the amount of NO release. The levels of TBARS, an indirect marker of oxidative stress in the RVLM, were higher in the AdiNOS group than in the Adβgal group. DHE staining, an oxidative fluorescent dye, detects in situ superoxide in the RVLM, and the intensity of the staining was greater in AdiNOS-transfected rats than in Adβgal-transfected rats. In addition, microinjection of tempol decreased blood pressure in the AdiNOS group, but not in the nontreated group. Furthermore, intracisternal infusion of tempol markedly attenuated the pressor response induced by AdiNOS transfection. In addition, the increased TBARS levels after AdiNOS transfection were significantly attenuated. Taken together, these results suggest that oxidative stress in the RVLM is increased in AdiNOS-transfected rats, and this increase might contribute to the pressor response evoked by iNOS transfection. NO might be trapped by superoxide anions. In support of this idea, we recently reported that increased reactive oxygen species in the RVLM contribute to the neural mechanisms of hypertension.[30]

An important finding of the present study was that blood pressure was increased after transfection of AdiNOS. The time course of the change in blood pressure was consistent with that of iNOS protein expression levels. This increase in blood pressure was nearly abolished by microinjection of aminoguanidine or SMT, a selective iNOS inhibitor, and partly inhibited by microinjection of L-NMMA, a nonselective NOS inhibitor. These results suggest that the pressor response that occurred after iNOS gene transfer was mediated by iNOS. If this is the case, then what caused the pressor response after iNOS production? We previously demonstrated that blood pressure decreased after transfection of AdeNOS into the RVLM.[19] eNOS and nNOS are constitutive NOS. Microinjection of L-NMMA in rats transfected with Adβgal elicited the pressor response, suggesting that NO produced by endogenous NOS in the RVLM, mainly nNOS, decreases blood pressure. In contrast, microinjection of aminoguanidine into the RVLM in Adβgal-transfected rats did not alter blood pressure, suggesting that endogenous iNOS in the RVLM does not affect blood pressure, at least in normotensive rats. In support of this finding, we demonstrated that expression levels of iNOS protein in the brain of WKY are very low compared with the aorta and heart and with stroke-prone spontaneously hypertensive rats.[13] HR did not change despite the fact that blood pressure was increased after iNOS gene transfection. This might be attributable to inhibition of the baroreflex control of HR. Blood pressure also returned to the control level after iNOS transfection into the RVLM, indicating that the cytotoxic effects of NO produced by iNOS in the present study are reversible. We transfected iNOS bilaterally into the RVLM. If RVLM neurons were irreversibly damaged, blood pressure would be expected to decrease to the level produced by spinal transection.[8]

The effects of NO in the RVLM on blood pressure regulation are controversial. NO in the RVLM is reported to reduce blood pressure by inhibiting sympathetic nerve activity;[6,12,47,48] but opposite results have also been reported.[49–51] In addition, it was reported that NO elicits a biphasic response that depends on the dose injected.[43] Most of these studies, however, were performed in anesthetized animals, and only acute effects of NO donors or nonselective NO blockers were examined. It is possible that NO donors such as sodium nitroprusside produce reactive oxygen species. To exclude the above-mentioned limitations, we demonstrated that transfection of adenovirus encoding constitutive eNOS in the RVLM reduces blood pressure via inhibition of the sympathetic nervous system and this effect is probably attributable to an increase in γ-amino-butyric acid (GABA) in the RVLM in conscious rats.[19] We used eNOS instead of nNOS, which is normally abundant in the CNS, because the purpose of that study was to increase NO production from constitutively expressed NOS. In support of these findings, a similar finding was obtained in the paraventricular nucleus of the hypothalamus[52] and RVLM.[16]

Recently, the contribution of nNOS or iNOS in the RVLM to blood pressure regulation was examined in propofol-anesthetized rats.[42] A selective inhibitor of nNOS, 7-nitroindazole, or selective antagonists of iNOS, aminoguanidine, N^6-(L-iminoethyl)-L-lysine, or SMT, were used in that study. The nNOS inhibitor reduced blood pressure and iNOS antagonists increased blood pressure, suggesting that endogenous NO produced by nNOS increases blood pressure and that produced by iNOS decreases blood pressure.[34] In a subsequent study, they explained that the different blood pressure responses evoked by nNOS and iNOS were attributable to differences in the amount of release of an excitatory neurotransmitter, L-glutamate, and an inhibitory neurotransmitter, GABA.[53] We do not yet have a clear explanation for the differences between their results and ours. Therefore, in the present study, we performed iNOS gene transfer into the RVLM in awake rats to clarify the role of iNOS in the RVLM. Our results raise another possibility that NO produced by iNOS enhances the production of reactive oxygen species, which influences the neuronal activity of the RVLM neurons.[30,53] Increased and sustained NO levels might lead to the formation of superoxide anions that react with NO to form peroxynitrite.[54] In support of this suggestion, nitrotyrosine staining in the RVLM was observed after transfection of AdiNOS as a peroxynitrite footprint. Indeed, lipopolysaccharide-induced NO generation results in an increase in oxidative stress in the rat liver and kidney and is inhibited by iNOS inhibitors.[55]

In summary, the present studies demonstrate that overexpression of iNOS in the RVLM elicits hypertension by activating the sympathetic nervous system, and these effects might be mediated by an increase in oxidative stress in the RVLM. An increase in iNOS expression levels occurs in some pathophysiological states, such as hypertension, heart failure, and endotoxin shock, and in aging.[22–28,41] Thus, it is conceivable that the increase in iNOS expression levels in the brain, particularly in the RVLM, occurs in those conditions, thereby modulating central sympathetic outflow resulting in blood pressure changes.

Acknowledgments

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture (C13670721, C15590757), and by grants for research on auto-

nomic nervous system and hypertension from Kimura Memorial Heart Foundation/Pfizer Japan, Inc. We thank Drs Donald D. Heistad and Beverly L. Davidson (The University of Iowa Gene Transfer Vector Core, supported by the National Institutes of Health Grants and the Carver Foundation) for vector preparation.

References

1. Krukoff TL. Central actions of nitric oxide in regulation of autonomic functions. *Brain Res Rev.* 1999;30:52–65.
2. Persson PB. Modulation of cardiovascular control of mechanisms and their interaction. *Physiol Rev.* 1996;76:193–244.
3. Zanzinger J. Role of nitric oxide in the neural control of cardiovascular function. *Cardiovasc Res.* 1999;43:639–649.
4. Patel KP, Li Y-F, Hirooka Y. Role of nitric oxide in central sympathetic outflow. *Exp BiolMed.* 2001;226:814–824.
5. Zhang K, Mayhan WG, Patel KP. Nitric oxide within the paraventricular nucleus mediates changes in renal sympathetic nerve activity. *Am J Physiol Regul Integr Comp Physiol.* 1997;273:R864–R872.
6. Tseng CJ, Liu HY, Lin HC, Ger LP, Tung CS, Yen MH. Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension.* 1996;27:36–42.
7. Lawrence AJ. Nitric oxide as a modulator of medullary pathways. *Clin Exp Pharmacol Physiol.* 1997;24:760–763.
8. Dampney RAL. Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev.* 1994;74:323–364.
9. Guyenet PG. Role of the ventral medulla oblongata in blood pressure regulation. In: Loewy AD, Spyer KM, eds. *Central Regulation of Autonomic Functions.* New York: Oxford University Press;1990: 145–167.
10. Pilowski PM, Goodchild AK. Baroreceptor reflex pathways and neurotransmitters: 10 years on. *J Hypertens.* 2002;20:1675–1688.
11. Kishi T, Hirooka Y, Ito K, Sakai K, Shimokawa H, Takeshita A. Cardiovascular effects of overexpression of endothelial nitric oxide synthase in the rostral ventrolateral medulla in stroke-prone spontaneously hypertensive rats. *Hypertension.* 2002;39:264–268.
12. Kagiyama S, Tsuchihashi T, Abe I, Fujishima M. Enhanced depressor response to nitric oxide in the rostral ventrolateral medulla of spontaneously hypertensive rats. *Hypertension.* 1998;31:1030–1034.
13. Kishi T, Hirooka Y, Mukai Y, Shimokawa H, Takeshita A. Atorvastatin causes depressor and sympatho-inhibitory effects with upregulation of nitric oxide synthases in stroke-prone spontaneously hypertensive rats. *J Hypertens.* 2003;21:379–386.
14. Kishi T, Hirooka Y, Kimura Y, Sakai K, Ito K, Shimokawa H, Takeshita A. Overexpression of eNOS in RVLM improves impaired baroreflex control of heart rate in SHRSP. *Hypertension.* 2003;41:255–260.
15. Hirooka Y, Shigematsu H, Kishi T, Kimura Y, Ueta Y, Takeshita A. Reduced nitric oxide synthase in the brainstem contributes to enhanced sympathetic drive in rats with heart failure. *J Cardiovasc Pharmacol.* 2003;42:S111–S115.
16. Wang Y, Patel KP, Cornish KG, Channon KM, Zucker IH. nNOS gene transfer to RVLM improves baroreflex function in rats with chronic heart failure. *Am J Physiol.* 2003;285:H1660–H1667.
17. Hirooka Y. Adenovirus-mediated gene transfer into the brain stem to examine cardiovascular function: role of nitric oxide and Rho-kinase. *Prog Biophys Mol Biol.* 2004;84:233–249.
18. Hirooka Y, Kishi T, Sakai K, Shimokawa H, Takeshita A. Effect of overproduction of nitric oxide in the brain stem on the cardiovascular response in conscious rats. *J Cardiovasc Pharmacol.* 2003;41: S119–S126.
19. Kishi T, Hirooka Y, Sakai K, Shigematsu H, Shimokawa H, Takeshita A. Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release. *Hypertension.* 2001;38:896–901.
20. Hirooka Y, Sakai K, Kishi T, Ito K, Shimokawa H, Takeshita A. Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. *Hypertens Res.* 2003;26:325–331.
21. Sakai K, Hirooka Y, Matsuo I, Eshima K, Shigematsu H, Shimokawa H, Takeshita A. Overexpression of eNOS in NTS causes hypotension and bradycardia in vivo. *Hypertension.* 2000;36:1023–1028.
22. Chou T-C, Yen M-H, Li C-Y, Ding Y-A. Alterations of nitric oxide synthase expression with aging and hypertension in rats. *Hypertension.* 1998;31:643–648.
23. Hong H-J, Loh S-H, Yen M-H. Suppression of the development of hypertension by the inhibitor of inducible nitric oxide synthase. *Br J Pharmacol.* 2000;131:631–637.
24. Feng Q, Lu X, Jones DL, Shen J, Arnold JMO. Increased inducible nitric oxide synthase expression contributes to myocardial dysfunction and higher mortality after myocardial infarction in mice. *Circulation.* 2001; 104:700–704.
25. Yang B, Larson DF, Watson RR. Modulation of iNOS activity in age-related cardiac dysfunction. *Life Sci.* 2004;75:655–667.
26. Horinaka S, Kobayashi N, Mori Y, Yagi H, Onoda M, Matsuoka H. Expression of inducible nitric oxide synthase, left ventricular function and remodeling in Dahl salt-sensitive hypertensive rats. *Int J Cardiol.* 2003; 91:25–35.
27. Massion PB, Feron O, Balligand J-L. Nitric oxide and cardiac function: ten years after, and coming. *Circ Res.* 2003;93:388–398.
28. Briones AM, Alonso MJ, Hernanz R, Miguel M, Salaices M. Alterations of nitric oxide pathway in cerebral arteries from spontaneously hypertensive rats. *J Cardiovasc Pharmacol.* 2002;39:378–388.
29. Kato N, Yanaka K, Hyodo K, Homma K, Nagase S, Nose T. Stable nitroxide Tempol ameliorates brain injury by inhibiting lipid peroxidation in a rat model of transient focal cerebral ischemia. *Brain Res.* 2003;979: 188–193.
30. Kishi T, Hirooka Y, Kimura Y, Ito K, Shimokawa H, Takeshita A. Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats. *Circulation.* 2004;109:2357–2362.
31. Chu Y, Heistad DD. Gene transfer to blood vessels using adenoviral vectors. *Methods Enzymol.* 2002;346:253–276.
32. Gunnett CA, Lund DD, Brooks II RM, Faraci FM, Heistad DD. NO-dependent vasorelaxation is impaired after gene transfer of inducible NO-synthase. *Arterioscler Thromb Vasc Biol.* 2001;21:1281–1287.
33. Ooboshi H, Chu Y, Rios CD, Faraci FM, Davidson BL, Heistad DD. Altered vascular function after adenovirus-mediated overexpression of endothelial nitric oxide synthase. *Am J Physiol.* 1997;273: H265–H270.
34. Yamada K, Nabeshima T. Simultaneous measurement of nitrite and nitrate levels as indices of nitric oxide release in the cerebellum of conscious rats. *J Neurochem.* 1997;68:1234–1243.
35. Matsuo I, Hirooka Y, Hironaga K, Eshima K, Shigematsu H, Shihara M, Sakai K, Takeshita A. Glutamate release via NO production evoked by NMDA in the NTS enhances hypotension and bradycardia in vivo. *Am J Physiol.* 2001;280:R1285–R1291.
36. Zimmerman MC, Lazartigues E, Sharma RV, Davisson RL. Hypertension caused by angiotensin II infusion involves increased superoxide production in the central nervous system. *Circ Res.* 2004;95:210–216.
37. Gao L, Wang W, Li Y-L, Schultz HD, Liu D, Cornish KG, Zucker IH. Superoxide mediates sympathoexcitation in heart failure: roles of angiotensin II and NAD(P)H oxidase. *Circ Res.* 2004;95:937–944.
38. Kagiyama S, Tsuchihashi T, Abe I, Matsumura K, Fujishima M. Central infusion of L-arginine or superoxide dismutase does not alter arterial pressure in SHR. *Hypertens Res.* 2000;23:339–343.
39. Ito K, Hirooka Y, Kishi T, Kimura Y, Kaibuchi K, Shimokawa H, Takeshita A. Rho/Rho-kinase pathway in the brainstem contributes to hypertension caused by chronic nitric oxide synthase inhibition. *Hypertension.* 2004;43:156–162.
40. Sato K, Miyakawa K, Takeya M, Hattori R, Yui Y, Sunamoto M, Ichimori Y, Ushio Y, Takahashi K. Immunohistochemical expression of inducible nitric oxide synthase (iNOS) in reversible endotoxic shock studied by a novel monoclonal antibody against rat iNOS. *J Leukoc Biol.* 1995;57:36–44.
41. Murphy S, Simmons ML, Agullo L, Garcia A, Feinstein DL, Galea E, Reis DJ, Minc-Golomb D, Schwartz JP. Synthesis of nitric oxide in CNS glial cells. *TINS.* 1993;16:323–328.
42. Chan SHH, Wang L-L, Wang S-H, Chan JYH. Differential cardiovascular responses to blockade of nNOS or iNOS in rostral ventrolateral medulla. *Br J Pharmacol.* 2001;133:606–614.
43. Morimoto S, Sasaki S, Miki S, Kawa T, Nakamura K, Itoh H, Nakata T, Takeda K, Nakagawa M, Fushiki S. Nitric oxide is an excitatory modulator in the rostral ventrolateral medulla in rats. *Am J Hypertens.* 2000; 13:1125–1134.
44. Xia Y, Roman LJ, Masters BSS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. *J Biol Chem.* 1998;273:22635–22639.

45. Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proc Natl Acad Sci U S A*. 1997;94:6954–6958.
46. Wu F, Wilson JX, Tyml K. Ascorbate inhibits iNOS expression and preserves vasoconstrictor responsiveness in skeletal muscle of septic mice. *Am J Physiol*. 2003;285:R50–R56.
47. Kugiyama S, Tsuchihashi T, Abe I, Fujishima M. Cardiovascular effects nitric oxide in the rostral ventrolateral medulla of rats. *Brain Res*. 1997; 757:155–158.
48. Zanzinger J, Czachurski J, Seller H. Inhibition of basal and reflex-mediated sympathetic activity in the RVLM by nitric oxide. *Am J Physiol*. 1995;268:R958–R962.
49. Hirooka Y, Polson JW, Dampney RA. Pressor and sympathoexcitatory effects of nitric oxide in the rostral ventrolateral medulla. *J Hypertens*. 1996;14:1317–1324.
50. Martins-Pinge MC, Baraldi-Passy I, Lopes OU. Excitatory effects of nitric oxide within the rostral ventrolateral medulla of freely moving rats. *Hypertension*. 1997;30:704–707.
51. Chan SHH, Wang L-L, Chan JYH. Differential engagements of glutamate and GABA receptors in cardiovascular actions of endogenous nNOS or iNOS at rostral ventrolateral medulla of rats. *Br J Pharmacol*. 2003;138:584–593.
52. Li YF, Roy SK, Channon KM, Zucker IH, Patel KP. Effect of in vivo gene transfer of nNOS in the PVN on renal nerve disharge in rats. *Am J Physiol Heart Circ Physiol*. 2002;282:H594–H601.
53. Zanzinger J. Mechanisms of action of nitric oxide in the brain stem: role of oxidative stress. *Auton Neurosci*. 2002;98:24–27.
54. Boczkowski J, Lisdero CL, Lanone S, Samb A, Carreeas MC, Boveris A, Aubier M, Poderoso JJ. Endogenous peroxynitrite mediates mitochondrial dysfunction in rat diaphragm during endotoxemia. *FASEB J*. 1999;13:1637–1647.
55. Zhang C, Walker LM, Hinson JA, Mayeux PR. Oxidant stress in rat liver after lipopolysaccharide administration: effect of inducible nitric-oxide synthase inhibition. *J Pharmacol Exper Ther*. 2000;293: 968–972.

Am J Physiol Heart Circ Physiol 288: H1179–H1185, 2005.
First published October 21, 2004; doi:10.1152/ajpheart.00045.2004.

Pronounced HR variability after exercise in inferior ischemia: evidence that the cardioinhibitory vagal reflex is invoked by exercise-induced inferior ischemia

Nobuhiro Tahara,[1] Hiroshi Takaki,[2] Atsushi Taguchi,[1] Kazuhiro Suyama,[1] Takashi Kurita,[1] Wataru Shimizu,[1] Shunichi Miyazaki,[1] Toru Kawada,[2] and Kenji Sunagawa[2]

[1]*Division of Cardiology, Department of Medicine, National Cardiovascular Center, and* [2]*Department of Cardiovascular Dynamics, National Cardiovascular Center Research Institute, Osaka, Japan*

Submitted 22 January 2004; accepted in final form 19 October 2004

Tahara, Nobuhiro, Hiroshi Takaki, Atsushi Taguchi, Kazuhiro Suyama, Takashi Kurita, Wataru Shimizu, Shunichi Miyazaki, Toru Kawada, and Kenji Sunagawa. Pronounced HR variability after exercise in inferior ischemia: evidence that the cardioinhibitory vagal reflex is invoked by exercise-induced inferior ischemia. *Am J Physiol Heart Circ Physiol* 288: H1179–H1185, 2005. First published October 21, 2004; doi:10.1152/ajpheart.00045.2004.—Potent cardioinhibitory vagal reflex resulting in bradycardia and hypotension has been observed under particular conditions of transmural inferior ischemia and its reperfusion, such as those observed with acute infarction. However, whether exercise-induced ischemia with ST depressions that is subendocardial and that might be recurrently experienced in daily activities can evoke this reflex remains unknown. In patients with exercise-induced ST depressions due to either inferior [right coronary artery stenosis (RCA), $n = 52$] or anterior ischemia [left anterior descending artery stenosis (LAD), $n = 51$], we evaluated postexercise vagal activity (from 0 to 6 min) by the time constant of heart rate (HR) decay and HR variability by 30-s averages of the absolute values of successive RR interval differences (ΔRR). Exercise parameters were similar between groups. The time constant was slightly but significantly shorter in RCA than LAD patients (79 ± 24 vs. 93 ± 29 s, $P < 0.01$). More significantly, ΔRR early after exercise (0.5–2.5 min) was approximately twofold greater in RCA than LAD patients (from +76 to +118%, $P < 0.001$), indicating pronounced vagal activity stimulated by inferior ischemia. Revascularization prolonged the time constant ($P < 0.05$) and attenuated recovery ΔRR in RCA patients ($P < 0.05$, $n = 10$) but did not change both parameters in LAD patients ($n = 12$). As well as acute inferior infarction, exercise-induced inferior subendocardial ischemia, which might recurrently occur in daily activities, activates the cardioinhibitory reflex. These new findings must be taken into account in interpreting vagal activity in patients with coronary artery disease.

heart rate variability; vagus nerve; coronary artery disease

EXPERIMENTAL STUDIES in animals have demonstrated that excitation of vagal sensory nerve endings from myocardial ischemia involving the inferoposterior wall of the left ventricle activates potent cardioinhibitory reflex resulting in bradycardia and hypotension (7, 24). In humans, similar observations have been made under particular conditions of severe transmural inferior ischemia and its reperfusion, such as those that occur with myocardial infarction, vasospastic angina, or angioplasty of the right coronary artery (12, 15, 21, 22, 28). For example, in the first hour of the onset of acute myocardial infarction, patients with inferior infarction often (~75%) show sinus bradycardia and/or hypotension, which generally responds to intravenous administration of atropine. This observation contrasts with that in patients with anterior infarction, about 50% of whom show sinus tachycardia and/or hypertension (15).

Despite these well-recognized clinical observations, little attention has been paid to the question as to whether this reflex could be evoked by exercise-induced ischemia that is usually subendocardial with the manifestation of ST depressions and that might be recurrently experienced during daily activities. If it does occur in this condition, this hitherto-unrecognized possibility must be taken into account in the clinical interpretation of vagal activity in patients with coronary artery disease (CAD). It is widely accepted that the higher the estimated measure of vagal activity, the better the patient status and the prognosis according to various reports examining the clinical significance of estimating vagal activity with the use of heart rate (HR) variability (HRV) analysis from 24-h Holter recording (1, 11, 13, 23a) or HR recovery after exercise testing (5, 18, 26). It is possible that vagal activity may be adversely augmented under a certain pathological condition, i.e., in the presence of inducible inferior ischemia, and that vagal estimation may be erroneously interpreted in patients with inducible inferior ischemia. In view of the diagnostic utility of exercise testing, the identification of the pronounced vagal activity induced by exercise may serve as an additive measure for detecting and localizing the presence of inferior ischemia.

On the basis of these considerations, the present study was designed to test the hypothesis that inferior ischemia, even that evoked by physiological stress such as exercise, may invoke the cardioinhibitory reflex, which would in turn influence postexercise HR decay and HRV through a reflex enhancement of vagal activity. The postexercise condition may more readily unmask this phenomenon than during exercise, because vagal activity is physiologically depressed during exercise but markedly reactivated after exercise (2, 20), although this reflex should be activated both during exercise-induced ischemia and after postexercise reperfusion. Thus we compared HR decay and HRV after exercise in patients with exercise-induced inferior ischemia and those with anterior ischemia and then evaluated the effects of revascularization on these parameters.

METHODS

Study population. From consecutive patients who underwent both coronary angiography and conventional treadmill exercise ECG testing within 3 wk for the evaluation of CAD, a total of 103 patients who were documented to have either inducible inferior ischemia due to

Address for reprint requests and other correspondence: H. Takaki, Dept. of Cardiovascular Dynamics, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan (E-mail: htakaki@res.ncvc.go.jp).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Table 1. *Patient characteristics*

	RCA	LAD
n	52	51
Age, yr	60±10	63±8
Men/women, *n*	45/7 (87/13)	45/6 (88/12)
Previous MI, *n*	25 (48)	26 (51)
LVEF, %	53±11	52±9
Hypertension, *n*	30 (58)	28 (55)
Diabetes mellitus, *n*	16 (31)	18 (35)
Medication		
β-Blockers, *n*	21 (40)	23 (45)
Ca antagonists, *n*	30 (58)	33 (65)
Nitrates, *n*	34 (65)	30 (59)

Values are means ± SD; *n*, no. of subjects. Values in parentheses are percentages. RCA, right coronary artery stenosis; LAD, left anterior descending coronary artery stenosis; MI, myocardial infarction; LVEF, left ventricular ejection fraction by contrast left ventriculography.

right coronary artery (RCA) stenosis (n = 52, RCA group) or anterior ischemia due to left anterior descending artery (LAD) stenosis (n = 51, LAD group) were enrolled in the study in a prospective fashion (Table 1). Significant coronary stenosis was defined as >50% luminal narrowing. All had significant exercise-induced ST segment depressions on treadmill ECG. Fifty-one (50%) patients had previous myocardial infarction. The majority (75%) had a single-vessel disease of either RCA stenosis (69%) or LAD stenosis (84%). In 24 patients with multiple vessel disease, exercise single-photon emission computed tomographic thallium-201 scintigraphy was performed to confirm that exercise-inducible ischemia was exclusively localized to either the inferior or anterior wall of the left ventricle. Exclusion criteria included the presence of atrial fibrillation, frequent premature beats (>5 beats/min) during the exercise test, and exercise-induced ST segment elevations (≥1.0 mm).

Clinical characteristics, including age, sex, prevalence of prior infarction, left ventricular ejection fraction (by contrast left ventriculography), and drug regimens, were quite similar between the two groups (Table 1). β-Blockers, calcium antagonists, and nitrates were taken in 44 (43%), 63 (61%), and 64 (62%) patients, respectively. No patient was taking digitalis at the time of the study. Drug regimens were neither altered nor stopped for the exercise test. The study protocol was approved by the ethics committee of our institution. All patients gave informed consent to participate in the study.

Exercise test. Conventional symptom-limited or submaximal (up to 90% age-predicted maximum HR) graded treadmill exercise testing was performed using a commercially available treadmill system (Formula; Esaote, Italy) equipped with an analog-to-digital converter and hard disk according to our protocol (23), being similar to the modified Bruce protocol. ECGs in lead V_2, aV_F, and V_5 were continuously monitored from rest through the recovery period. Arterial blood pressure was measured at rest, at the end of each stage, peak exercise, and at 1, 2, 4, and 6 min after exercise by a sphygmomanometer. Throughout the test, eight leads of the ECG, including lead I, II, and V_{1-6}, were continuously digitized at 500 Hz (12 bit) and stored on a computer hard disk for subsequent analysis. The standard 12-lead ECGs were hardcopied at rest, at the end of each stage, at peak exercise, immediately after exercise, and at every minute of the recovery period. A significant ST segment depression was defined by the following criteria: *1*) a horizontal or downsloping ST segment displacement at J point ≥ 0.1 mV and 2) an upsloping ST segment displacement at 80 ms after J point ≥ 0.15 mV in at least three consecutive beats at peak exercise.

Assessment of revascularization effects. In 22 patients who subsequently received successful revascularization, we repeated the exercise test within 1 mo after the procedure. Eighteen patients received successful percutaneous transluminal coronary angioplasty either on the RCA (n = 8) or LAD (n = 10). Coronary artery bypass graft surgery was undertaken in the other four patients (RCA n = 2, LAD n = 2).

On the basis of consideration that a higher attainment of peak HR resulting from increased exercise capacity after the intervention (i.e., different exercise time) and its possible influences on vagal activity would make it difficult to estimate the true effect of revascularization on postexercise HR analysis, the exercise test after revascularization was terminated at the same exercise duration as that before the revascularization. Drug regimens were also kept constant.

Data analysis. Off-line analysis was performed on a personal computer using our custom-made software. We first determined the beat-by-beat RR intervals throughout the test from rest to recovery period by detecting the peak of R wave deflections. In patients with premature beats, we corrected RR intervals by linear interpolation with the previous and following beats.

Using the time series of RR intervals during recovery periods of 6 min, we computed the time constant of HR decay. We fitted the HR data to a monoexponential curve ($HR = A + Be^{-t/\tau}$, where A and B are constants, t is the elapsed time after peak exercise, and τ is the time constant of recovery) by nonlinear least-squares regression analysis. We then estimated the time course of HRV by time-domain and frequency-domain analysis as follows. Instead of parameters such as the standard deviation of the average interval between normal beats, which is substantially influenced by dynamic changes in overall trend, serial changes in HRV were assessed by 30-s averages of the absolute values of successive beat-to-beat differences in the RR interval (ΔRR). Serial changes in HRV were also evaluated by 30-s averages of the beat-to-beat percent changes [absolute successive differences relative to instantaneous RR interval (%ΔRR)] to eliminate the effect of the individual variation in HR.

Power spectral analysis of RR interval changes was also performed by fast Fourier transformation. We serially computed the spectrum of RR interval data of 1-min duration with 50% overlapping of each segment (0–1 min, 0.5–1.5 min, ... , 4.5–5.5 min, 5–6 min; 11 segments in all). The linear trend in the data was subtracted from the data set in each segment. A Blackman-Harris window was applied to reduce spectral leakage.

Statistical analysis. Data are presented as means ± SD. Serial changes in variables were evaluated by repeated-measures ANOVA followed by Scheffe's test for intergroup and intragroup comparisons. Student's unpaired and paired t-tests, linear regression analysis, multiple linear regression analysis, and χ^2-analysis were used when applicable. A P value of <0.05 was considered statistically significant.

RESULTS

All exercise tests were completed without any unfavorable events or serious complications. Exercise test parameters including exercise duration, resting and peak HR, resting and peak systolic blood pressure, the maximum magnitude of ST segment depression, and the occurrence of exercise-induced angina were consistently similar between the RCA and LAD groups (Table 2). Only peak HR tended to be lower in the RCA

Table 2. *Exercise test results*

	RCA	LAD
Exercise time, s	507±159	497±133
HR, beats/min		
Resting	65±12	65±11
Peak	126±22	135±21
SBP, mmHg		
Resting	129±13	130±18
Peak	158±24	160±20
ST depression, mm	−2.0±0.8	−1.7±1.5
Angina, *n*	22/30	25/26

Values are means ± SD. HR, heart rate; SBP, systolic blood pressure; *n*, no. of subjects.

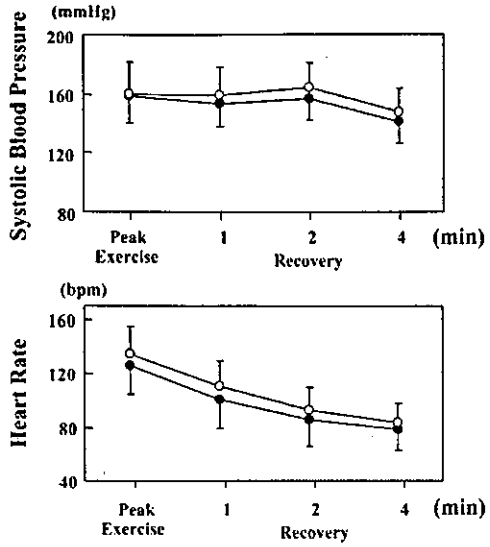

Fig. 1. Time course of systolic blood pressure (*top*) and heart rate [HR, in beats/min (bpm); *bottom*] in right coronary artery (RCA) stenosis (closed circles) and left anterior descending artery (LAD) stenosis (open circles) patients. No differences were observed in either parameter. Values are expressed as means ± SD.

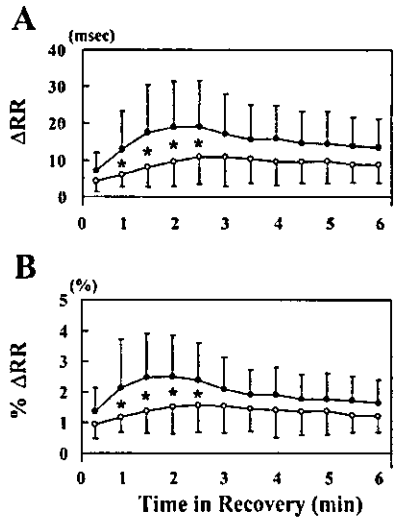

Fig. 3. Comparisons of the HRV time course expressed by ΔRR (*A*) and absolute successive differences relative to instantaneous RR interval (%ΔRR; *B*). Closed circles depict the RCA stenosis group, and open circles depict the LAD stenosis group. Values are expressed as means ± SD. *$P < 0.01$, RCA stenosis patients vs. LAD stenosis patients.

group (126 ± 22 beats/min) than in the LAD group (135 ± 21 beats/min), but this difference did not reach statistical significance.

Postexercise systolic blood pressure and HR. There was no significant difference between the groups with respect to postexercise systolic blood pressure and HR at 1, 2, and 4 min of recovery (Fig. 1).

Postexercise HR decay and HRV. Shown in Fig. 2 are representative examples of the time series of beat-by-beat HR and absolute value of the successive RR interval changes throughout the exercise test. A patient with RCA stenosis (Fig. 2, *left*) showed a transient increase in RR variability soon after the termination of exercise, whereas such findings were not observed in a patient with LAD stenosis (Fig. 2, *right*). The time constant was shorter in the former (92 s) than in the latter (112 s).

When pooled data were compared between the two groups, the time constant of postexercise HR decay was slightly but significantly shorter in RCA than LAD patients (79 ± 24 vs. 93 ± 29 s, $P < 0.01$). More significantly, postexercise HRV expressed by ΔRR (average over every 30 s) early after exercise was markedly greater in RCA than LAD patients for a period of 1.0–2.5 min ($P < 0.001$ for all; Fig. 3*A*). %ΔRR was similarly greater in RCA patients than in LAD patients in the same period of 1.0–2.5 min after exercise ($P < 0.001$ for 1.0–2.0 min and $P < 0.01$ for 2.5 min; Fig. 3*B*).

Figure 4, *top*, shows serial changes in the power spectrum of RR intervals in the recovery period analyzed in the same two patients shown in Fig. 2. As can be seen, unlike the patient with LAD stenosis (Fig. 4, *right*), the patient with RCA stenosis (Fig. 4, *left*) showed substantial amounts of power spectra in the frequency range between ~0.30 and 0.60 Hz. When these data were pooled (Fig. 4, *bottom*), total power within the frequency range between 0.25 and 0.60 Hz (corresponding to the respiratory rates after exercise) was significantly higher in the RCA group than in the LAD group in the four time window segments early after exercise, i.e., 0.5–1.5 min, 1.0–2.0 min,

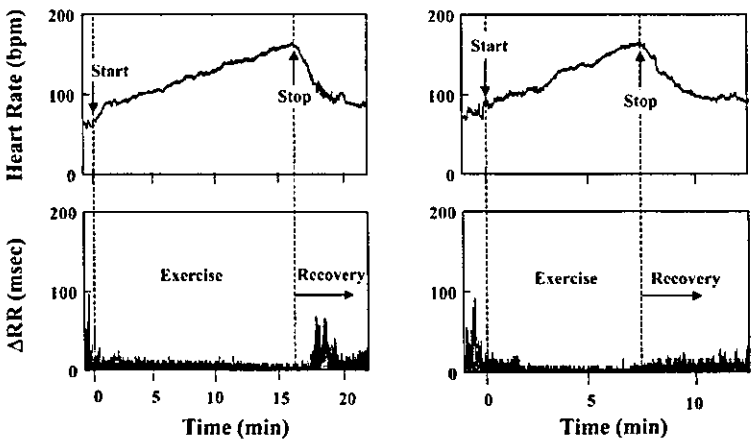

Fig. 2. Representative trends of beat-by-beat HR (*top*) and absolute values of successive beat-to-beat differences in the RR interval (ΔRR; *bottom*) from rest to recovery in a patient with RCA stenosis (*left*) and in a patient with LAD stenosis (*right*). A transient increase in HR variability (HRV) can be seen soon after exercise in the patient with RCA stenosis.

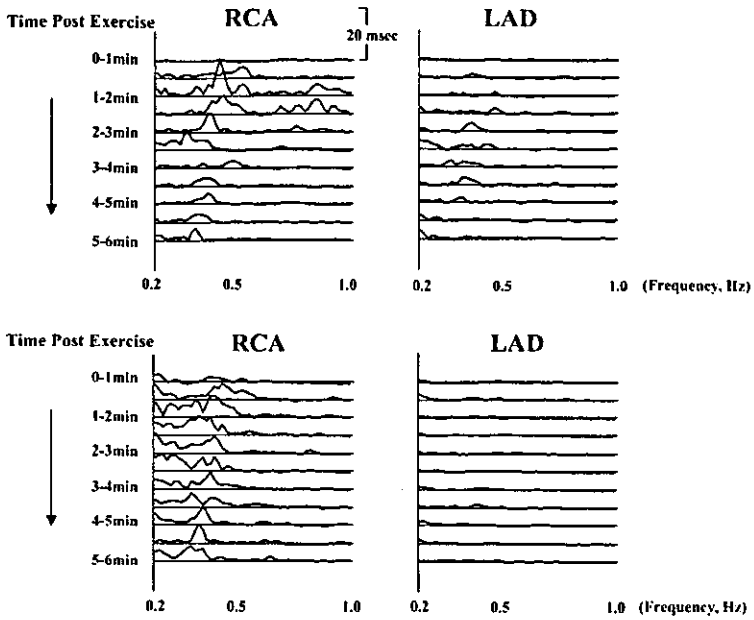

Fig. 4. *Top*: representative data of power spectrum analysis of HRV obtained in the same 2 patients shown in Fig. 2 (*left*, RCA stenosis patient; *right*, LAD stenosis patient). Horizontal lines depict the frequency (in Hz). The data series of power spectrum for 1 min are shown serially from the top (0–1 min) downward with time of recovery (overlapping 30 s). The spectral component of RR intervals for each segment is shown as the square root of the power (i.e., amplitude). *Bottom*: pooled data of power spectrum analysis in the RCA stenosis group (*left*; n = 52) and LAD stenosis group (*right*; n = 51).

1.5–2.5 min, and 2.0–3.0 min ($P < 0.005$ for the first 3 segments and $P < 0.02$ for 2.0- to 3.0-min segment).

Figure 5 shows scatterplots of ΔRR at 1.5 min (60–90 s) versus the time constant for all patients. There was a relatively close relationship ($r = -0.50$, $P < 0.001$) between these parameters. When ΔRR at 1.5 min (60–90 s) for each patient was plotted separately in RCA and LAD patients (Fig. 6), the prevalence of pronounced HRV (exceeding 12 ms) was much more frequently found in RCA patients (58%) than in LAD patients (16%, $P < 0.001$). Subgroup analysis of RCA patients showed that those with enhanced HRV were significantly younger (58 ± 11 vs. 64 ± 8 yr, $P < 0.01$) and had a significantly lower HR at rest (61 ± 10 vs. 70 ± 12 beats/min, $P < 0.01$) and peak HR (117 ± 19 vs. 139 ± 18 beats/min, $P < 0.01$) compared with those without this phenomenon. There was no significant difference with respect to sex, left ventricular ejection fraction, the use of cardiovascular drugs (β-blockers, calcium antagonists, or nitrates), prevalence of previous myocardial infarction, history of diabetes mellitus, exercise time, magnitude of ST depression, occurrence of exercise-induced angina, or resting ΔRR level. When multiple linear regression analysis that included clinical, angiographic, and exercise variables was used in the overall population, age ($P = 0.02$), resting HR ($P = 0.04$), and peak exercise HR ($P = 0.02$) together with the location of ischemia ($P < 0.0001$) were independently associated with ΔRR (60–90 s).

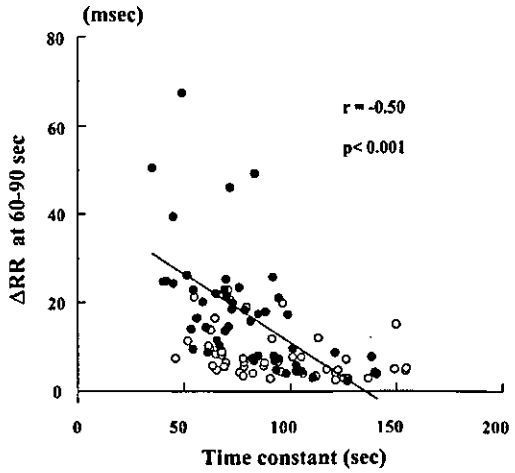

Fig. 5. Graph showing the relationship between ΔRR (60–90 s) and the time constant. Closed circles, RCA stenosis patient; open circles, LAD stenosis patient.

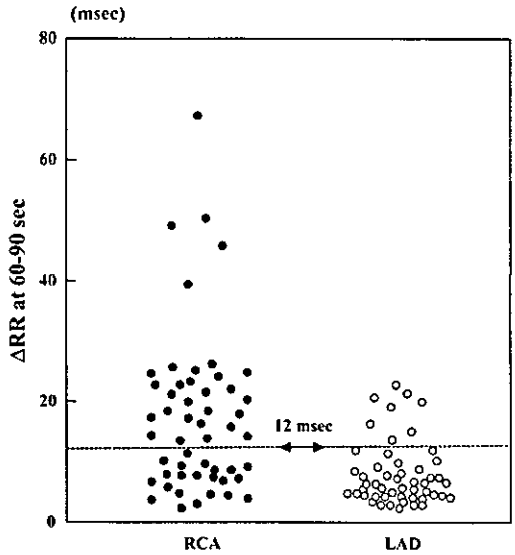

Fig. 6. Scatterplots of ΔRR at 60–90 s plotted separately for each patient in the RCA (*left*) and LAD (*right*) stenosis groups. Pronounced HRV (defined as ΔRR >12 ms; dotted line) was observed in 58% of RCA stenosis patients, whereas it was found in 16% of LAD stenosis patients ($P < 0.001$).

Effects of revascularization on postexercise HR decay and HRV. In either the RCA or LAD patient group, HR at both rest and the end of exercise were not significantly different before and after revascularization (note that the second test was terminated at the same duration as was achieved at the first test). In RCA patients, HR at 1 and 2 min of recovery were significantly higher (both $P < 0.05$) after than before revascularization, whereas no such significant differences were observed in LAD patients.

After revascularization, in RCA patients, ΔRR early after exercise was significantly attenuated (from 22 ± 14 to 9 ± 5 ms for 60–90 s, from 25 ± 12 to 11 ± 5 ms for 90–120 s, and from 27 ± 13 to 13 ± 5 ms for 120–150 s, all $P < 0.05$; Fig. 7). The time constant was concordantly prolonged (from 73 ± 21 to 96 ± 30 s, $P < 0.05$). Both parameters in RCA patients changed toward the same level as those in LAD patients, in whom both parameters remained unchanged after the revascularization procedure. There was no significant difference in systolic blood pressure at any time point in both RCA and LAD patient groups.

DISCUSSION

Although potent cardioinhibitory vagal reflex stimulated by inferior ischemia (the so-called Bezold-Jarisch reflex) has been recognized under particular conditions of transmural ischemia in animal and human studies (7, 12, 15, 21, 22, 24, 28), whether exercise-induced transient subendocardial ischemia could evoke this reflex has received little attention and has not been previously examined. The present study indicated that exercise-induced inferior ischemia with ST depressions reflecting subendocardial ischemia, which might be recurrently experienced in daily activities, activates the cardioinhibitory reflex as evidenced by a faster postexercise HR decay and more pronounced HRV in RCA patients compared with LAD patients. In addition, removal of inferior ischemia by revascularization prolonged the time constant and reduced pronounced HRV in the early recovery in RCA patients, whereas revascularization did not significantly change these parameters in LAD patients. These findings, indicative of the direct role of "localized inferior ischemia" on the appearance of this phenomenon, support the validity of our hypothesis that transmural severe ischemia is not a prerequisite for the manifestation of this reflex.

Estimation of vagal activity. Numerous previous studies have indicated the clinical importance of estimating the vagal activity regulating the cardiovascular system in patients with heart disease by noninvasive methods such as HRV analysis (1, 11, 13, 23a) and baroreflex sensitivity measurements with phenylephrine injection (3, 13, 14). After a report of Imai et al. (10) demonstrating that the rate of HR decay after exercise is a function of the reactivation of vagal activity, recent studies have shown the postexercise HR fall is a useful marker for predicting mortality in subjects with suspected CAD (5, 18, 26). In the present study, to assess the dynamic changes in vagal activity, serial HRV analysis during recovery was conducted by calculating ΔRR and %ΔRR every 30 s along with the evaluation of the HR decay (time constant). As a result, both HRV parameters in RCA patients markedly increased from 0.5 to 1 min of recovery, remained rather constant up to 2.5 min, and thereafter decreased nearly to the level of those in LAD patients. The group difference was more striking compared with that of the time constant. The characteristic overshooting of the HRV parameters suggests a transiently enhanced vagal activity as a "reperfusion reflex" after the resolution of exercise-induced ischemia. In agreement with its observed time course, frequency analysis also revealed a transient augmentation of power spectrum of RR intervals in high-frequency ranges early after exercise, supporting the validity of reperfusion-stimulated vagal overactivation.

It should be of importance that, in the present study, the exercise duration after revascularization was matched to that before the procedure. This is because a higher level of exercise intensity, as a consequence of the removal of critical stenosis, would alter the postexercise autonomic activity, possibly making it difficult to estimate the direct effects of revascularization.

Cardioinhibitory reflex activated by exercise-induced ischemia. To the best of our knowledge, no previous study has been systematically conducted to evaluate the possible role of exercise-induced ischemia with ST depressions on this reflex phenomenon. Miller et al. (16) reported on seven patients who developed sinus deceleration during exercise testing, all of whom had angiographically documented RCA lesions. The authors speculated the role of Bezold-Jarisch reflex in this mechanism and stated that the prevalence of deceleration during exercise appears to be very low, which is in agreement with our experiences in the exercise laboratory. Sinus deceleration during exercise may be an extreme example caused by an ischemia-mediated reflex (4, 6).

Thus this reflex phenomenon is presumably operative during exercise-induced ischemia as well as during postexercise reperfusion; however, we focused on postexercise HR dynamics for the following reasons. Because vagal activity is physiologi-

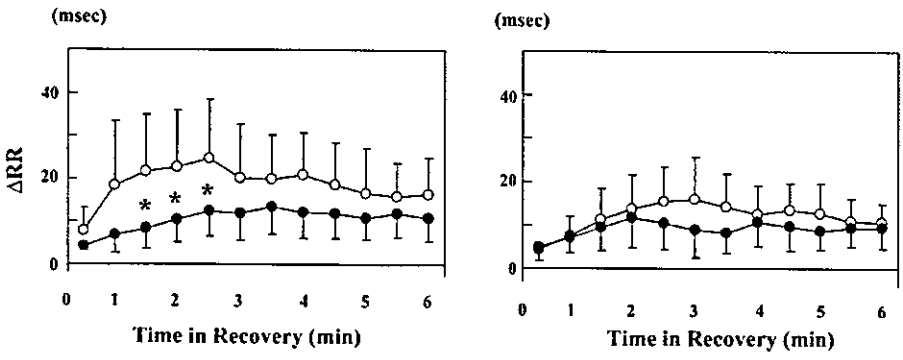

Fig. 7. Changes in the HRV (ΔRR) time course after revascularization in the RCA (*left*; $n = 52$) and LAD (*right*; $n = 51$) stenosis groups. Open circles depict the values before the procedure, and closed circles depict the values after the procedure. Values are expressed as means ± SD. *$P < 0.05$, before vs. after revascularization.

cally attenuated in proportion to the increase in exercise intensity, this reflex might be masked during exercise. In contrast, potent reactivation of vagal nerve activity after exercise may accelerate the appearance of this reflex under a higher vagal condition after exercise. In practice, several cases among RCA patients showed a pronounced HRV and marked impairment of HR increase even during exercise indicative of the operation of this reflex during exercise; however, we also found a markedly rapid HR decay and more profound increase in HRV during recovery almost without exception.

The physiological implication of this reflex, namely, what role this reflex may play, is unknown. The possibility that the reflex cardioprotectively works thorough the reduction in myocardial oxygen demand or that the resultant high vagal tone prevents the development of serious ventricular arrhythmias is of interest (9, 19); however, there are few available data to support this so far.

Pronounced HRV after exercise (defined as $\Delta RR > 12$ ms) was observed in 58% of RCA patients but in only 16% of LAD patients. These prevalences are very similar to those during the observation of the "bradycardia-hypotension" pattern observed in patients early after acute inferior and anterior myocardial infarction, respectively (27). The difference probably indicates that the vagal nerves involving this reflex are preferentially distributed in the inferior area but some mounts of fibers are distributed in the anterior area of the left ventricle.

In RCA patients, none of the clinical, angiographic, and exercise parameters differed between patients with and without this phenomenon except in regard to age, resting HR, and peak HR. All of these three parameters were independently associated with HRV early after exercise in our multiple regression analysis. Vagal activity is known to be strongly associated with age and resting HR. Thus it is suggested that the presence or absence of this phenomenon would depend on the basal level of vagal activity rather than other parameters such as severity of ischemia or the presence of previous myocardial infarction.

Clinical implications. The findings demonstrated in the present study should provide a new insight into the interpretation of estimated vagal activity in patients with CAD. It is widely accepted that autonomic imbalance, i.e., vagal withdrawal and coexisting sympathetic activation, is associated with poor prognosis and pathophysiology in various types of heart disease (25). In other words, we believe that the higher the vagal activity, the better the patient prognosis and status. However, present data suggest that this is not necessarily the case in some patients under certain conditions. For example, studies using Holter recordings showed that a considerable number of patients with documented CAD frequently experience episodes of transient myocardial ischemia in their daily life (17). In such patients, transient enhancement of HRV provoked by inferior ischemia may often occur, leading to an erroneous interpretation of HRV. In addition, there are several studies (5, 18, 26) that related a poor prognosis to attenuated HR recovery after exercise testing on the assumption that a fall in HR recovery immediately after exercise is a function of vagal reactivation. These findings might be true in the overall population; however, it should be noted that a rapid HR decrease after exercise may occur under a pathological condition through an ischemia-mediated cardioinhibitory reflex. HRV measures might be affected not by the patient status but by the presence of inferior ischemia.

We did not analyze postexercise parameters in subjects without CAD. This is because they should be capable of exercising far longer than our patients with exercise-induced ischemia, which may considerably influence the postexercise vagal activity. At present, we can consider that the vagal overactivation after exercise may be useful in predicting the presence of inferior ischemia when significant exercise-induced ST depressions are observed. It may also be useful in patients after angioplasty of RCA disease to predict restenosis or to confirm the therapeutic effects.

Study limitations. It is generally considered that anterior ischemia is more deleterious than inferior ischemia in terms of hemodynamics, leading to a more severe autonomic impairment, i.e., a more depressed vagal activity in LAD patients. Thus the observed differences in vagal activity between the groups might be caused not only by the cardioinhibitory reflex evoked by inferior ischemia but also by the differences in hemodynamic impairment. For this reason, we evaluated the changes in vagal parameters (time constant and ΔRR) after revascularization and found that these parameters were significantly altered in RCA patients but not in LAD patients. This strongly supports the notion that the differences in estimated vagal activity between the groups are determined primarily by the presence or absence of inferior located ischemia. Nevertheless, we cannot completely exclude the possible contribution of the different sympathetic tone between the groups, because anterior located ischemia, although rare, can stimulate this reflex.

In conclusion, as well as transmural myocardial ischemia with ST elevations such as that occurring with acute inferior myocardial infarction, exercise-induced transient inferior subendocardial ischemia with ST depressions, which might be recurrently experienced in daily activities, activates cardioinhibitory reflex by stimulating vagal nerve endings in humans. This hitherto-unknown findings must be taken into account in the estimation of vagal function conducted in various clinical settings, especially when evaluating patients with CAD.

GRANTS

This study was supported by Ministry of Health and Welfare of Japan Research Grant for Cardiovascular Diseases 11C-7, by Japan Society for the Promotion of Science Grant-In-Aid for Scientific Research C-11670730, and by the Program for Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety and Research.

REFERENCES

1. **Algra A, Tijssen JGP, Roelandt JRTC, Pool J, and Lubsen J.** Heart rate variability from 24-hour electrocardiography and the 2-year risk for sudden death. *Circulation* 88: 180–185, 1993.
2. **Arai Y, Saul JP, Albrecht P, Hartley LH, Lilly LS, Cohen RJ, and Colucci WS.** Modulation of cardiac autonomic activity during and immediately after exercise. *Am J Physiol Heart Circ Physiol* 256: H132–H141, 1989.
3. **Bigger JT Jr, La Rovere MT, Steinman RC, Fleiss JL, Rottman JN, Rolnitzky LM, and Schwartz PJ.** Comparison of baroreflex sensitivity and heart period variability after myocardial infarction. *J Am Coll Cardiol* 14: 1511–1518, 1989.
4. **Chokshi SK, Sarmiento J, Nazari J, Mattioni T, Zheutlin T, and Kehoe R.** Exercise-provoked distal atrioventricular block. *Am J Cardiol* 66: 114–116, 1990.
5. **Cole CR, Blackstone EH, Pashkow FJ, Snader CE, and Lauer MS.** Heart rate recovery immediately after exercise as a predictor of mortality. *N Engl J Med* 341: 1351–1357, 1999.

6. **Coplan NL, Morales MC, Romanello P, Wilentz JR, and Moses JW.** Exercise-related atrioventricular block: influence of myocardial ischemia. *Chest* 100: 1728–1730, 1991.
7. **Felder RB and Thames MD.** Interaction between cardiac receptors and sinoaortic baroreceptors in the control of efferent cardiac sympathetic nerve activity during myocardial ischemia in dogs. *Circ Res* 45: 728–736, 1979.
9. **Huikuri HV, Valkama JO, Airaksinen KEJ, Seppanen T, Kessler KM, Takkunen JT, and Myerburg RJ.** Frequency domain measures of heart rate variability before the onset of nonsustained and sustained ventricular tachycardia in patients with coronary artery disease. *Circulation* 87: 1220–1228, 1993.
10. **Imai K, Sato H, Hori M, Kusuoka H, Ozaki H, Yokoyama H, Takeda H, Inoue M, and Kamada T.** Vagally mediated heart rate recovery after exercise is accelerated in athletes but blunted in patients with chronic heart failure. *J Am Coll Cardiol* 24: 1529–1535, 1994.
11. **Kleiger RE, Miller JP, Bigger JT Jr, Moss AJ, and the Multicenter Postinfarction Research Group.** Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. *Am J Cardiol* 59: 256–262, 1987.
12. **Koren G, Weiss AT, Ben-David Y, Hasin Y, Luria MH, and Gotsman MS.** Bradycardia and hypotension following reperfusion with streptokinase (Bezold-Jarisch reflex): a sign of coronary thrombolysis and myocardial salvage. *Am Heart J* 112: 468–471, 1986.
13. **La Rovere MT, Bigger JT Jr, Marcus FI, Mortana A, and Schwartz PJ for the Autonomic Tone and Reflexes After Myocardial Infarcion Investigators.** Baroreflex sensibility and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. *Lancet* 351: 478–484, 1998.
14. **La Rovere MT, Specchia G, Mortara A, and Schwartz PJ.** Baroreflex sensitivity, clinical correlates, and cardiovascular mortality among patients with a first myocardial infarction: a prospective study. *Circulation* 78:816–824, 1988.
15. **Mark AL.** The Bezold-Jarisch reflex revisited: clinical implications of inhibitory reflexes originating in the heart. *J Am Coll Cardiol* 1: 90–102, 1983.
16. **Miller TD, Gibbons RJ, Squires RW, Allison TG, and Gau GT.** Sinus node deceleration during exercise as a marker of significant narrowing of the right coronary artery. *Am J Cardiol* 71: 371–373, 1993.
17. **Mulcahy D, Husain S, Zalos G, Rehman A, Andrews NP, Schenke WH, Geller NL, and Quyyumi AA.** Can ambulatory monitoring identify high-risk patients with stable coronary artery disease? *JAMA* 277: 318–324, 1997.
18. **Nishime EO, Cole CR, Blackstone EH, Pashkow FJ, and Lauer MS.** Heart rate recovery and treadmill exercise score as predictors of mortality in patients referred for exercise ECG. *JAMA* 284: 1392–1398, 2000.
19. **Pedretti R, Etro MD, Laporta A, Braga SS, and Caru B.** Prediction of late arrhythmic events after acute myocardial infarction from combined use of noninvasive prognostic variables and inducibility of sustained monomorphic ventricular tachycardia. *Am J Cardiol* 71: 1131–1141, 1993.
20. **Perini R, Orizio C, Comande A, Castellano M, Beschi M, and Veicsteinas A.** Plasma norepinephrine and heart rate dynamics during recovery from submaximal exercise in man. *Eur J Appl Physiol* 58:879–883, 1989.
21. **Prez-Gomez F, Martin de Dios R, Rey J, and Aguado AG.** Prinzmetal's angina: reflex cardiovascular response during episode of pain. *Br Heart J* 42: 81–87, 1979.
22. **Robertson RM and Robertson D.** The Bezold-Jarisch reflex: possible role in limiting myocardial ischemia. *Clin Cardiol* 4: 75–79, 1981.
23. **Sato I, Tomobuchi Y, Funahashi T, Ohe T, Kamakura S, Matsuhisa M, Haze K, and Shimomura K.** Poor responsiveness of heart rate to treadmill exercise in vasospastic angina. *Clin Cardiol* 8: 206–212, 1985.

23a. **Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology.** Heart rate variability: standards of measurement, physiology interpretation, and clinical use. *Circulation* 93: 1043–1065, 1996.

24. **Thames MD, Klopfenstein HS, Abboud FM, Mark AL, and Walker JL.** Preferential distribution of inhibitory cardiac receptors with vagal afferents to the inferoposterior wall of the left ventricle activated during coronary occlusion in the dog. *Circ Res* 43: 512–519, 1978.
25. **Tsuji H, Larson MG, Venditti FJ, Manders ES, Evans JC, Feldman CL, and Levy D.** Impact of reduced heart rate variability on risk for cardiac events: the Framingham Heart Study. *Circulation* 94: 2850–2855, 1996.
26. **Watanabe J, Thamilarasan M, Blackstone EH, Thomas JD, and Lauer MS.** Heart rate recovery immediately after treadmill exercise and left ventricular systolic dysfunction as predictors of mortality: the case of stress echocardiography. *Circulation* 104: 1911–1916, 2001.
27. **Webb SW, Adgey AAJ, and Pantridge JF.** Autonomic disturbance at onset of acute myocardial infarction. *Br Med J* 3: 89–92, 1972.
28. **Wei JY, Markis JE, Malagold M, and Braunwald E.** Cardiovascular reflexes stimulated by reperfusion of ischemic myocardium in acute myocardial infarction. *Circulation* 67: 796–801, 1983.

Essential Role of Vascular Endothelial Growth Factor and Flt-1 Signals in Neointimal Formation After Periadventitial Injury

Qingwei Zhao, Kensuke Egashira, Ken-ichi Hiasa, Minako Ishibashi, Shujiro Inoue, Kisho Ohtani, Chunyan Tan, Masabumi Shibuya, Akira Takeshita, Kenji Sunagawa

Objective—Vascular endothelial growth factor (VEGF) is upregulated after arterial injury. Its role in the pathogenesis of neointimal formation after periadventitial injury, however, has not been addressed.

Methods and Results—Expression of VEGF and its receptors but not that of placental growth factor markedly increased with the development of neointimal formation in hypercholesterolemic mice after cuff-induced periarterial injury. Transfection with the murine soluble Flt-1 (sFlt-1) gene to block VEGF in vivo in mice inhibited early inflammation and later neointimal formation. The sFlt-1 gene transfer did not affect plasma lipid levels but attenuated increased expression of VEGF, Flt-1, Flk-1, monocyte chemoattractant protein-1, and other inflammation-promoting factors. Mice with Flt-1 kinase deficiency also displayed reduced neointimal formation.

Conclusions—Inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. VEGF might promote neointimal formation by acting as a proinflammatory cytokine. (*Arterioscler Thromb Vasc Biol.* 2004;24:2284-2289.)

Key Words: remodeling ■ growth substances ■ inflammation ■ arteriosclerosis ■ gene therapy

Neointimal formation is a major cause of restenosis after coronary intervention.[1,2] Vascular endothelial growth factor (VEGF) and its receptors (VRGFR-1: Flt-1, VEGFR-2: Flk-1) are upregulated in vascular inflammatory and proliferative disorders such as atherosclerosis and restenosis.[3–6] VEGF is thought to protect the artery from such disorders by inducing endothelial regeneration and improving endothelial function.[7] VEGF gene transfer or administration of its protein induces endothelial regeneration and attenuates neointimal formation after endothelial injury.[7–9] VEGF is reported to inhibit leukocyte infiltration through hemeoxygenase-1.[10] There is still considerable debate, however, over the role of VEGF in the development of neointimal formation after injury.[11,12] Emerging evidence suggests that VEGF causes or promotes the development of atherosclerosis or neointimal formation after injury. VEGF induces migration and activation of monocytes,[13] adhesion molecules,[14] or monocyte chemoattractant protein-1 (MCP-1)[15] through its receptor Flt-1. Moreover, administration of VEGF protein to hypercholesterolemic animals enhances atherogenesis by inducing monocyte infiltration and activation.[16] In addition, VEGF might promote migration of vascular smooth muscle cells though Flt-1.[17,18] Angiogenesis inhibitors are shown to reduce intimal neovascularization and plaque growth in hyperlipidemic mice.[19]

One major reason for the inconsistent reports regarding the role of VEGF might be because there are no selective VEGF inhibitors tested. The only known endogenous VEGF inhibitor is a soluble form of the VEGF receptor-1, Flt-1 (sFlt-1).[20] This isoform is mainly expressed by vascular endothelial cells and can inhibit VEGF activity by directly sequestering VEGF and by functioning as a dominant-negative inhibitor.[20] We and others previously demonstrated that intramuscular transfection of the sFlt-1 gene blocks VEGF signaling and thus quenches VEGF activity in vivo.[21,22] Therefore, sFlt-1 gene transfer can be used as an inhibitor against VEGF and its receptors (Flt-1, Flk-1). In addition, Flt-1 tyrosine kinase–deficient mice can be used to determine the role of Flt-1 signals.[23]

In this study, we investigated the role of VEGF and Flt-1 signals in the pathogenesis of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. Several animal models for evaluation of neointimal formation after injury have been reported, including balloon injury, wire injury, chemical injury, and cuff injury, among others. The ideal animal model for human neointimal forma-

Original received October 21, 2003; final version accepted September 22, 2004.

From the Department of Cardiovascular Medicine (Q.Z., K.E., K.H., M.I., S.I., K.O., C.T., A.T., K.S.), Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and the Department of Genetics (M.S.), Institute of Medical Science, University of Tokyo, Japan.

Consulting Editor for this article was Peter Libby, MD, Brigham and Women's Hospital, Boston, Mass.

Correspondence to Kensuke Egashira, MD, PhD, Department of Cardiovascular Medicine, Graduate School of Medical Science, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail egashira@cardiol.med.kyushu-u.ac.jp

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000147161.42956.80

tion is uncertain. The cuff model was chosen because cuff placement in the presence of hypercholesterolemia offers the advantage of inducing reproducible site-controlled neointimal formation and stenosis.[24,25] In addition, the cuff-induced injury triggers vascular inflammation and induces neointimal lesions that are partly similar to the restenotic and atherosclerotic lesions observed in humans.[24,25] Our present data provide direct evidence suggesting that inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury.

Methods

Expression Vector

The 3.3-kb mouse sFlt-1 gene was obtained from a mouse lung cDNA library[26] and cloned into the BamHI(5′) and NotI(3′) sites of the eukaryotic expression vector plasmid cDNA3 (Invitrogen).

Experimental Animals

The study protocol was reviewed and approved by the Committee on Ethics for Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines of the American Physiological Society. A part of this study was performed at the Kyushu University Station for Collaborative Research.

Apolipoprotein E–deficient (apoE-KO) and wild-type mice (8 to 10 weeks old, n=5 to 9 each group) with a genetic background of C57BL/6J were purchased from The Jackson Laboratory (Bar Harbor, Me) and fed with commercial standard chow. Placement of cuff and gene transfer were performed as previously described.[21,27] A nonconstrictive polyethylene cuff (1.5 mm long; PE20, 0.38-mm inner diameter, 1.09-mm outer diameter) was placed loosely around the left femoral artery. Either empty plasmid or sFlt-1 plasmid (300 μg/100 μL PBS) was injected into the right femoral muscle using a 27-gauge needle immediately and 10 days after cuff placement. To enhance transgene expression, these animals received electroporation at the injected site immediately after injection.[21,27–29] It has been shown that electroporation-mediated gene transfer is useful to introduce genes into muscle tissues in vivo with no serious tissue injury.[30] To determine the role of flt-1 signals, Flt-1 tyrosine kinase–deficient mice with a genetic background of C57BL/6J were used.[23]

In Vivo Matrigel Plug Assay

An in vivo matrigel plug assay was used to determine the effect of sFlt-1 gene transfer on VEGF activity.[21,27] Matrigel matrix alone (300 μL) or mixed with recombinant VEGF protein (100 ng/mL) was injected subcutaneously into the flanks of C57BL/6J mice. The matrigels were then removed 7 or 14 days after injection, and angiogenesis and inflammation were examined by histopathologic analysis.

Histopathology, Immunohistochemistry, and Morphometry

Mice were anesthetized with pentobarbital, and the femoral artery was harvested, fixed overnight in 3.7% formaldehyde in PBS, and paraffin-embedded.[27] Serial cross sections (5 μm thick) throughout the entire length of the cuffed femoral artery were used for histological analysis. Cryosections were made from 2 mice in each condition. All sections were routinely stained with hematoxylin-eosin or van Gieson. Mac-3 (PharMingen) staining was used to detect monocytes/macrophages, and CD3 (Santa Cruz Biotechnology) was used for T cells. Proliferating cell nuclear antigen (PCNA; Santa Cruz Biotechnology) was used to detect vascular proliferation. An antibody against von Willebrand factor (vWF; Sigma Chemical Co) was used to mark endothelial cells. Antibodies against VEGF (Santa Cruz Biotechnology) and placental growth factor (PlGF; R&D Systems Inc) were also used. Indirect immunofluorescence double-staining with matched primary and fluorescein-conjugated secondary antibodies was used to stain for colocalization with VEGF receptors in smooth muscle cells or monocytes as follows: Rabbit anti-mouse Flt-1 (Santa Cruz Biotechnology), rabbit anti-mouse Flk-1 (Santa Cruz Biotechnology), rat anti-mouse Mac-3, anti–α-smooth muscle actin (α-SMA; Boehringer Mannheim Corp), anti-rabbit IgG conjugated with fluorescein isothiocyanate or rhodamine, and anti-rat IgG conjugated with fluorescein isothiocyanate or rhodamine (Santa Cruz Biotechnology).

Ten equally-spaced cross sections were examined in all mice to quantify intimal lesions. Using image analysis software, the total cross-sectional medial area was measured between the external and internal elastic lamina; the total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Plasma Measurements

Plasma total cholesterol and triacylglycerol levels were determined with commercially available kits (Wako Pure Chemicals). Plasma concentrations of sFlt-1, VEGF, and PlGF were measured by the use of ELISA kit (R&D Systems Inc).

RT-PCR and RNAse Protection Assay

RNA was prepared from the pooled samples (5 to 7 arteries for 1 sample).[21] First-strand DNA was synthesized using reverse transcriptase with random hexamers from 1 μg total RNA in a 20-μL reaction volume according to the manufacturer's protocol (GeneAmp RNA polymerase chain reaction Kit; Perkin–Elmer). Primers used for amplification of VEGF were 5′-GGA TCC ATG AAC TTT CTG CT-3′ and 5′-GAA TTC ACC GCC TCG GCT TGT C-3′ with expected sizes of 654, 582, and 450 bp for the 3 VEGF isoforms (VEGF 188, 164, and 120, respectively). Primers for PlGF were 5′-CCC ACA CCC AGC TCA CGT ATT TA-3′ and 5′-TCC CCT CTA CAT GCC TTC AAT GC-3′. Primers for Flk-1 were 5′-ACT GCA GTG ATT GCC ATG TTC T-3′ and 5′-GCT CAT CCA AGG GCA ATT CAT-3′. Primers for the internal control, β-actin, were 5′-ATG GAT GAC GAT ATC GCT-3′ and 5′-ATG AGG TAG TCT GCT AGG T-3′ with an expected product of 550 bp.

RNAse protection assays were performed using 5 μg total RNA with 2 custom template sets according to the manufacturer's protocol (PharMingen).[27]

Statistical Analysis

Data are expressed as the mean±SE. Statistical analysis of differences was compared by ANOVA. Post hoc analyses were performed using Bonferroni correction for multiple comparison tests. $P<0.05$ was considered to be statistically significant.

Results

In Vivo Matrigel Plug Assay in ApoE-KO Mice

Seven days after injection of matrigel, there were significant angiogenic (number of CD31-positive cells per mm^2) and inflammatory (number of Mac3-positive cells per mm^2) reactions in the matrigel plugs containing recombinant VEGF protein compared with matrigel alone. Soluble Flt-1 gene transfer but not injection of an empty plasmid suppressed both the angiogenic and inflammatory reactions to VEGF to a level similar to that of matrigel plugs without VEGF (Figure I, available online at http://atvb.ahajournals.org). This suppression of angiogenic and inflammatory reactions to VEGF was noted on day 14 but not on day 21 of sFlt-1 gene transfer (data not shown).

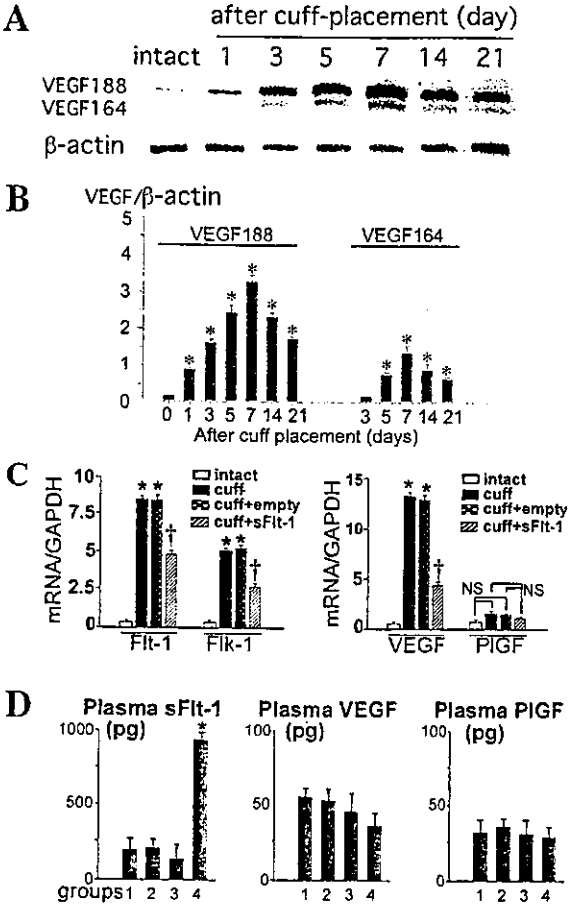

Figure 1. Gene expression VEGF, Flt-1, Flk-1, and PlGF in cuffed femoral artery. A, Time course of VEGF mRNA levels (RT-RCR) and expression of arterial VEGF and β-actin mRNA after cuff placement. mRNA levels were assessed at the indicated times. This is a representative assay from 5 separate experiments. B, Densitometric analysis of data in A. Expression of VEGF mRNA in each sample was normalized by β-actin mRNA expression in the same sample. N=5 for each bar. *$P<0.01$ vs control intact artery. C, Gene expressions of Flt-1, Flk-1, PlGF, and VEGF (RT-PCR) in femoral arteries before or 7 days after cuff placement/sFlt-1 gene transfer. *$P<0.01$ vs intact control. D, Plasma levels of sFlt-1, PlGF, and VEGF 7 days after cuff placement in 4 animal groups: (1) untreated control, (2) mice with cuff alone, (3) cuff+empty plasmid, and (4) cuff+sFlt-1 plasmid. N=6 for each. *$P<0.01$ vs control group.

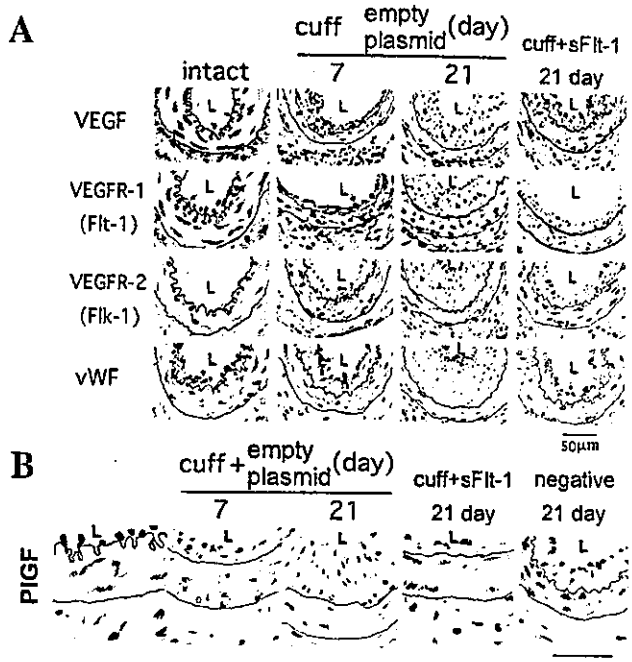

Figure 2. Immunostaining of VEGF, Flt-1, Flk-1, and PlGF in cuffed femoral artery. A, Cross sections of intact or cuffed femoral arteries were stained immunohistochemically against VEGF, VEGF receptor 1 (Flt-1), VEGF receptor 2 (Flk-1), or vWF 7 and 21 days after cuff placement in empty plasmid group. Immunohistochemical sections of cuffed arteries on day 21 in sFlt-1 group are also shown. Black lines indicate internal and external elastin laminas. L indicates lumen. Scale bar=50 μm. B, Immunohistochemical staining of PlGF in femoral arteries before or after cuff placement/sFlt-1 gene transfer. Black lines indicate internal or external elastin laminas. L indicates lumen. Scale bar=50 μm.

Gene Expression and Immunoreactivity in ApoE-KO Mice

The mRNA levels of 2 VEGF isoforms (188 and 164) markedly increased after cuff placement, whereas they were undetectably low in control intact artery (Figure 1A and 1B). Peak expression was observed on day 7. VEGF 121 mRNA was undetectable before and after cuff placement. Gene expression of Flt-1, Flk-1, VEGF, and PlGF was also increased on day 7 (Figure 1C). Plasma concentrations of sFlt-1, VEGF, and PlGF were measured on day 7 in several groups of animals (Figure 1D). Plasma sFlt-1 was increased in sFlt-1 transfection group. There was no significant change in plasma VEGF nor in PlGF among groups (Figure 1D).

Immunohistochemical staining indicated that compared with faint staining in the control artery, VEGF increased in the vicinity of inflammatory lesions (mononuclear cell infiltration) in the intima and adventitia on day 7 and in cells of 3 layers of cuffed artery on day 21 (Figure 2A). The endothelial layer, as detected by vWF staining, was preserved before and after cuff placement (Figure 2A). No detectable increase in PlGF staining was observed before and after cuff placement (Figure 2B).

Both Flk-1 and Flt-1 were undetectable, except in endothelial layers in control intact arteries, but both were increased in the intima, media, and adventitia 7 and 21 days after cuff placement (Figure 2A). Compared with Flt-1 staining, Flk-1 staining was less impressive on day 7 but was apparently noted on day 21. To localize VEGF receptors, immunofluorescent double-staining was performed (Figure 3). On day 7, α-SMA–positive cells in the media and neointima expressed little Flk-1, whereas they did express Flt-1 (Figure 3A). Also, some α-SMA–positive cells in the adventitia (possibly adventitial myofibroblasts) expressed Flt-1. Mac-3 positive cells recruited to the neointima, media, and adventitia expressed VEGF and Flt-1. On day 21, most α-SMA–positive cells in the neointima and media expressed VEGF and its receptors (Figure 3B).

Time Course of Development of Neointimal Hyperplasia in ApoE-KO Mice

As published,[24,27,31,32] within 7 days of cuff placement, mononuclear leukocytes, most of which were Mac3-positive monocytes, were recruited into the adventitia, media, and

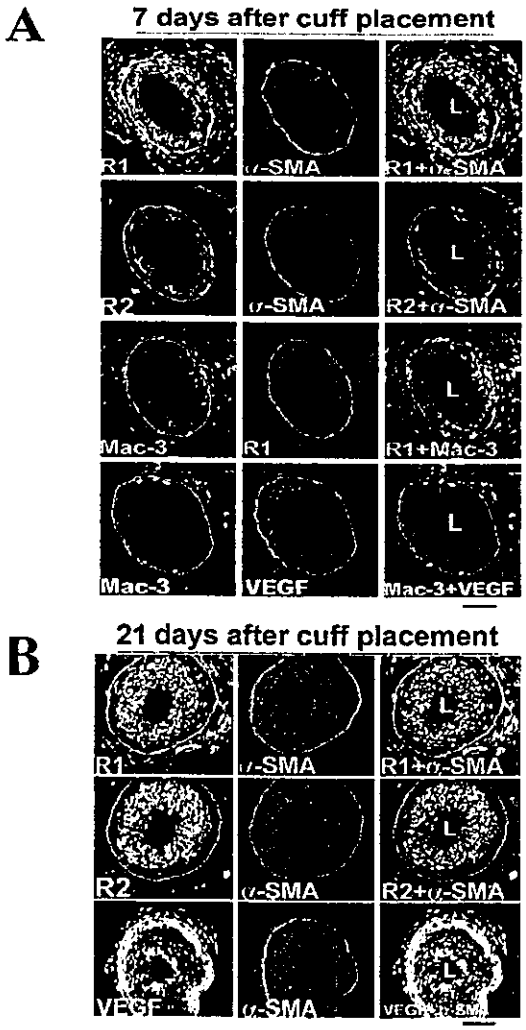

Figure 3. Immunofluorescence double-staining of VEGF receptors, monocytes, and α-SMA in cuffed femoral artery. A, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1, green) and α-SMA (red), with Flk-1 (VEGF-R2, green) and α-SMA (red), and with Mac-3 (green) and Flt-1 (VEGF-R1, red) 7 days after cuff placement. Scale bar=50 μm. B, Micrographs of cuffed femoral arteries doubly stained with Flt-1 (VEGF-R1) and α-SMA, Flk-1 (VEGF-R2) and α-SMA, and VEGF and α-SMA in the cuffed femoral arteries 21 days after cuff placement. Single fluorescence-positive cells were stained green or red, whereas double-positive cells were stained yellow. White lines indicate external elastin laminas. L indicates lumen. Scale bar=50 μm.

intima (Figure 3). After 7 days, neointimal lesions developed and became thick over time (Figure 4A). Monocyte infiltration declined spontaneously and α-SMA–positive cells appeared predominantly in the neointima. On day 21, significant neointimal formation with luminal stenosis developed (Figure 4A). Endothelial staining with vWF antibody showed that no significant neointimal neovascularization was observed during the course of experiments (Figure 2A).

Effects of Soluble Flt-1 Gene Transfer on Cuff-Induced Neointimal Hyperplasia in ApoE-KO Mice

As we previously reported,[27] Mac3-positive monocytes/macrophages and PCNA-positive cells were detected mainly in

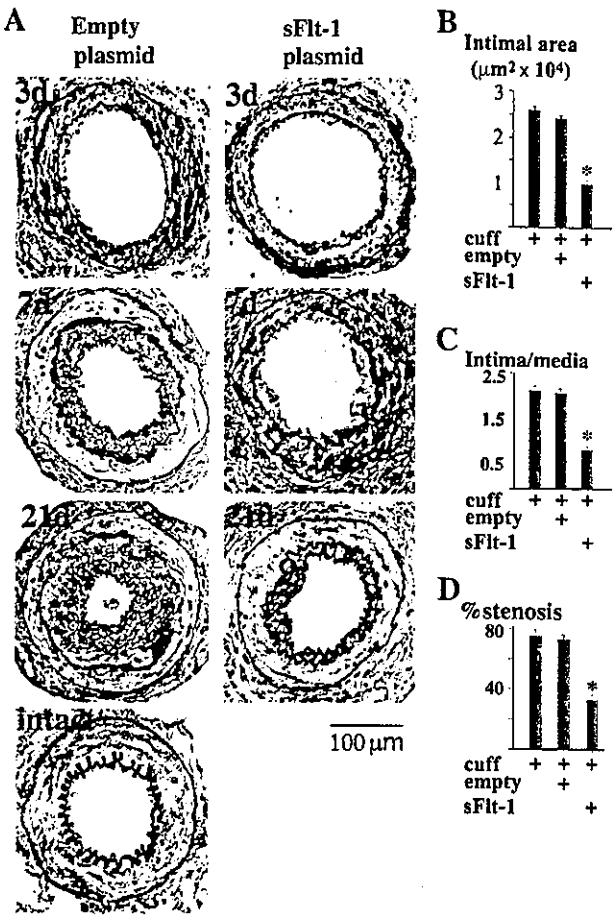

Figure 4. Histopathology of cuffed femoral artery. A, Time course of cuff injury–induced neointimal formation and effect of sFlt-1 gene transfer. Micrographs of cross sections of control (intact) and cuffed arteries stained with van Gieson Elastica on days 3, 7, and 21 are shown. Scale bar=100 μm. B through D, Effects of sFlt-1 gene transfer on neointimal thickening (B), intima/media ratio (C), and % stenosis (D) 21 days after cuff placement. *$P<0.01$ vs cuff only and cuff+empty plasmid group.

the adventitia and intima. There was markedly less inflammation (Mac3-positive cells) and proliferation (the PCNA index) in sFlt-1–transfected mice than in empty plasmid-transfected mice on day 7 (Figure II, available online at http://atvb.ahajournals.org). There was no detectable change in the number of CD3-positive T cells in empty plasmid or sFlt-1–transferred mice (Figure II). The sFlt-1 gene transfer significantly reduced neointimal formation (increases in neointimal area, intima/media ratio, and luminal stenosis) 21 days after cuff placement (Figure 4A through 4D).

Because sFlt-1 gene transfer markedly reduced monocyte-mediated inflammation, gene expression of a battery of inflammatory cytokines, chemokines, and chemokine receptors was examined by RNAse protection assays (Figure III, available online at http://atvb.ahajournals.org) or by RT-PCR (Figure 1C) 7 days after cuff placement. The sFlt-1 gene transfer did not affect gene expression of RANTES, macrophage inflammatory protein-1α, transforming growth factor-β, macrophage inflammatory protein-2, and PlGF, but prevented or attenuated the increased gene expression of CCR1, interleukin-6, CCR2, MCP-1, Flt-1, CXCR2, eotaxin,

vascular cell adhesion molecule-1, intercellular adhesion molecule-1, Flk-1, and VEGF. The sFlt-1 gene transfer reduced increased immunostainings against VEGF, Flt-1, and Flk-1, but did not affect staining against vWF (Figure 2A).

Time course of plasma concentrations of sFlt-1 after sFlt-1 gene transfer was determined. Plasma sFlt-1 levels before and 3, 7, and 14 days after sFlt-1 transfection were 467±37, 1037±132 ($P<0.01$ versus baseline), 927±215 ($P<0.01$), and 649±83 pg/mL ($P<0.05$, n=6 each), indicating that sFlt-1 was released from the transfected muscle.

Plasma Lipid Levels in ApoE-KO Mice

Total cholesterol and triacylglycerol levels were 503±11 and 38±6 mg/dL in the control group, 512±16 and 40±5 mg/dL in the empty plasmid group, and 497±10 and 39±3 mg/dL in the sFlt-1 group, indicating that the observed effects of sFlt-1 gene transfer were not caused by changes in serum lipid levels.

Effects of Flt-1 Tyrosine Kinase Deficiency on Neointimal Hyperplasia

Wild-type and Flt-1 tyrosine kinase–deficient mice were fed a high-fat diet for 2 weeks, and cuff was placed as mentioned above. Mice received a high-fat diet for an additional 3 weeks. Neointimal formation was noted 21 days after cuff placement in wild-type mice. Compared with wild-type mice, Flt-1 tyrosine kinase–deficient mice displayed reduced neointimal formation (Figure IV, available online at http://atvb.ahajournals.org). Total cholesterol levels at 5 weeks of high-fat diet were 520±21 and 511±18 mg/dL in wild-type and Flt-1 tyrosine kinase–deficient mice, respectively.

Discussion

This study is the first to demonstrate the essential role of VEGF and Flt-1 signals in the development of neointimal formation after cuff-induced periadventitial injury in hypercholesterolemic mice. VEGF is conventionally thought to be an endothelial cell–specific growth factor and to attenuate vascular disease by inducing endothelial proliferation and regeneration mainly through the endothelial type 2 receptor Flk-1.[7] Recent evidence, however, suggests that functional Flt-1 and Flk-1 are expressed in injured arterial wall cells other than endothelial cells. In this study, Flt-1, Flk-1, and VEGF were increased in lesional monocytes and medial smooth muscle cells at early stages and in neointimal and medial smooth muscle cells at later stages. Flt-1 is demonstrated to act as an important mediator of chemotaxis through vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and MCP-1.[13–15] We demonstrated that sFlt-1 gene transfer reduced the early inflammatory and proliferative changes and thus attenuated the development of neointimal formation. It is speculated, therefore, that VEGF might cause inflammation (monocyte recruitment and activation) and proliferation through Flt-1–mediated signals and thus cause neointimal formation after cuff-induced periadventitial injury. In addition, Flt-1 in smooth muscle cells is reported to mediate migration and proliferation in vitro.[18,33] An alternative interpretation is that VEGF directly caused migration and proliferation of smooth muscle cells resulting in neointimal formation. In any way, our present finding of Flt-1 tyrosine kinase–deficient mice suggests the involvement of Flt-1–related signals in the pathogenesis of neointimal formation after periadventitial injury.

Periadventitial inflammation has a major role in the pathogenesis of cuff-induced neointimal formation.[27,32] To gain insight into the mechanism of VEGF-mediated inflammation and neointimal formation, we assessed gene expression of various inflammatory genes. The sFlt-1 gene transfer attenuated increased gene expression of inflammatory cytokines, adhesion molecules, chemokines, and chemokine receptors. Yamada et al[34] showed that MCP-1 is essential in VEGF-induced angiogenesis, vascular leakage, and inflammation. An essential role of MCP-1 in the development of neointimal formation after arterial injury has also been reported.[27–29,35,36] The sFlt-1 gene transfer attenuated increased VEGF, Flk-1, and Flt-1 gene expression, indicating that VEGF regulates its activity by an autocrine loop mechanism within diseased arterial wall cells, such as smooth muscle cells, endothelial cells, and lesional monocytes. A positive feedback effect of VEGF is supported by previous studies that demonstrated enhanced VEGF production by monocytes through Flt-1 stimulation.[37] Therefore, sFlt-1 gene transfer might attenuate cuff-induced neointimal formation mainly by suppressing inflammation (monocyte recruitment and activation).

This study has potentially significant clinical implications. Blockade of VEGF by sFlt-1 gene transfer can be an attractive anti-VEGF therapy for inflammatory vascular disease and other inflammatory disorders. The efficacy of this strategy for experimental tumor angiogenesis has already been tested.[22] Luttun et al[38] recently reported that treatment with anti–Flt-1 antibody attenuated the development of experimental tumor angiogenesis, arthritis, and atherosclerosis. One limitation of the present finding is that the pathogenesis of neointimal formation after periadventitial injury differs from that after endothelial injury/denudation and from that in humans. The endothelial integrity is preserved in cuff-induced periadventitial injury. For clinical application of our findings to human vascular disease, future studies are needed to examine the efficacy and safety of anti-VEGF strategy with sFlt-1 in experimental atherosclerosis and restenosis.

In conclusion, inflammatory changes mediated by VEGF and Flt-1 signals play an important role in the pathogenesis of neointimal formation after cuff-induced periadventitial injury. These data support the notion that VEGF promote neointimal formation by acting as a proinflammatory cytokine after cuff-induced periadventitial injury.

Acknowledgments

This study was supported by Grants-in-Aid for Scientific Research (14657172, 14207036) from the Ministry of Education, Science, and Culture by Health Science Research Grants (Comprehensive Research on Aging and Health and Research on Translational Research), from the Ministry of Health, Labor, and Welfare, and by the Program for Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research, Tokyo, Japan.

References

1. Libby P, Ganz P. Restenosis revisited: new targets, new therapies. *N Engl J Med.* 1997;337:418–419.

2. Topol EJ, Serruys PW. Frontiers in interventional cardiology. *Circulation.* 1998;98:1802–1820.
3. Shibata M, Suzuki H, Nakatani M, Koba S, Geshi E, Katagiri T, Takeyama Y. The involvement of vascular endothelial growth factor and flt-1 in the process of neointimal proliferation in pig coronary arteries following stent implantation. *Histochem Cell Biol.* 2001;116:471–481.
4. Ruef J, Hu ZY, Yin LY, Wu Y, Hanson SR, Kelly AB, Harker LA, Rao GN, Runge MS, Patterson C. Induction of vascular endothelial growth factor in balloon-injured baboon arteries. A novel role for reactive oxygen species in atherosclerosis. *Circ Res.* 1997;81:24–33.
5. Chen YX, Nakashima Y, Tanaka K, Shiraishi S, Nakagawa K, Sueishi K. Immunohistochemical expression of vascular endothelial growth factor/vascular permeability factor in atherosclerotic intimas of human coronary arteries. *Arterioscler Thromb Vasc Biol.* 1999;19:131–139.
6. Inoue M, Itoh H, Ueda M, Naruko T, Kojima A, Komatsu R, Doi K, Ogawa Y, Tamura N, Takaya K, Igaki T, Yamashita J, Chun TH, Masatsugu K, Becker AE, Nakao K. Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. *Circulation.* 1998;98:2108–2116.
7. Baumgartner I, Isner JM. Somatic gene therapy in the cardiovascular system. *Annu Rev Physiol.* 2001;63:427–450.
8. Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: a novel nonangiogenic cardiovascular role for vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol.* 2000;20:1512–1520.
9. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. *Cardiovasc Res.* 2001;49:568–581.
10. Bussolati B, Ahmed A, Pemberton H, Landis RC, Di Carlo F, Haskard DO, Mason JC. Bifunctional role for VEGF-induced heme oxygenase-1 in vivo: induction of angiogenesis and inhibition of leukocytic infiltration. *Blood.* 2004;103:761–766.
11. Isner JM. Still more debate over VEGF. *Nat Med.* 2001;7:639–641.
12. Ware JA. Too many vessels? Not enough? The wrong kind? The VEGF debate continues. *Nat Med.* 2001;7:403–404.
13. Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood.* 1996;87:3336–3343.
14. Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem.* 2001;276:7614–7620.
15. Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes.* 1999;48:1131–1137.
16. Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD. Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med.* 2001;7:425–429.
17. Grosskreutz CL, Anand-Apte B, Duplaa C, Quinn TP, Terman BI, Zetter B, D'Amore PA. Vascular endothelial growth factor-induced migration of vascular smooth muscle cells in vitro. *Microvasc Res.* 1999;58:128–136.
18. Ishida A, Murray J, Saito Y, Kanthou C, Benzakour O, Shibuya M, Wijelath ES. Expression of vascular endothelial growth factor receptors in smooth muscle cells. *J Cell Physiol.* 2001;188:359–368.
19. Moulton KS, Heller E, Konerding MA, Flynn E, Palinski W, Folkman J. Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation.* 1999;99:1726–1732.
20. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun.* 1996;226:324–328.
21. Zhao Q, Egashira K, Inoue S, Usui M, Kitamoto S, Ni W, Ishibashi M, Hiasa Ki K, Ichiki T, Shibuya M, Takeshita A. Vascular endothelial growth factor is necessary in the development of arteriosclerosis by recruiting/activating monocytes in a rat model of long-term inhibition of nitric oxide synthesis. *Circulation.* 2002;105:1110–1115.
22. Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY, Siegal GP, Mao X, Bett AJ, Huckle WR, Thomas KA, Curiel DT. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci U S A.* 1998;95:8795–8800.
23. Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A.* 1998;95:9349–9354.
24. Lardenoye JH, Delsing DJ, de Vries MR, Deckers MM, Princen HM, Havekes LM, van Hinsbergh VW, van Bockel JH, Quax PH. Accelerated atherosclerosis by placement of a perivascular cuff and a cholesterol-rich diet in ApoE*3Leiden transgenic mice. *Circ Res.* 2000;87:248–253.
25. von der Thusen JH, van Berkel TJ, Biessen EA. Induction of rapid atherogenesis by perivascular carotid collar placement in apolipoprotein E-deficient and low-density lipoprotein receptor-deficient mice. *Circulation.* 2001;103:1164–1170.
26. Kondo K, Hiratsuka S, Subbalakshmi E, Matsushime H, Shibuya M. Genomic organization of the flt-1 gene encoding for vascular endothelial growth factor (VEGF) receptor-1 suggests an intimate evolutionary relationship between the 7-Ig and the 5-Ig tyrosine kinase receptors. *Gene.* 1998;208:297–305.
27. Egashira K, Zhao Q, Kataoka C, Ohtani K, Usui M, Charo IF, Nishida K, Inoue S, Katoh M, Ichiki T, Takeshita A. Importance of monocyte chemoattractant protein-1 pathway in neointimal hyperplasia after periarterial injury in mice and monkeys. *Circ Res.* 2002;90:1167–1172.
28. Usui M, Egashira K, Ohtani K, Kataoka C, Ishibashi M, Hiasa K, Katoh M, Zhao Q, Kitamoto S, Takeshita A. Anti-monocyte chemoattractant protein-1 gene therapy inhibits restenotic changes (neointimal hyperplasia) after balloon injury in rats and monkeys. *FASEB J.* 2002;16:1838–1840.
29. Mori E, Komori K, Yamaoka T, Tanii M, Kataoka C, Takeshita A, Usui M, Egashira K, Sugimachi K. Essential role of monocyte chemoattractant protein-1 in development of restenotic changes (neointimal hyperplasia and constrictive remodeling) after balloon angioplasty in hypercholesterolemic rabbits. *Circulation.* 2002;105:2905–2910.
30. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation in vivo. *Nat Biotechnol.* 1998;16:867–870.
31. Moroi M, Zhang L, Yasuda T, Virmani R, Gold HK, Fishman MC, Huang PL. Interaction of genetic deficiency of endothelial nitric oxide, gender, and pregnancy in vascular response to injury in mice. *J Clin Invest.* 1998;101:1225–1232.
32. Wu L, Iwai M, Nakagami H, Li Z, Chen R, Suzuki J, Akishita M, de Gasparo M, Horiuchi M. Roles of angiotensin II type 2 receptor stimulation associated with selective angiotensin II type 1 receptor blockade with valsartan in the improvement of inflammation-induced vascular injury. *Circulation.* 2001;104:2716–2721.
33. Wang H, Keiser JA. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. *Circ Res.* 1998;83:832–840.
34. Yamada M, Kim S, Egashira K, Takeya M, Ikeda T, Mimura O, Iwao H. Molecular mechanism and role of endothelial monocyte chemoattractant protein-1 induction by vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol.* 2003;23:1996–2001.
35. Egashira K. Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-1. *Hypertension.* 2003;41:834–841.
36. Ohtani K, Usui M, Nakano K, Kohjimoto Y, Kitajima S, Hirouchi Y, Li XH, Kitamoto S, Takeshita A, Egashira K. Antimonocyte chemoattractant protein-1 gene therapy reduces experimental in-stent restenosis in hypercholesterolemic rabbits and monkeys. *Gene Ther.* 2004;11:1273–1282.
37. Bottomley MJ, Webb NJ, Watson CJ, Holt L, Bukhari M, Denton J, Freemont AJ, Brenchley PE. Placenta growth factor (PlGF) induces vascular endothelial growth factor (VEGF) secretion from mononuclear cells and is co-expressed with VEGF in synovial fluid. *Clin Exp Immunol.* 2000;119:182–188.
38. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med.* 2002;8:831–840.

Extracorporeal Cardiac Shock Wave Therapy Markedly Ameliorates Ischemia-Induced Myocardial Dysfunction in Pigs in Vivo

Takahiro Nishida, MD; Hiroaki Shimokawa, MD; Keiji Oi, MD; Hideki Tatewaki, MD; Toyokazu Uwatoku, MD; Kohtaro Abe, MD; Yasuharu Matsumoto, MD; Noriyoshi Kajihara, MD; Masataka Eto, MD; Takehisa Matsuda, PhD; Hisataka Yasui, MD; Akira Takeshita, MD; Kenji Sunagawa, MD

Background—Prognosis of ischemic cardiomyopathy still remains poor because of the lack of effective treatments. To develop a noninvasive therapy for the disorder, we examined the in vitro and vivo effects of extracorporeal shock wave (SW) that could enhance angiogenesis.

Methods and Results—SW treatment applied to cultured human umbilical vein endothelial cells significantly upregulated mRNA expression of vascular endothelial growth factor and its receptor Flt-1 in vitro. A porcine model of chronic myocardial ischemia was made by placing an ameroid constrictor at the proximal segment of the left circumflex coronary artery, which gradually induced a total occlusion of the artery with sustained myocardial dysfunction but without myocardial infarction in 4 weeks. Thereafter, extracorporeal SW therapy to the ischemic myocardial region (200 shots/spot for 9 spots at 0.09 mJ/mm^2) was performed (n=8), which induced a complete recovery of left ventricular ejection fraction (51±2% to 62±2%), wall thickening fraction (13±3% to 30±3%), and regional myocardial blood flow (1.0±0.2 to 1.4±0.3 $mL \cdot min^{-1} \cdot g^{-1}$) of the ischemic region in 4 weeks (all $P<0.01$). By contrast, animals that did not receive the therapy (n=8) had sustained myocardial dysfunction (left ventricular ejection fraction, 48±3% to 48±1%; wall thickening fraction, 13±2% to 9±2%) and regional myocardial blood flow (1.0±0.3 to 0.6±0.1 $mL \cdot min^{-1} \cdot g^{-1}$). Neither arrhythmias nor other complications were observed during or after the treatment. SW treatment of the ischemic myocardium significantly upregulated vascular endothelial growth factor expression in vivo.

Conclusions—These results suggest that extracorporeal cardiac SW therapy is an effective and noninvasive therapeutic strategy for ischemic heart disease. (***Circulation.* 2004;110:3055-3061.**)

Key Words: angiogenesis ■ contractility ■ hibernation ■ ischemia ■ regional blood flow

Prognosis of ischemic cardiomyopathy without an indication for coronary intervention or coronary artery bypass grafting still remains poor because medication is the only therapy to treat the disorder.[1] Thus, it is imperative that an effective and noninvasive therapy for ischemic cardiomyopathy be developed. Although no medication or procedure used clinically has shown efficacy in replacing myocardial scar with functioning contractile tissue, it could be possible to improve the contractility of the hibernating myocardium by inducing angiogenesis.

It recently has been suggested that shock wave (SW) could enhance angiogenesis in vitro.[2] SW is a longitudinal acoustic wave, traveling with the speed in water of ultrasound through body tissue. It is a single pressure pulse with a short needle-like positive spike <1 μs in duration and up to 100 MPa in amplitude, followed by a tensile part of several microseconds with lower amplitude.[3] SW is known to exert the "cavitation effect" (a micrometer-sized violent collapse of bubbles inside and outside the cells)[3] and recently has been demonstrated to induce localized stress on cell membranes that resembles shear stress.[4] If SW-induced angiogenesis could be reproduced in vivo, it would provide a unique opportunity to develop a new angiogenic therapy that would not require invasive procedures such as open-chest surgery or catheter intervention. Therefore, the present study was designed to examine the possible beneficial effects of SW on ischemia-induced myocardial dysfunction in a porcine model of chronic myocardial ischemia in vivo.

Methods

This study was reviewed by the Committee on Ethics in Animal Experiments of Kyushu University and was carried out under the

Received May 5, 2003; de novo received June 2, 2004; accepted June 17, 2004.

From the Departments of Cardiovascular Surgery (T.N., H.T., N.K., M.E., H.Y.), Cardiovascular Medicine (K.O., H.S., T.U., K.A., Y.M., A.T., K.S.), and Biomedical Engineering (T.M.), and the 21st Century COE Program on Lifestyle-Related Diseases (H.S., T.M.), Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan.

Correspondence to Hiroaki Shimokawa, MD, PhD, Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail shimo@cardiol.med.kyushu-u.ac.jp

Circulation is available at http://www.circulationaha.org

DOI: 10.1161/01.CIR.0000148849.51177.97

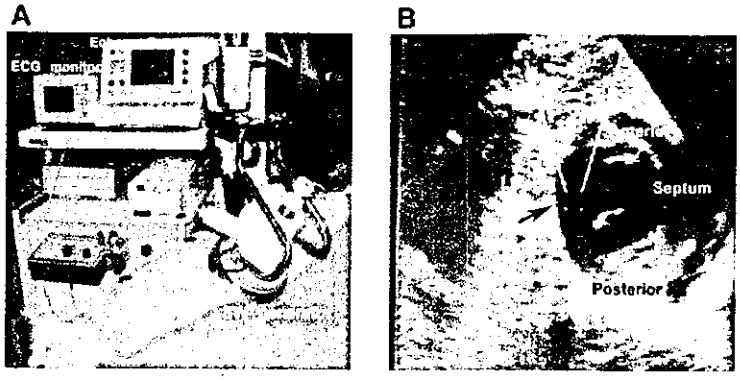

Figure 1. Extracorporeal cardiac SW therapy in action in a pig chronically instrumented with an ameroid constrictor. A, The machine is equipped with a SW generator and in-line echocardiography. The SW generator is attached to the chest wall when used. B, The SW pulse is easily focused on the ischemic myocardium under the guidance of echocardiography (black arrow).

Guidelines for Animal Experiments of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Effect of SW on Human Umbilical Vein Endothelial Cells in Vitro

We purchased single-donor human umbilical vein endothelial cells (HUVECs) (Clonetics, Walkersville, Md) and cultured them in a complete endothelial medium (EBM-2 BulletKit, Clonetics). HUVECs were subcultured and used at passages 3 to 5 and were maintained in EBM-2. Twenty-four hours before the SW treatment, HUVECs (1×10^5) were resuspended in a 2-mL tube with EBM (Clonetics). We treated the HUVECs with 500 shots of SW at 4 different energy levels (0 [control], 0.02, 0.09, 0.18, and 0.35 mJ/mm^2) and stored them for 24 hours in the same medium before RNA extraction.

Ribonuclease Protection Assay

We analyzed equal amounts of mRNA by ribonuclease protection assay by means of the RiboQuant multiprobe template (PharMingen). Briefly, we hybridized RNA overnight with a ^{32}P-labeled RNA probe, which previously had been synthesized from the template set. We digested single-stranded RNA and free probe by ribonuclease A and T1. We then analyzed protected RNA on a 5% denaturing polyacrylamide gel. We analyzed several angiogenic factors, including vascular endothelial growth factor (VEGF) and its receptor, *fms*-like tyrosine kinase (Flt)-1, and angiopoietin and its receptor, tie-1, either by means of an NIH image or by means of autoradiography and subsequent quantification by densitometry (Alpha Innotech). For quantification, we normalized the signals for each sample of the blot with the corresponding signals of the housekeeping genes GAPDH and L32.

Porcine Model of Chronic Myocardial Ischemia

A total of 28 domestic pigs (25 to 30 kg in body weight) were used in this study. We anesthetized the animals with ketamine (15 mg/kg IM) and maintained anesthesia with an inhalation of 1.5% isoflurane for implantation of an ameroid constrictor, SW treatment, and euthanization. We opened the chest, suspended the pericardium and the left atrial appendage, revealed the left circumflex coronary artery (LCx), and put an ameroid constrictor around the proximal LCx to gradually induce a total occlusion of the artery in 4 weeks without causing myocardial infarction.[5,6] We also confirmed histologically that no myocardial necrosis had developed in the present porcine model (data not shown). This model is widely used to examine the effect of an angiogenic therapy in the ischemic hibernating myocardium.[5,6]

Extracorporeal Cardiac SW Therapy to Chronic Ischemic Myocardium

On the basis of the in vitro experiment, we applied a low energy of SW (0.09 mJ/mm2, ≈10% of the energy for the lithotripsy treatment) to 9 spots in the ischemic region (200 shots/spot) with the guidance of an echocardiogram equipped within a specially designed SW generator (Storz Medical AG) (Figure 1A). We were able to focus SW in any part of the heart under the guidance of echocardiography (Figure 1B). We applied SW to the ischemic myocardium in an R-wave–triggered manner to avoid ventricular arrhythmias. We performed the SW treatment (n=8) at 4 weeks after the implantation of an ameroid constrictor 3 times within 1 week, whereas animals in the control group (n=8) received the same anesthesia procedures 3 times a week but without the SW treatment. Because the SW treatment only requires the gentle compression of the generator to the chest wall, it is unlikely that this handling itself enhances angiogenesis in the ischemic myocardium.

Coronary Angiography and Left Ventriculography

After systemic heparinization (10 000 U/body), we performed coronary angiography and left ventriculography in a left oblique view with the use of a cineangiography system (Toshiba Medical). We semiquantitatively evaluated the extent of collateral flow to the LCx by the graded Rentrop score (0, no visible collateral vessels; 1, faint filling of side branches of the main epicardial vessel without filling the main vessel; 2, partial filling of the main epicardial vessel; 3, complete filling of the main vessel).[7] We also counted the number of visible coronary arteries in the LCx region. To compare the extent of collateral development at a given time, we selected the frame in which the whole left anterior descending coronary artery was first visualized.

Echocardiographic Evaluation

We performed epicardial echocardiographic studies at ameroid implantation (baseline) and at 4 and 8 weeks after the implantation of the constrictor (Sonos 5500, Agilent Technology). We calculated wall thickening fraction (WTF) by using the following formula: WTF = 100 × (end-systolic wall thickness − end-diastolic wall thickness) /end-diastolic wall thickness. We measured WTF when pigs were sedated, with and without dobutamine loading (15 $\mu g \cdot kg^{-1} \cdot min^{-1}$). Dobutamine was infused continuously from the ear vein, and WTF was measured after the hemodynamic condition was stabilized (in ≈5 minutes).

Measurement of Regional Myocardial Blood Flow

We evaluated regional myocardial blood flow (RMBF) with colored microspheres (Dye-Trak, Triton Technology) at ameroid implantation (baseline) and at 4 and 8 weeks after implantation.[8] We injected microspheres through the left atrium and aspirated a reference arterial blood sample from the descending aorta at a constant rate of 20 mL/min for 60 seconds using a withdrawal pump. We extracted microspheres from the left ventricular (LV) wall and blood samples by potassium hydroxide digestion, extracted the dyes from the spheres with dimethylformamide (200 μL), and determined their concentrations by spectrophotometry.[8] We calculated myocardial blood flow ($mL \cdot min^{-1} \cdot g^{-1}$) of the endocardial and epicardial lateral LV wall (the LCx region).

Analysis of Cardiac Enzymes

We measured serum concentrations of cardiac troponin T and creatinine kinase (CK)–MB by using chemiluminescence immuno-

assay before the SW treatment and at 4, 5 (2 hours after the SW treatment), and 8 weeks after ameroid implantation.

Factor VIII Staining

We treated paraffin-embedded sections with a rabbit anti–factor VIII antibody (N1505, Dako, Copenhagen, Denmark). We counted the number of factor VIII–positive cells in the endocardial and epicardial wall in 10 fields of the LCx region in each heart at 400× magnification.

Real-Time Polymerase Chain Reaction

To examine the effect of SW treatment on the ischemic myocardium in vivo, the animals with an ameroid constrictor were euthanized 1 week after the SW treatment. Total RNA was isolated from rapidly frozen ischemic LV wall (LCx region) after 3 SW treatments and was reverse transcribed. Quantification of VEGF and its receptor Flt-1 was performed by amplification of cDNA with an ABI Prism 7000 real-time thermocycler.

Western Blot Analysis for VEGF

We performed Western blot analysis for VEGF. Western blot analysis for VEGF was performed with and without 3 SW treatments. Three sections from the ischemic LV wall (LCx region) were measured. The regions containing VEGF proteins were visualized by electrochemiluminescence Western blotting luminal reagent (Santa Cruz Biotechnology). The extent of the VEGF was normalized by that of β-actin.

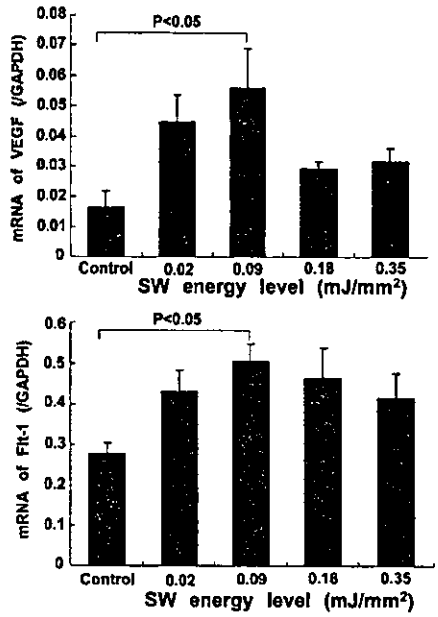

Figure 2. SW treatment upregulated mRNA expression of VEGF (A) and Flt-1 (B) in HUVECs in vitro with a maximum effect noted at 0.09 mJ/mm². Results are expressed as mean±SEM (n=10 each).

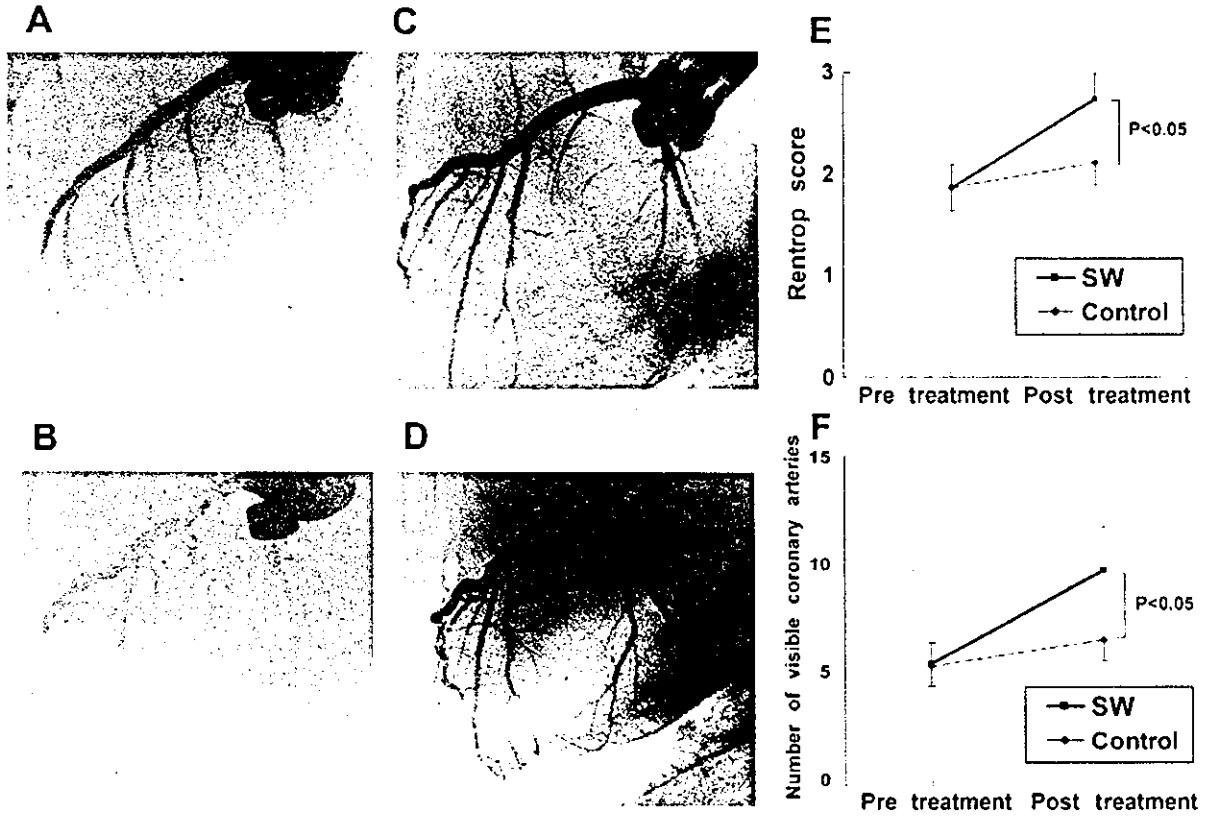

Figure 3. Extracorporeal cardiac SW therapy enhances coronary angiogenesis in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LCx was totally occluded and was perfused via collateral vessels with severe delay in both the control group (A) and the SW group (before SW therapy) (C). B and D, Four weeks after the first coronary angiography, no significant change in coronary vessels was noted in the control group (B), whereas a marked development of visible coronary vessels was noted in the SW group (D). E and F, Four weeks after the first coronary angiography, no significant increase in the Rentrop score (E) or visible coronary arteries from LCx (F) was noted in the control group, whereas increased Rentrop score and a marked development of visible coronary vessels were noted in the SW group. Results are expressed as mean±SEM (n=8 each).

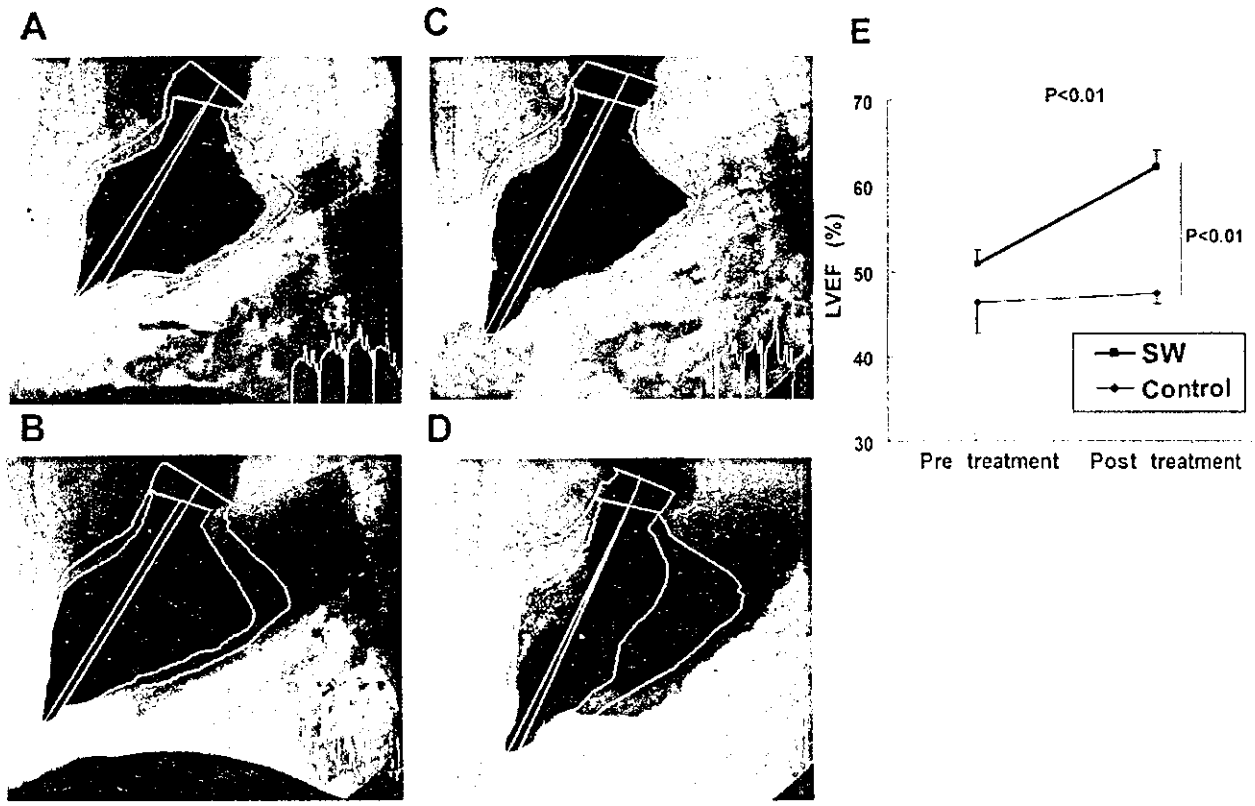

Figure 4. Extracorporeal cardiac SW therapy improves ischemia-induced myocardial dysfunction in vivo. A and C, Four weeks after the implantation of an ameroid constrictor, LV wall motion of the LCx (posterolateral) region was reduced in both the control (A) and the SW group (before the SW therapy) (C). B and D, Four weeks after the first left ventriculography, no significant change in LV wall motion was noted in the control group (B), whereas marked recovery was noted in the SW group (D). E, The SW therapy normalized left ventricular ejection fraction in the SW group but not in the control group. Results are expressed as mean±SEM (n=8 each).

Statistical Analysis

Results are expressed as mean±SEM. We determined statistical significance by analysis of variance for multiple comparisons. A value of $P<0.05$ was considered to be statistically significant.

Results

Effect of SW on mRNA Expression of VEGF and Flt-1 in HUVECs

SW treatment significantly upregulated mRNA expression of VEGF and its receptor Flt-1 in HUVECs, with a maximum effect noted at 0.09 mJ/mm^2 (Figure 2).

Effects of Extracorporeal Cardiac SW Therapy on Angiogenesis and Ischemia-Induced Myocardial Dysfunction

Four weeks after ameroid implantation, coronary angiography demonstrated a total occlusion of the LCx, which was perfused via collateral vessels with severe delay in both the control (Figure 3A) and the SW groups (Figure 3C). At 8 weeks after ameroid implantation (4 weeks after SW therapy), the SW group (Figure 3D), but not the control group (Figure 3B), had a marked development of coronary collateral vessels in the ischemic LCx region, an increased Rentrop score (Figure 3E), and an increased number of visible coronary arteries in the region (Figure 3F). Similarly, at 4 weeks, left ventriculography demonstrated an impaired left ventricular ejection fraction in both groups (Figure 4A, 4C, and 4E), whereas at 8 weeks, left ventricular ejection fraction was normalized in the SW group but remained impaired in the control group (Figure 4B, 4D, and 4E).

Effects of Extracorporeal Cardiac SW Therapy on Regional Myocardial Function and Myocardial Blood Flow

We serially measured WTF of the LCx region (lateral wall of the LV) by epicardiac echocardiography. At 4 weeks, we observed a significant reduction in WTF (%) in both groups (13±2 in the control group and 13±3 in the SW group; Figure 5A). At 8 weeks, however, the SW treatment markedly improved WTF in the SW group (30±3) but not in the control group (9±2) under control conditions (Figure 5A). Under dobutamine-loading conditions, which mimicked exercise conditions, WTF was further reduced at 4 weeks after the ameroid implantation in both groups (16±3 in the control and 18±2 in the SW groups), however, at 8 weeks, WTF was again markedly ameliorated only in the SW group (31±2) but not in the control group (16±4) (Figure 5B).

At 4 weeks, RMBF in the endocardium and epicardium (mL · min^{-1} · g^{-1}) was equally decreased in both groups (1.0±0.3 and 0.9±0.2 in the control group and 1.0±0.2 and 0.9±0.2 in the SW group, respectively). The SW treatment again improved RMBF in the endocardium (0.6±0.1 in the