cf-PWV ($p < 0.01$).

Conclusions These findings suggest that aging and accumulating smoking habit, which might induce excessive oxidative stress, are involved in ROCK activity in the vasculature, leading to an increase in aortic stiffness in humans. (J Am Coll Cardiol 2007;49:698–705) © 2007 by the American College of Cardiology Foundation

The small guanosine triphosphatase (GTPase) Rho works as a switch and plays an important role in various cellular physiologic functions, including actomyosin-based cellular processes such as cell adhesion, migration, motility, cytokinesis, and contraction, all of which may be involved in the pathogenesis of atherosclerosis (1). There is growing evidence that Rho-associated kinase (ROCK) (also known as Rho-kinase), the immediate downstream target of the small GTP-binding protein Rho, contributes to endothelial dysfunction and vascular disease (2–6). Indeed, recent clinical evidence has demonstrated that ROCK is significantly activated in patients with coronary vasospasm (7), hypertension (8), and stable-effort angina (9) and even in current smoking subjects (10,11). ROCK, therefore, is becoming a new therapeutic target in cardiovascular disease. ROCK physiologically plays a key role in vasoconstriction. It activates myosin light chain kinase by phosphorylation of the myosin-binding subunit in myosin light chain phosphatase, leading to contraction of vascular smooth muscle cells (VSMC) (12,13). Thus, the vasoconstriction mediated by ROCK is dependent on Ca^{2+} sensitization but independent of Ca^{2+} concentration. Earlier studies have known that vascular dysfunction, including endothelial and VSMC dysfunction, is associated with cardiovascular risk factors such as aging, smoking habit, and oxidative stress (14,15), but little is known about the underlying correlations of

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ROCK with cardiovascular risk factors. In addition, there is no clinical evidence of a relationship between ROCK and oxidative stress, although recent studies have revealed a relationship between endothelial dysfunction and ROCK (3,4,11,16) and between endothelial dysfunction and oxidative stress (17,18). Several recent studies have provided evidence that reactive oxygen species activate the Rho/ROCK pathway (19,20). Indeed, we previously showed that there is a significant relationship between endothelial dysfunction and increased ROCK activity in young current smokers, which might be considered as a human model of excess oxidative stress compared with young nonsmokers (11). Taken together, these findings indicate that reactive oxygen species may play an important role in activation of ROCK in humans.

Epidemiologic and clinical studies have shown that impaired aortic stiffness, which can be assessed noninvasively by measurement of pulse wave velocity (PWV), is an independent marker to estimate subjects with cardiovascular disease (21,22). The PWV depends on arterial wall structure, mainly collagen and elastin, and arterial function (23). The elastic properties of the aorta and central arteries are the major determinants of systemic arterial impedance, and PWV measured along the aortic and aortoiliac pathway is the most clinically relevant.

To determine the roles of ROCK and oxidative stress in the pathogenesis of impaired aortic stiffness, we evaluated whether oxidative stress is related to ROCK activity and subsequently to aortic stiffness in humans.

Methods

Subjects. We studied 51 healthy male subjects (mean age 45.6 ± 3.0 years). Subjects with a history of hypertension, hypercholesterolemia, or diabetes mellitus were excluded. Normal blood pressure was defined as systolic blood pressure of <140 mm Hg and diastolic blood pressure of <85 mm Hg. The results of physical and routine laboratory examinations of the subjects were normal. None of the subjects were taking oral antioxidant vitamins or vasoactive drugs. Current smokers were defined as any who had smoked at least 1 pack-year. One pack-year was defined as 20 cigarettes per day for 1 year. All of the smokers (35.2 ± 5.2 pack-years) had a current smoking history of more than 5 years and abstained from smoking for at least 3 h before the forearm blood flow (FBF) measurements. We defined nonsmokers as those who had never smoked. 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.

Procedures. Forearm vascular response to sodium nitroprusside (SNP) (Maluishi Pharmaceutical Co., Tokyo, Japan) alone, acetylcholine (ACh) (Daiichi Pharmaceutical Co., Tokyo, Japan) alone, and fasudil (Asahi Chemical Industries, Tokyo, Japan) alone and after co-infusion of N^G-monomethyl-L-arginine (L-NMMA) (Sigma Chemical Co., St. Louis, Missouri), were evaluated. The study began at 8:30 AM with the subjects in the fasting condition. A 23-gauge polyethylene catheter (Hakkow Co., Tokyo, Japan) was inserted into the left brachial artery for the infusion of each drug and for the recording of arterial pressure with an AP-641G pressure transducer (Nihon Kohden Co., Tokyo, Japan) under local anesthesia (1% lidocaine). Another catheter was inserted into the left deep antecubital vein to obtain blood samples.

Abbreviations and Acronyms

ACh = acetylcholine
cf-PWV = carotid-femoral pulse wave velocity
FBF = forearm blood flow
L-NMMA = N^G-monomethyl-L-arginine
MDA-LDL = malondialdehyde-modified low-density lipoprotein
PWV = pulse wave velocity
ROCK = Rho-associated kinase
SNP = sodium nitroprusside
VSMC = vascular smooth muscle cells

After 30 min in the supine position, we measured basal FBF and arterial blood pressure. Then forearm vascular response to SNP, a direct vasodilator of VSMCs, ACh, an endothelium-dependent vasodilator, and fasudil, a specific ROCK inhibitor, on forearm hemodynamics were measured. The SNP (0.75, 1.5, or 3.0 μg/min), ACh (3.75 or 7.5 μg/min), and fasudil (3, 10, or 30 μg/min) were infused intra-arterially for 5 min at each dose. The FBF was measured during the last 2 min of the infusion. The infusions of SNP, ACh, and fasudil were carried out in a randomized fashion. Each study proceeded after the FBF returned to baseline.

After a 30-min rest period, L-NMMA, an inhibitor of nitric oxide synthase, was infused intra-arterially at a dose of 8 μmol/min for 5 min, and fasudil was administered 5 min after the initiation of L-NMMA.

To evaluate the relationship among ROCK activity, oxidative stress, and aortic stiffness, we studied 35 healthy male subjects (mean age 45.0 ± 3.6 years), whose prespecified entry criteria are identical to that described in the preceding. Number of pack-years smoked in smokers was 29.0 ± 6.6 pack-years. The PWV was measured in the supine position after 15 minutes of bed rest, and then forearm vasodilative responses to SNP alone, ACh alone, and fasudil alone and after co-infusion of L-NMMA, were evaluated in a manner identical to that described in the preceding text.

Measurement of FBF. The FBF was measured with a mercury-filled Silastic strain-gauge plethysmography (EC-5R, D. E. Hokanson, Issaquah, Washington) as previously described (11,18).

Measurement of PWV. Aortic compliance was assessed noninvasively on the basis of Doppler ultrasound measurements of PWV along the descending thoracoabdominal aorta as previously published and validated (24). Briefly,

carotid-femoral PWV (cf-PWV), an index of arterial stiffness, was determined by 2 pressure sensors, placed on the right femoral and left carotid arteries to record each pulse wave simultaneously, and the time lag (t) between the notches of the 2 waves, using a pulse wave velocimeter (Form PWV/ABI, model BP-203RPE, Colin Co., Aichi, Japan). The distance (D) between the 2 recording sensors was calculated automatically from the value of individual height. The PWV value was calculated as PWV = D/t. The PWV was measured for 5 consecutive pulses, and averages were used for analysis. The observer was blind to the form of the examination.

Analytical methods. Routine chemical methods were used to determine serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose, and insulin. The serum concentration of malondialdehyde-modified low-density lipoprotein (MDA-LDL) was assayed by ELISA (antiMDA-LDL antibody, SRL Co., Atsugi, Japan). Fasting concentrations of insulin and glucose were used to determine homeostatic model of assessment (HOMA) parameters of insulin resistance using a program based on the HOMA algorithm (insulin resistance = insulin/22.5$e^{-\ln \text{glucose}}$), as previously described (25).

Statistical analysis. Results are presented as mean ± SEM. Values of $p < 0.05$ were considered to indicate statistical significance. Comparisons of time-course curves of variables during the infusion of SNP alone, ACh alone, and fasudil alone and after co-infusion of L-NMMA, were analyzed by analysis of variance (ANOVA) for repeated measures with Bonferroni correction to baseline. The ROCK activity was expressed as the percentage in the ratio of fasudil-stimulated maximal FBF relative to the immediately preceding basal FBF. Spearman rank correlation was used to compare ROCK activity before or after co-infusion of L-NMMA with age, body mass index (BMI), systolic blood pressure, diastolic blood pressure, heart rate, serum concentration of total cholesterol, HDL cholesterol, triglycerides, mean fasting glucose, insulin, MDA-LDL, and number of pack-years smoked. The analysis was also used to compare the ratio of SNP-stimulated maximal FBF to basal FBF or ACh-stimulated maximal FBF to basal FBF (endothelial function) with the variables and to compare the PWV with the variables in which endothelial function and ROCK activity before or after L-NMMA were added. Multivariate analysis using multiple stepwise regression was performed to determine the significant correlation of ROCK activity before or after co-infusion of L-NMMA and of PWV with variables which showed a p value of <0.1 in Spearman correlation analysis. Multivariate analysis was performed with the Statistical Analysis System program package (SAS Institute, Cary, North Carolina). The data were analyzed using the software package StatView V (SAS Institute) and Super ANOVA (Abacus Concepts, Berkley, California).

Table 1 Baseline Clinical Characteristics in the Subjects

Variable	Subjects (n = 51)
Age (yrs)	45.6 ± 3.0
Body mass index (kg/m^2)	23.5 ± 0.4
Systolic blood pressure (mm Hg)	123.6 ± 1.4
Diastolic blood pressure (mm Hg)	65.5 ± 1.4
Heart rate (beats/min)	62.5 ± 1.1
Total cholesterol (mmol/l)	4.70 ± 0.10
HDL cholesterol (mmol/l)	1.33 ± 0.06
Triglyceride (mmol/l)	1.03 ± 0.03
Mean fasting glucose (mmol/l)	5.1 ± 0.1
Serum insulin (pmol/l)	73.9 ± 16.6
Insulin resistance (HOMA index)	3.17 ± 0.81
Current smoker	27
Number of pack-yrs smoked	35.2 ± 5.2
Basal FBF (ml/min/100 ml tissue)	7.2 ± 0.4

All results are presented as mean ± SEM.
FBF = forearm blood flow; HDL = high-density lipoprotein; HOMA = homeostatic model assessment.

Results

Clinical characteristics. The baseline clinical characteristics of the subjects are summarized in Table 1.

Effects of FBF responses to SNP, ACh, and fasudil. Intra-arterial infusion of SNP significantly increased FBF in a dose-dependent manner by ANOVA for repeated measurements (6.1 ± 0.3 ml/min to 31.1 ± 1.4 ml/min per 100 ml tissue; $p < 0.01$) (Fig. 1). Intra-arterial infusion of ACh also significantly increased FBF in a dose-dependent manner by ANOVA for repeated measurements (7.7 ± 0.6 ml/min to 16.6 ± 1.5 ml/min per 100 ml tissue; $p < 0.01$) (Fig. 2). Intra-arterial infusion of fasudil significantly increased FBF in a dose-dependent manner alone and after co-infusion of L-NMMA by ANOVA for repeated measurements (7.0 ± 0.4 ml/min to 12.3 ± 0.6 ml/min per 100 ml tissue, 5.2 ± 0.4 ml/min vs. 10.3 ± 0.7 ml/min per 100 ml tissue, respectively; $p < 0.01$) (Fig. 3). No significant change was observed in arterial blood pressure or heart rate during intra-arterial infusion of any drugs.

Correlations with ROCK activity before and after L-NMMA. ROCK was significantly correlated with age ($r = 0.64$; $p < 0.001$), systolic blood pressure ($r = 0.44$; $p < 0.01$), serum concentration of total cholesterol ($r = 0.41$; $p < 0.01$), and number of pack-years smoked ($r = 0.52$; $p < 0.001$). The ROCK activity after co-infusion of L-NMMA was significantly correlated with age ($r = 0.64$; $p < 0.01$), systolic blood pressure ($r = 0.40$; $p < 0.01$), serum concentration of total cholesterol ($r = 0.47$; $p < 0.01$), and number of pack-years smoked ($r = 0.49$; $p < 0.01$). The ROCK activity before or after co-infusion of L-NMMA was not correlated with other parameters. The correlations between ROCK activity before or after co-infusion of L-NMMA and variables are summarized in Table 2. Stepwise multiple regression analysis was carried out to identify the independent predictors of ROCK activity before or after co-infusion

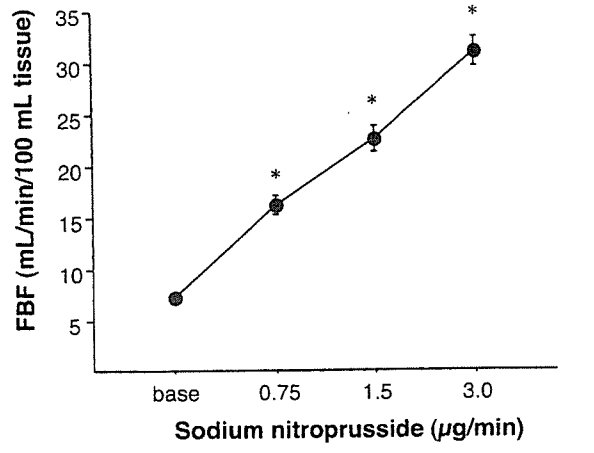

Figure 1 **FBF Response to SNP**

Results are presented as mean ± SEM. The probability value refers to a comparison of time course curves by analysis of variance for repeated measurements. *p < 0.01 versus basal forearm blood flow (FBF) assessed by Bonferroni correction. SNP = sodium nitroprusside.

of L-NMMA. For multiple regression analysis, variables showing a p value of <0.1 in Spearman correlation analysis were selected; age, systolic blood pressure, serum concentration of total cholesterol, and number of pack-years smoked were entered as candidates for independent variables. Stepwise multiple regression analysis revealed that age (standardized r = 0.36) and number of pack-years smoked (standardized r = 0.39) were independent predictors of

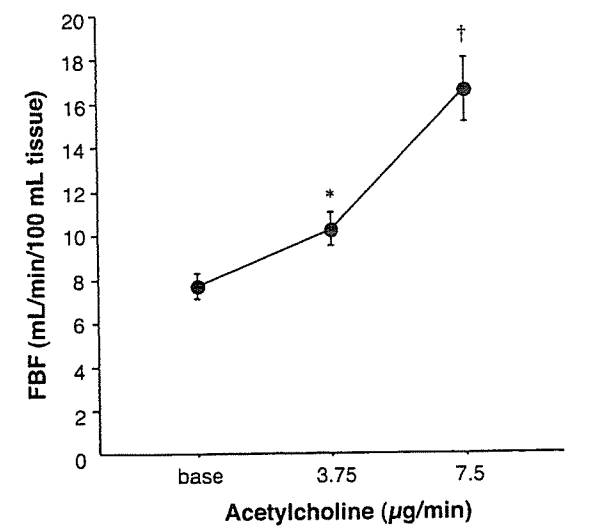

Figure 2 **FBF Response to ACh**

Results are presented as mean ± SEM. The probability value refers to a comparison of time course curves by analysis of variance for repeated measurements. *p < 0.05; †p < 0.01 versus basal forearm blood flow (FBF) assessed by Bonferroni correction. ACh = acetylcholine.

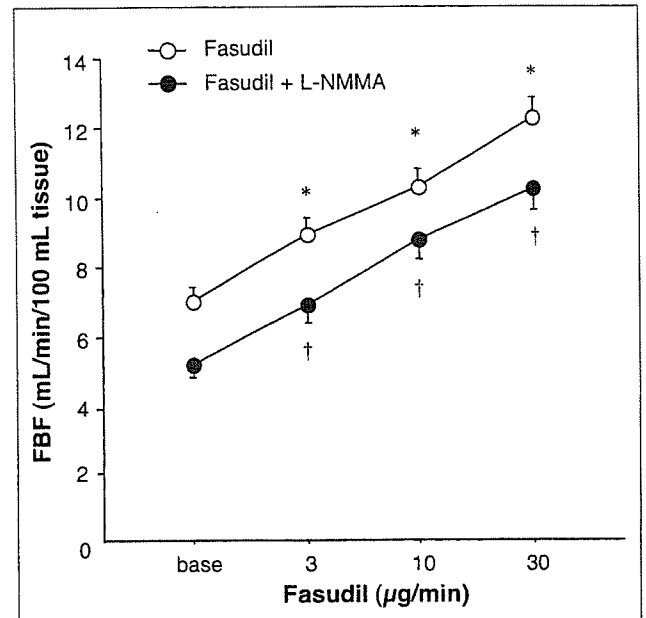

Figure 3 **FBF Response to Fasudil Alone and After Co-Infusion of L-NMMA**

Results are presented as mean ± SEM. The probability value refers to a comparison of time course curves by analysis of variance for repeated measurements in each group. *p < 0.01 versus basal forearm blood flow (FBF) of fasudil alone; †p < 0.01 versus basal FBF after co-infusion of N^G-monomethyl-L-arginine (L-NMMA) assessed by Bonferroni correction.

ROCK activity (multiple R^2 = 0.43; p < 0.01) and that age (standardized r = 0.34) and number of pack-years smoked (standardized r = 0.35) were independent predictors of ROCK activity after co-infusion of L-NMMA (multiple R^2 = 0.37; p < 0.01). The serum concentration of MDA-LDL was significantly correlated with ROCK activity before and after co-infusion of L-NMMA (r = 0.57 and r = 0.51, respectively; p < 0.01).

Correlations with maximal FBF response to SNP. There was no variable that was significantly correlated with the ratio of SNP-stimulated maximal FBF to basal FBF (Table 3). The serum concentration of MDA-LDL was also not significantly correlated with FBF response to SNP (r = −0.04; p = 0.81).

Correlations with endothelial function. Endothelial function was significant correlated with age (r = −0.55; p < 0.01), serum concentration of total cholesterol (r = −0.67; p < 0.01), and number of pack-years smoked (r = −0.59; p < 0.01) (Table 4). The serum concentration of MDA-LDL was also significantly correlated with endothelial function (r = −0.64; p < 0.01).

Correlations with cf-PWV. The cf-PWV was significantly correlated with age (r = 0.88, p < 0.01), systolic blood pressure (r = 0.42; p < 0.05), serum concentration of total cholesterol (r = 0.47; p < 0.01), number of pack-years smoked (r = 0.38; p < 0.05), endothelial function (r = −0.54; p < 0.01), ROCK activity (r = 0.67; p < 0.01), and ROCK activity after co-infusion of L-NMMA (r = 0.75;