Abbreviations and Acronyms

AS	= aortic stenosis
BNP	= B-type natriuretic peptide
CHF	= congestive heart failure
DHF	= diastolic heart failure
EDP	= end-diastolic pressure
EDWS	= end-diastolic wall stress
EF	= ejection fraction
HF	= heart failure
LV	= left ventricle/ventricular
LVEDVI	= left ventricular end-diastolic volume index
LVMI	= left ventricular mass index
SHF	= systolic heart failure
SWS	= systolic wall stress

with HF of various etiologies. Accordingly, in the present study, we evaluated plasma BNP levels in 160 consecutive patients presenting with HF of various etiologies including diastolic HF.

METHODS

Patients. Among the patients referred to our National Cardiovascular Center Hospital between October 2003 and December 2004, we included in this study those admitted with congestive heart failure (CHF) consecutively. Patients who did not undergo LV catheterization or had renal dysfunction (serum creatinine >2.0 mg/dl) were excluded. A sample of 160 patients was obtained. For all participants, cardiac catheterization and echocardiograms were performed at a compensated CHF stage (before discharge), and plasma BNP was measured on the day before cardiac catheterization. The clinical characteristics of these patients are listed in Table 1.

BNP assay. Blood was collected into tubes containing EDTA, and plasma BNP was measured using a validated and commercially available immunoassay kit (Tosoh Co. Ltd., Japan).

Cardiac catheterization. Left ventricular pressure was recorded with a 5-F pigtail catheter connected to a fluid-filled transducer. Left ventricular volume and EF were determined with left ventriculography with contrast medium using Kennedy's formula.

Echocardiography. Echocardiographic examinations were performed with a Sonos 5500 machine equipped with a 2.5-MHz probe. M-mode images were obtained to measure left atrial and ventricular dimensions (14). The left ventricular mass index (LVMI) was estimated from the formula of Devereux et al. (15). The severity of mitral regurgitation was quantified on a semicontinuous scale from none (0) to moderately severe (3+). In patients with sinus rhythm, the pulsed Doppler transmitral flow velocity was recorded to measure a ratio of peak mitral E-wave velocity to peak mitral A-wave velocity (E/A ratio) and the deceleration time of the mitral E-wave velocity.

On the basis of hemodynamic and echocardiographic data, end-diastolic and systolic meridional wall stresses (WS) were calculated. These were obtained by using the formula: $WS = 0.334 \times P(LVID)/WT(1 + WT/LVID)$, where P = LV pressure (i.e., peak systolic pressure or EDP, which was obtained during cardiac catheterization), LVID = left ventricular internal dimension, and WT = wall thickness (16). In the present study, the posterior wall thickness was used to assess WT regardless of regional wall motion abnormalities. In the analysis of the interobserver reproducibility of the posterior wall thickness measurement in 48 patients with CHF, a high degree of the reproducibility was

Table 1. Patient Characteristics

	Total	SHF	DHF	p Value
n	160	98	62	
Women	31	25	40	0.052
Age, yrs	66.8 ± 1.0	66.3 ± 1.3	67.7 ± 1.6	0.485
BMI, kg/m^2	22.9 ± 0.3	22.8 ± 0.4	23.1 ± 0.4	0.684
NYHA functional class ≥2	32	37	24	0.138
HT	71	61	87	0.001
DM	35	36	34	0.946
HLP	53	49	58	0.338
AF	18	17	19	0.912
Etiology				
DCM	18	30	0	
ISCM or OMI	29	44	6	
HHD	26	9	53	
VHD	26	17	40	
Medications				
ACEI or ARB	70	77	57	0.013
Beta-blocker	51	54	46	0.397
Diuretics	60	71	42	0.001
BNP, pg/ml	282 ± 23	379 ± 33	129 ± 13	<0.001

Values are mean ± SEM or %.

ACEI = angiotensin-converting enzyme inhibitor; AF = atrial fibrillation; ARB = angiotensin receptor blocker; BMI = body mass index; DCM = dilated cardiomyopathy; DHF = diastolic heart failure; DM = diabetes mellitus; HHD = hypertensive heart disease; HLP = hyperlipidemia; HT = hypertension; ISCM = ischemic cardiomyopathy; NYHA = New York Heart Association; OMI = old myocardial infarction; SHF = systolic heart failure; VHD = valvular heart disease.

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Table 2. Echocardiographic and Hemodynamic Parameters

	Total (n = 160)	SHF (n = 98)	DHF (n = 62)	p Value
FS, %	27 ± 1	20 ± 1	38 ± 1	<0.001
LVEDD, mm	57 ± 1	61 ± 1	50 ± 1	<0.001
LVMI, g/m^2	166 ± 4	179 ± 5	145 ± 6	<0.001
LAD, mm	45 ± 1	45 ± 1	44 ± 1	0.779
E/A	1.3 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	0.024
EF, %	41.5 ± 1.1	32.0 ± 0.9	56.4 ± 0.5	<0.001
LVEDVI, ml/m^2	106 ± 4	125 ± 15	76 ± 2	<0.001
LVSP, mm Hg	134 ± 3	124 ± 3	151 ± 4	<0.001
LVEDP, mm Hg	14.9 ± 0.4	15.0 ± 0.6	14.8 ± 0.5	0.829

Values are mean ± SEM.

EF = ejection fraction; E/A = ratio of peak mitral E-wave velocity to peak mitral A-wave velocity; FS = fractional shortening; LAD = left atrial dimension; LVEDD = left ventricular end-diastolic dimension; LVEDP = left ventricular end-diastolic pressure; LVEDVI = left ventricular end-diastolic volume index; LVMI = left ventricular mass index; LVSP = left ventricular peak systolic pressure. Other abbreviations as in Table 1.

found with an intraclass correlation coefficient value 0.830 (95% confidence interval 0.609 to 0.925), and absolute difference was small (mean ± SD; 0.01 ± 1.16 mm). Also, adequate M-mode images were not available in three patients, and they were excluded in the present study.

Statistical analysis. Comparisons between groups were made using chi-square analysis for proportions and unpaired Student *t* tests for continuous variables. Linearity of a relationship between two variables was assessed by linear regression analysis; $p < 0.05$ was considered significant. Results were expressed as mean ± SEM.

RESULTS

Patient characteristics. Clinical characteristics of the group of 160 patients are summarized in Table 1. Mean age was 66.8 ± 1.0 years (range 20 to 87 years), and 31% of the patients were women. In all, 98 patients had HF symptoms with an LV EF of ≤50%. These comprised the systolic heart failure group (SHF). The diastolic heart failure group (DHF) was comprised of 62 patients with preserved systolic function (LV EF >50%). Mean age and body mass index did not differ significantly between SHF and DHF groups, while there was a trend of more female patients in DHF. A history of hypertension and etiologies of dilated cardiomyopathy and ischemic cardiomyopathy/old myocardial infarction were more prevalent in SHF. Patients with SHF were more likely to be taking angiotensin-converting enzyme inhibitors or angiotensin receptor blockers and diuretics.

Geometric and functional parameters obtained by echocardiography or cardiac catheterization are shown in Table 2. In total patients, mean EF was 41.5 ± 1.1% (range 13% to 66%), and mean LVMI and LV end-diastolic volume index (LVEDVI) were 166 ± 4 g/m^2 and 106 ± 4 ml/m^2, respectively.

Correlations of plasma BNP to echocardiographic and hemodynamic parameters. Scatter plots of plasma BNP levels (dependent variable) against some echocardiographic and hemodynamic parameters (independent) are shown in Figure 1. There were strong correlations between LV EF, LVEDVI or LV end-systolic volume index, or LV EDP and plasma BNP (coefficient of correlation; $r^2 = 0.325$, 0.343, 0.421, and 0.328, respectively). There were weak correlations with parameters of transmitral Doppler flow $r^2 = 0.201$ and 0.101 for E/A and deceleration time, respectively. In contrast, LVMI and left atrial diameter did not show significant correlations with BNP levels. Although LV systolic wall stress (SWS) calculated by echocardiographic and hemodynamic parameters showed a modest correlation ($r^2 = 0.277$), a correlation of BNP with LV EDWS was much more robust ($r^2 = 0.887$).

Although age, gender, and atrial fibrillation were not significantly associated, body mass index (BMI) and New York Heart Association functional class ≥II were associated with BNP levels ($p < 0.001$ in both).

Comparison between SHF and DHF. Plasma BNP levels were significantly higher in SHF than in DHF (median [interquartile range]; 267 [136 to 583] and 105 [64 to 146] pg/ml, respectively, $p < 0.001$); however, EDP levels did not show any differences as shown in Figure 2 and Table 2. Other parameters such as SWS, EDWS, LV end-diastolic dimension, LVMI, LVEDVI, and LV peak systolic pressure were significantly higher in SHF than in DHF ($p < 0.001$). Scatter plots in patients with SHF and DHF are demonstrated in Figures 3A and 3B and Figures 3C and 3D, respectively. End-diastolic wall stress showed a better correlation with BNP ($r^2 = 0.704$) than EDP ($r^2 = 0.143$) in DHF as well as in SHF.

Subanalysis in patients without local wall motion abnormality. It is conceivable that this estimation of wall stress did not accurately reflect the entire non-uniform LV wall stress in patients with regional asynergy in LV wall motion or with variation in segmental LV wall thickness. In the present study, 83% of patients with ischemic cardiomyopathy or old myocardial infarction and 28% with dilated cardiomyopathy had regional wall motion abnormalities. Therefore, a subanalysis was performed for patients without local wall motion abnormality (n = 105). As a result, an even stronger correlation was obtained as shown ($r^2 = 0.919$). A correlation in patients with regional wall motion abnormality (n = 55) was still strong ($r^2 = 0.820$).

DISCUSSION

Heterogeneity of BNP levels among individuals with HF can cause some confusion in interpreting results. It has been unclear why some patients with LV EF <35% have BNP levels in the normal range whereas others exhibit extremely elevated levels, and why some patients with isolated diastolic dysfunction (i.e., with normal EF) show a similar increase of plasma BNP as do the patients with severe systolic dysfunction. One of the answers to the question has been the change of EDP levels in the LV (6). Another recent report has demonstrated that heterogeneity of BNP levels in patients with systolic HF reflects the severity of diastolic abnormality, right ventricular function, and mitral regurgi-

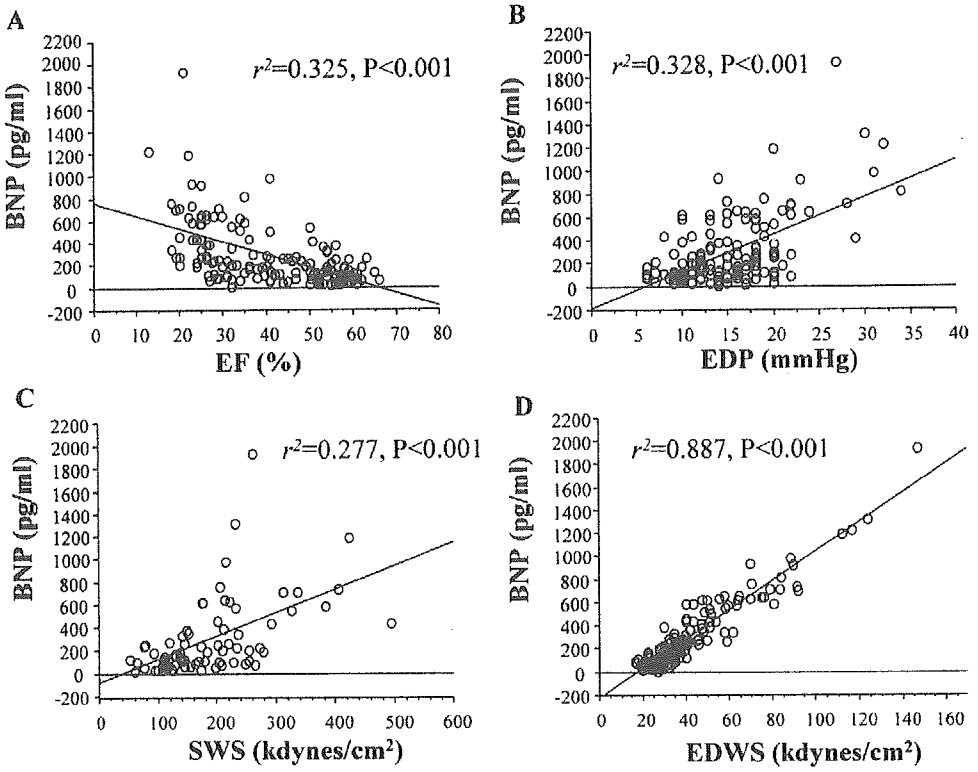

Figure 1. Correlation between B-type natriuretic peptide (BNP) and left ventricular functional parameters in all 160 patients. **(A)** Left ventricular ejection fraction (EF) (%). **(B)** End-diastolic pressure (EDP) (mm Hg). **(C)** End-systolic wall stress (SWS) (kdynes/cm^2). **(D)** End-diastolic wall stress (EDWS) (kdynes/cm^2).

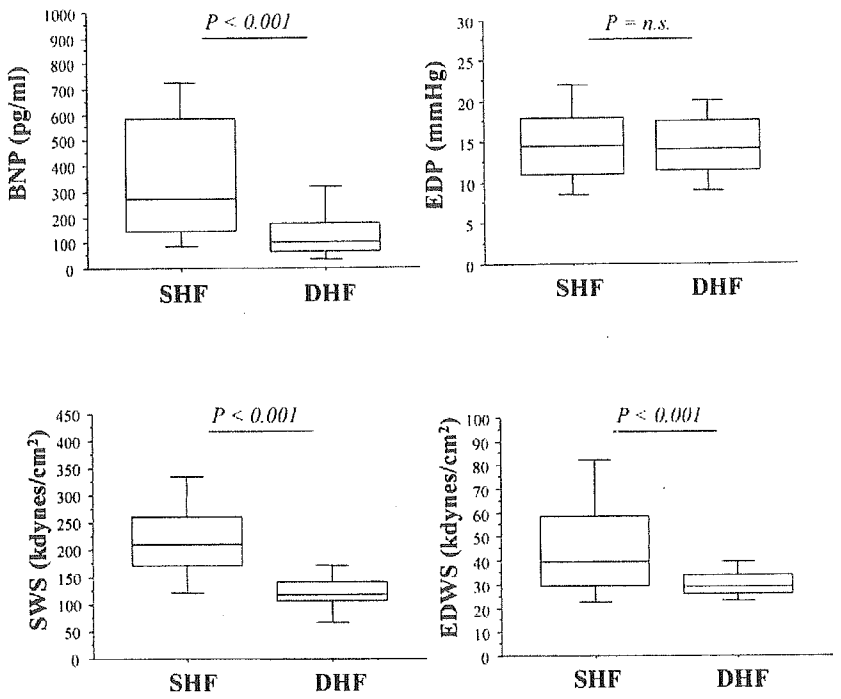

Figure 2. Differences of B-type natriuretic peptide (BNP) and left ventricular functional parameters between systolic heart failure (SHF) (n = 98) and diastolic heart failure (DHF) (n = 62). The **box** defines the interquartile range with the median indicated by the **crossbar**. The **error bars** indicate the 10th and 90th percentiles. EDP = end-diastolic pressure (mm Hg); EDVI = end-diastolic volume index (ml/m^2); EDWS = end-diastolic wall stress (kdynes/cm^2); SWS = end-systolic wall stress (kdynes/cm^2).

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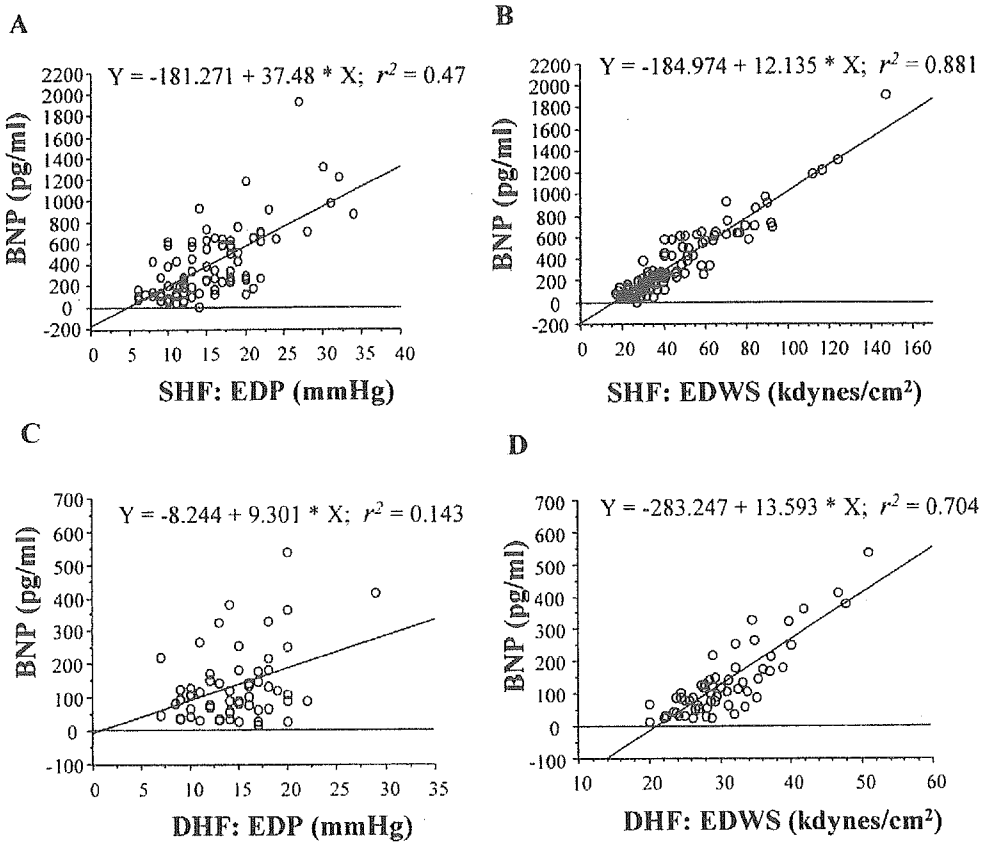

Figure 3. Correlation between B-type natriuretic peptide (BNP) and left ventricular functional parameters in 98 patients with systolic heart failure (SHF) **(A and B)** and in 62 patients with diastolic heart failure (DHF) **(C and D)**; **(A and C)** end-diastolic pressure (EDP) (mm Hg) and **(B and D)** end-diastolic wall stress (EDWS) (kdynes/cm^2).

tation in addition to LV EF, age, and renal function (7). The present study demonstrates the significance of LV EDWS in the regulation of BNP in patients with HF in general. This was true not only in patients with SHF but also with DHF. Although correlation analysis suggested a relationship between other parameters of LV geometry and function including EDP and plasma BNP levels, the correlation between LV EDWS and BNP was the most robust (r^2 = 0.887). Many studies including ours have shown that BNP levels correlate well with changes in filling pressures during tailored therapy (6,17), while O'Neill et al. (18) recently reported that plasma BNP might not correlate closely with changes in intracardiac filling pressures. In any case, plasma BNP levels are not uniform across different patients with the same LVEDP (i.e., interindividual heterogeneity), and this may be because BNP is determined more by EDWS than by filling pressure. Left ventricular EDWS might account for the wide variations that they observed in patients with HF.

The present result suggests that LV EDWS may regulate BNP secretion in humans. Indeed, experiments using cultured neonatal rat ventricular cells showed that cardiac myocytes are able to respond to mechanical stretch by increasing BNP secretion and gene expression (11). Wiese et al. (19), using isolated human myocardium, have also demonstrated that, while the isometric contraction mode did not have any influence on BNP expression, diastolic overstretch increased BNP gene expression in a time-dependent manner. This implies that diastolic stretch (i.e., preload rather than afterload) seems to be the mechanical factor responsible for the induction of BNP expression and may be the reason that in the present study LV EDWS shows a better correlation with the plasma BNP levels than does LV SWS. Furthermore, in vitro studies have implicated the contributions of local paracrine and autocrine factors in the stretch-induced BNP activation (11). Local angiotensin II was shown to play a critical role in the development of stretch-induced cardiac hypertrophy and to at least partly regulate mechanical load-induced BNP expression. Recently, in addition to stimuli such as myocyte stretching and neurohumoral activation, acute myocardial hypoxia has been reported to increase cardiac BNP gene transcription and raise the plasma proBNP concentration in an animal study (20). This mechanism may explain the increase in plasma BNP in patients with acute coronary syndromes and myocardial infarction (21). In the present study, because such patients with acute ischemia were not included, the correlation between LV EDWS and plasma BNP might actually be stronger.

Myocardial wall stress is one of the primary determinants of myocardial oxygen consumption (22). Cardiac decompensation is thought to result when the feedback loop that normalizes wall stress to abnormal loading of the heart dysfunctions. The increased wall stress may act directly or indirectly via cellular mediators such as angiotensin, endothelin, inflammatory cytokines, reactive oxygen species, and matrix metalloproteinase to orchestrate a variety of molecular and cellular remodeling events determining the structural and functional properties of the myocardium and, ultimately, the rate of disease progression (23–27). Therefore, usefulness of plasma BNP levels in predicting morbidity and mortality accurately in patients with chronic HF may be explained by the relationship between the LV EDWS and BNP. Many other factors, such as age, gender, body mass, genetics, etc., are also known to affect plasma BNP levels. However, the demonstration of the link between the hemodynamics (LV EDWS) and neurohormonal factor (BNP) may support the usefulness of BNP-guided treatment of HF. Although more randomized studies are needed, pharmacotherapy guided by BNP levels is intriguing and promising (3).

There are several methods to estimate the wall stress, and we used a formula based on M-mode echocardiographic variables (16). This method may have several limitations. For example, when there is regional asynergy in LV wall motion and variation in local LV wall thickness, the estimate may not reflect the entire non-uniform LV wall stress correctly. To test this possibility, we analyzed the data of the patients without LV asynergy demonstrated by echocardiogram and LV ventriculography. We obtained an even better correlation. Interestingly, a correlation in patients with a local wall motion abnormality was still strong (r^2 = 0.820). There are several other limitations to our study. Echocardiography and blood sampling were typically performed the day before cardiac catheterization. This time lag could have influenced the results. A further limitation is that the study population was composed of the patients who were in stable condition and could tolerate LV cardiac catheterization; thus, patients who could not bear cardiac catheterization (e.g., patients with New York Heart Association functional class IV HF) were excluded.

In the present study, we demonstrated that plasma BNP levels strongly reflect EDWS in the LV more than any other parameter previously reported. In addition, EDWS accurately accounts for the increase in plasma BNP levels even in patients with diastolic HF. The relationship of LV EDWS to plasma BNP may give a better understanding to the interindividual heterogeneity of plasma BNP levels and its clinical utility in the diagnosis and management of HF.

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CASE REPORT

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Postinfarction cardiac rupture despite immediate reperfusion therapy in a patient with severe aortic valve stenosis

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Abstract A 74-year-old woman with severe aortic valve stenosis (AS) was admitted to our hospital because of dyspnea on exertion. On day 2, she developed acute anterior wall myocardial infarction (MI) with ST elevation. Tissue plasminogen activator (tPA) was administered 10 min after the onset of chest pain, and emergency percutaneous coronary intervention was performed to induce coronary reperfusion after another 50 min. Five hours after MI onset, however, she suddenly went into electromechanical dissociation and died from cardiac rupture. This is the first case report of postinfarct cardiac rupture with severe AS occurring in spite of instituting immediate reperfusion therapy. High intraventricular pressure may be a critical risk factor for cardiac rupture in patients with AS complicated with acute MI. Further studies are required to clarify the risk and benefit of tPA administration before percutaneous coronary intervention and the necessity of the emergency correction of AS to prevent cardiac rupture.

Key words Cardiac rupture · Acute myocardial infarction · Aortic valve stenosis · Reperfusion therapy

Introduction

Cardiac rupture occurs in 1.5%–8% of patients with acute myocardial infarction (MI) and is involved in 5%–24% of in-hospital deaths due to MI.[1] The risk factors for cardiac rupture are a first transmural MI, anterior wall MI, advanced age, female gender, the absence of collaterals, a history of hypertension, and recurrent chest pain.[2–5] Here we report on a patient with severe aortic valve stenosis (AS) who developed acute MI complicated with blow-out type cardiac rupture.

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Case report

A 74-year-old woman with hypertension and diabetes was admitted complaining of increasing dyspnea on exertion. Her blood pressure was 116/85 mmHg on admission and there was no jugular venous distention or peripheral edema present. However, an S4 and a grade III systolic ejection murmur at the right second rib interspace near the right border of the sternum were audible. Electrocardiography showed a normal sinus rhythm at a rate of 82 beats/min with strain T waves in leads I, aV_L, and V_{4-6} (Fig. 1a), and a chest X-ray showed prominence of the left ventricle, with a cardiothoracic ratio of 57% and mild congestion in the upper lobes. Echocardiography also revealed severe AS, left ventricular hypertrophy, and global hypokinesia, with a fractional shortening of 21%. The estimated pressure gradient across the left ventricular outflow was 177 mmHg and the aortic valve area was 0.3 cm^2. The patient was treated with 20 mg of intravenous furosemide and soon became free from dyspnea.

On day 2, she suffered sudden chest pain while at rest. Electrocardiography and emergency echocardiography indicated anterior wall MI (Fig. 1b). Tissue plasminogen activator (tPA; monteplase, 1 600 000 units) was administered 10 min after the onset of chest pain, intravenous nitroglycerin and heparin were given, and emergency coronary angiography was started. It was subsequently determined that the proximal left anterior descending coronary artery was occluded. Percutaneous coronary intervention (PCI) was thus performed and a metallic stent (Bx Velocity Stent with Hepacoat, 3.0 × 23 mm, Cordis, Miami, FL, USA) was inserted after predilation was carried out using a same-size balloon catheter (Maverick^2 Monorail Balloon Catheter, Boston Scientific, Natick, MA, USA). Coronary flow to the left anterior descending artery was re-established 1 h after the onset of chest pain (Fig. 2), although a distal embolic

occlusion was found at the distal end of the left anterior descending artery at the end of the PCI.

The patient's systolic blood pressure was kept strictly below 120 mm Hg during and following the PCI via the infusion of intravenous nitroglycerin. Her total creatine kinase (CK), CK-MB, and CK-MB% 4 h from the onset were 4999 U/l, 238 U/l, and 4.8%, respectively. Five hours after the onset, she suddenly lost consciousness. Electrocardiography showed electromechanical dissociation, and echocardiography showed pericardial effusion with cardiac tamponade (Fig. 3). Cardiac rupture was suggested, and she underwent emergency sternotomy and open cardiac massage, while at the same time emergency percutaneous cardiopulmonary support was initiated. Despite immediate resuscitation, the patient died.

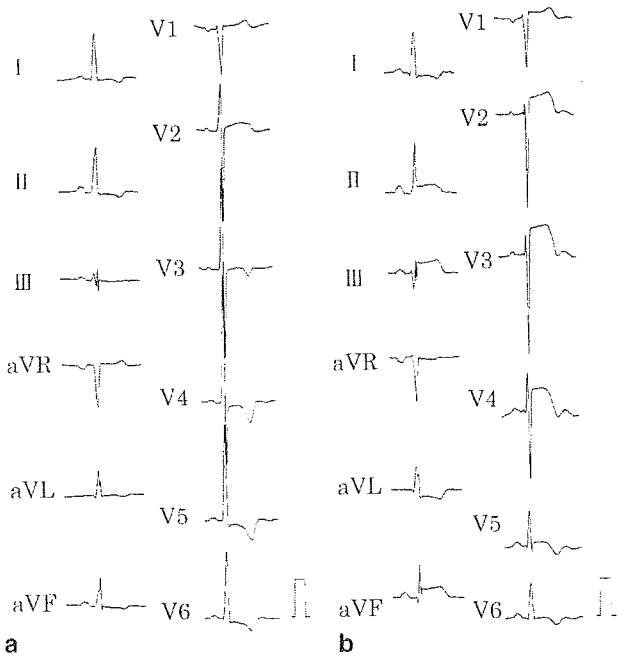

Fig. 1. Electrocardiograms of the patient. **a** The electrocardiogram on admission showed strain T waves in leads I, aV_L, and V_{4-6}. **b** ST segment elevation was observed in leads II, III, aV_F, and V_{1-5} at the onset of chest pain

Discussion

Although the patient underwent reperfusion therapy immediately after the onset of MI, she could not be rescued from catastrophic cardiac rupture, which occurred 5 h after the onset of chest pain. Blow-out rupture is characterized by the rapid development of hemodynamic collapse associated with sinus bradycardia and slow atrioventricular junctional rhythm (i.e., electromechanical dissociation), and is usually fatal. It was also difficult to keep her alive although she was subjected to full resuscitation immediately after the appearance of hemodynamic collapse.

The patient had several risk factors for postinfarct cardiac rupture such as a history of hypertension, female gender, first transmural MI, anterior wall MI, and advanced age, and these factors may have contributed to the catastrophic event. In addition to these traditional risks, she had severe AS. Several case reports have shown that postinfarct cardiac rupture occurs in patients with coexisting severe AS.[6,7] In the presence of AS, the left ventricular wall is

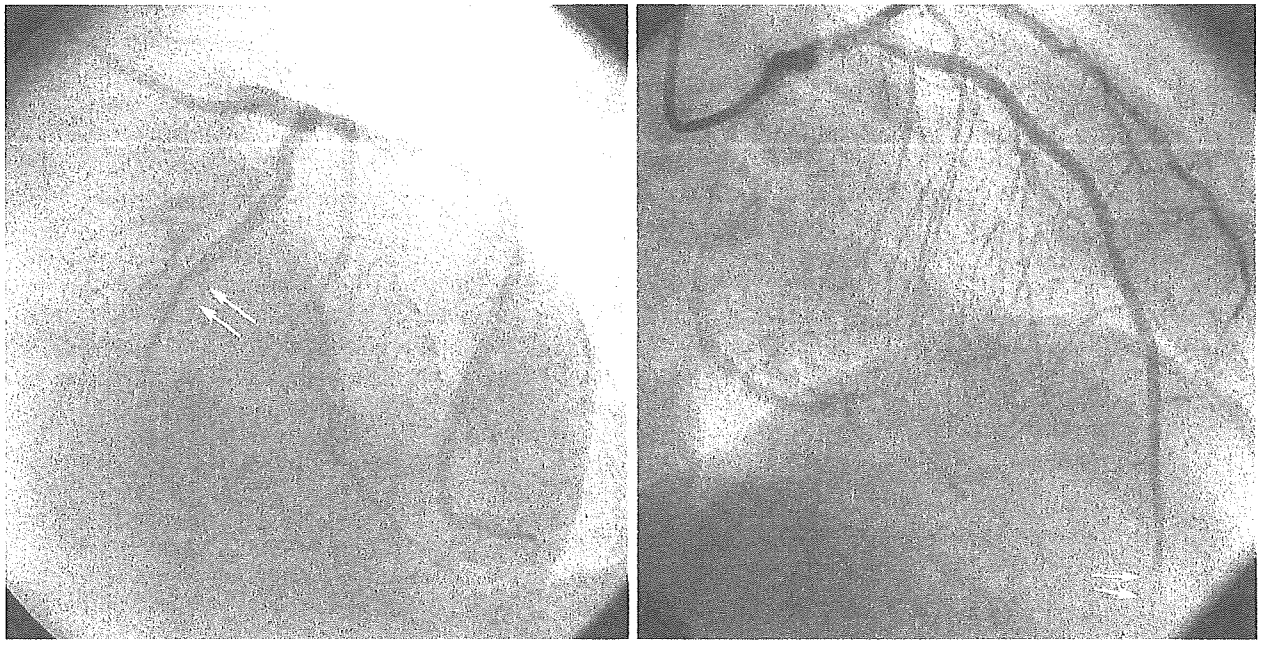

Fig. 2. **a** Coronary angiography showing an occlusion of the proximal left anterior descending coronary artery (*arrows*). **b** Coronary reperfusion was achieved in the left anterior descending artery, although a distal embolic occlusion was present (*arrows*)

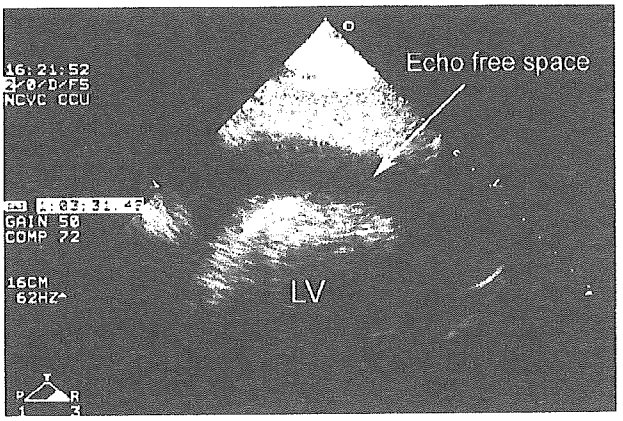

Fig. 3. Echocardiography showing pericardial effusion with findings of cardiac tamponade, indicating cardiac rupture (*arrows*). *LV*, left ventricle

subjected to an increased systolic pressure load. Furthermore, if the infarct size is small, the overall contractile strength of the left ventricle is preserved, thereby generating a high intracavitary pressure in the presence of a vulnerable infracted myocardium. High pressure in the left ventricle subsequently exhausts the infracted muscle and leads to cardiac rupture even though the peripheral blood pressure is normal. In the present case, the estimated gradient across the valve was beyond 170 mm Hg. In this situation, arterial blood pressure reduction induced by vasodilatory drugs was of no help for the compromised infarcted myocardium that had been exposed to high pressures. Cardiac rupture was inevitable even though the peripheral blood pressure had been kept strictly as low as possible during and after reperfusion therapy. Therefore, the presence of severe AS is a critical risk factor that accelerates cardiac rupture, in addition to conventional risk factors.

Generally, early recanalization reduces mortality in patients with acute MI. The PACT Trial showed that the combination therapy of short-acting reduced-dose thrombolysis and immediate planned rescue angioplasty facilitates greater LV function preservation with no significant differences in adverse events compared with primary PCI.[8] Therefore, early PCI facilitated by reduced-dose thrombolytic therapy is a beneficial and favorable strategy. On the other hand, the administration of thrombolytic drugs may increase the incidence of early cardiac rupture. In GISSI-1, the increased number of deaths during the first 6 h among patients treated with intravenous streptokinase was largely attributed to heart failure and electromechanical dissociation, and the latter was potentially a manifestation of cardiac rupture.[9] An excess of cardiac rupture events within the first 48 h was also reported in ISIS-2.[10]

Two peaks exist for the incidence of cardiac rupture after the onset of acute MI, where an early peak occurs within the first 72 h and a late peak occurs after 5–14 days.[1,11,12] Different mechanisms may be responsible for these peaks. In patients with early-phase rupture, there is hardly any thinning of the infarcted area, whereas late-phase rupture generally develops in already expanded infarcted tissue. Thrombolytic therapy may enhance the degree of early-phase rupture, although it decreases the degree of late-phase rupture and the overall death rate. The LATE study showed that, among patients treated within 12 h, the proportion of rupture deaths in the tPA group was higher than in the placebo group.[13] A large registry of these events in the United States also showed that death from cardiac rupture occurs earlier in patients treated with thrombolytic therapy, with a clustering of events within 24 h of drug administration.[14] Reperfusion may contribute to significant intramyocardial hemorrhage, which dissects through the infarcted myocardium, thus contributing to early cardiac rupture. In contrast, several studies recently found that primary direct angioplasty reduces the risk of rupture compared with thrombolysis for acute MI.[15,16] The present case had many risk factors of cardiac rupture, and the administration of tPA before PCI might have further accelerated the development of rupture no matter how early it could have been administered.

Case reports exist on patients who experienced post-MI cardiac rupture in the presence of severe AS, and who were rescued by surgical treatment.[7,17–19] However, they all had a subacute type (i.e., oozing) of cardiac rupture. No case of abrupt, catastrophic (i.e., blow-out) rupture has ever been rescued. This is the first report showing that post-MI cardiac rupture with severe AS can occur in spite of the use of immediate reperfusion therapy. Medical treatment involving immediate thrombolytic therapy followed by PCI and strict blood pressure control may have limitations in patients with severe AS. However, it remains unclear whether primary PCI alone is adequate or should be followed by the emergency correction of severe AS by aortic valve replacement or aortic valvloplasty. Further studies are thus necessary to determine an optimal treatment strategy.

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Biphasic Action of Inducible Nitric Oxide Synthase in a Hindlimb Ischemia Model

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Summary **We investigated the influence of inducible nitric oxide synthase (iNOS) on acute ischemic injury and chronic angiogenesis. In a hindlimb ischemia model, NO produced by endothelial NO synthase (eNOS) reduces ischemic injury and promotes angiogenesis. However, the effect of the large amounts of NO generated by induced iNOS is unclear. Experimental groups of mice were as follows: (1) wild-type group (Wild), (2) iNOS-knockout group (iNOS-KO), and (3) aminoguanidine-treated wild-type group (Wild + AG), which received aminoguanidine from day 0 to day 3 after ischemia. Acute ischemic injury was evaluated by measuring the plasma CK value and ischemic score. Chronic angiogenesis was evaluated by microangiography and with a non-contact type Doppler blood flowmeter on day 3. Compared with the Wild group (251 ± 34.7 IU/l), the CK value was significantly elevated in the iNOS-KO (497 ± 126.7 IU/l) and Wild + AG (587.2 ± 128.7 IU/l) groups. The ischemic score was significantly decreased in the iNOS-KO (92%) and Wild ± AG (66.6%) groups compared with the Wild group (23%). Blood flow was significantly increased in the iNOS-KO group (58.7 ± 15.3%) compared with the Wild (38.1 ± 15.9%) and Wild ± AG (43.5 ± 9.8%) groups in the chronic stage. Microangiography revealed a significantly increased number of blood vessels in the iNOS-KO (0.29 ± 0.02) group compared with the Wild (0.12 ± 0.01) and Wild + AG (0.15 ± 0.02) groups. Our findings indicate that NO generated by iNOS has a biphasic action, reducing acute ischemic injury and inhibiting angiogenesis in the chronic stage.**

Key Words: **angiogenesis, ischemia, nitric oxide synthase**

Introduction

The incidence of refractory peripheral arterial disease is increasing rapidly in developed countries [*1*]. When peripheral arterial disease becomes severe, not only is the quality of life of patients impaired, but also their prognosis is poor [*2*]. Consequently new therapies, including angiogenic treatment with vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and fibroblast growth factor-4 (FGF-4) gene transduction or bone marrow cells, have been developed, with some success [*3-8*].

NO is produced by NO synthase and has multiple bioactivities, including vasodilating, anti-platelet-aggregating

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and anti-microbial activities [*9*]. Among the three NO synthase isoforms, neuronal NO synthase (nNOS) is found in the central nerve system, and iNOS is induced in smooth-muscle cells and inflammatory cells in various diseases, such as endotoxemia or ischemia, while eNOS is found in vascular endothelial cells [*10*].

NO generally has a cytoprotective action on hindlimb ischemia [*11–14*]. During ischemic injury, eNOS is upregulated and iNOS is induced. It is reasonable that NO produced by eNOS reduces acute ischemic injury and induces angiogenesis, as it has been shown to have a vasodilatory action [*15–17*]. Induced iNOS produces large amounts of NO [*18*], but the effect of NO generated by iNOS in hindlimb ischemia remains unclear.

In this experiment, we examined the contributions of iNOS to the acute phase of ischemic injury and to angiogenesis in the chronic phase of ischemia in a mouse hindlimb ischemia model, using iNOS knockout mice and wild-type mice treated with aminoguanidine (a selective inhibitor of inducible nitric oxide synthese in macrophages)[*19–21*] in the acute phase.

Materials and Methods

Mice

All mice used in experiments were male, 2 to 3 months of age, weighing 18 to 26 g each. Wild-type (Wild) 129 SvEv mice were purchased from CLEA, Japan. iNOS –/– mice, with a mixed C57BL/6J × 129 SvEv genetic background, were obtained from Merck & Co, Inc.. INOS +/+ mice were obtained by crossing 129 SvEv mice with C57BL/6J mice twice. INOS –/– and iNOS +/+ strains have similar genetic backgrounds of 75% C57BL/6J and 25% 129/SvEv [*22*]. For the pharmacologically iNOS-inhibited group, Wild mice were given aminoguanidine (AG; Sigma, 50 mg/kg, i.p., KI value of 55 micro M and a $K_{inact\ max}$ value of 0.09 min^{-1}) [*23*] 24 hr before operation and daily for 3 days postoperatively [*24, 25*]. The animals were maintained in a pathogen-free barrier facility with a 12-hour light/dark cycle and had free access to food and water. Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and hindlimb ischemia was created by ligation of the left common iliac artery and external iliac artery and resection of the femoral artery [*26, 27*]. Mice were killed 7 days (acute phase) or 14 to 21 days (chronic phase) after surgery [*28*]. The study was approved by the Animal Care Committee of Tokai University.

Evaluation of acute ischemic injury

Serum CK value—To estimate skeletal muscle injury, CK release was estimated in the effluent collected from the infraorbitalis vein on day 3. Plasma was obtained through centrifugation of the whole blood for 10 minutes at 12000 g at 4°C. Plasma was collected and CK was assayed by SRL Co..

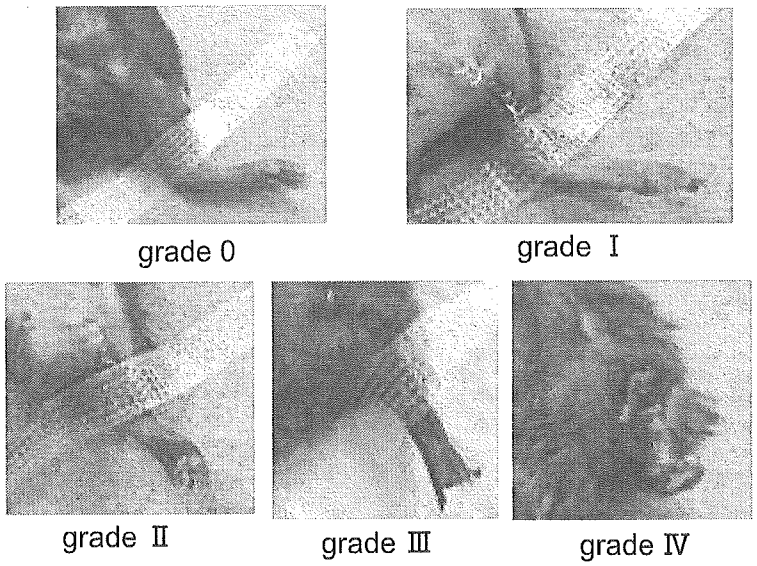
Ischemic score—On day 7, the degree of ischemic insult in the limb was macroscopically evaluated by using graded morphological scales for tissue necrosis (grade 0 to IV): grade 0: absence of necrosis: grade I, necrosis only of toes; grade II, necrosis extending to dorsum of a foot; grade III, necrosis extending to crus; grade IV, necrosis extending to a thigh or complete necrosis (Fig. 1).

Evaluation of chronic angiogenesis

Non-contact type laser Doppler measurement—We employed laser Doppler flowmetry (LDF), a non-nvasive technique for measuring tissue blood flow [*16, 29*], using a FLO-N1 device (OMEGAWAVE, Japan), which delivers light generated by a semiconductor laser diode operating at a wavelength of 780 nm, with a maximum accessible power of 3 mW. Briefly, the skin was removed so that only deep muscle blood flow would be measured, and the probe (ST-N probe, OMEGAWAVE, Japan) was placed on 4 points of the femoral muscles. Blood flow was expressed as ml/min/100 g. The contralateral hindlimb served as an internal control.

Sequential microangiography in in vivo—A PE-10 (10-gauge polyethylene) catheter was placed in the right common carotid artery of a mouse fixed on a board (1.0 mm thick) in the standing position under general anesthesia. Sequential images of the hind limb were obtained by the injection of non-ionic contrast material (1 ml/s for 2 s, Iopamidol, Nihon Schering, Tokyo, Japan) via the arterial catheter [*30*] on day 0 and day 14. Monochromatic synchrotron radiation with an energy level of 33.3 keV was obtained with a silicon crystal from beamlines NE5 and BL-14 at the High Energy Accelerator Research Organization, Tsukuba, Japan. To improve contrast resolution, subtraction images were created in the computer from the digital images obtained immediately before and during contrast material injection [*31*]. Angiogenesis was evaluated in terms of vessel density and assigned an angiographic score [*28, 32, 33*]. The ischemic signal in the acute phase is a critical factor inducing angiogenesis [*34*], and angiogenesis increases in proportion to the degree of ischemia [*35, 36*]. Therefore, angiogenesis should be compared among groups with comparable severity of acute ischemic injury. For this reason, we compared results among groups using only animals with grade I ischemic score (refer to Figure 3).

FITC gel angiography—To visualize microvessel networks, the FITC-gelatin conjugate fluorescence injection method (dialyzed FITC, 30 mg/mL conjugated gelatin solution) was employed [*37*]. Mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a PE-10 (10-gauge polyethylene) catheter was placed in the right common carotid artery. The FITC gelatin solution (20 ml) was injected into the catheter



grade	necrotic area
0	none
I	only toes
II	extending to dorsum pedis
III	extending to crus
IV	extending to thigh or complete necrosis

Fig. 1. The grading of necrosis in ischemic hindlimb. The ischemic limb was macroscopically evaluated by using a graded morphological scale for tissue necrosis area (grade 0 to IV).

(1 ml/min) and the right common carotid vein was cut. After complete perfusion, the left leg were resected and immediately fixed in ice-cold graded paraformaldehyde (4%). A confocal laser scanning microscopy (CLSM) system (LSM-410, Carl Zeiss, Jena, Germany), equipped with a 488-nm argon laser (for FITC), was employed on thick sections (1–2 mm) to visualize microvessel networks in detail [*38*]. After computer-assisted 3-D imaging of microvessel networks by the CLSM system, the images were stored on hard disk memory or a magnetic optical disk, EDM-230C (Sony, Tokyo, Japan) and were printed with a digital Pictrostat 400 (Fuji Film Co/Ltd., Tokyo, Japan).

Statistical Analysis

Data are presented as mean values ± SD. Differences were assessed by using one-way ANOVA with Tukey's post test.

Results

Acute ischemic injury

Serum CK value—Firstly, we measured serum CK value to evaluate the acute ischemic injury in the three experimental groups. In the control (Wild) group, the serum CK value was 251 ± 34.7 IU/l. The serum CK values in the Wild + AG group (587.2 ± 128.7 IU/l) and iNOS-KO group (497 ± 126.7 IU/l) were significantly higher than that in the Wild group (Figure 2).

Ischemic score on day 7—The ischemic scores in the iNOS-KO group (92%) and the Wild + AG group (66.6%) were significantly higher than that in the Wild group (23%) (Figure 3). Percentages of grade I in the Wild, iNOS-KO and Wild + AG groups were 23%, 25% and 33%, respectively.

Chronic angiogenesis

Laser Doppler (non contact type) measurement—The

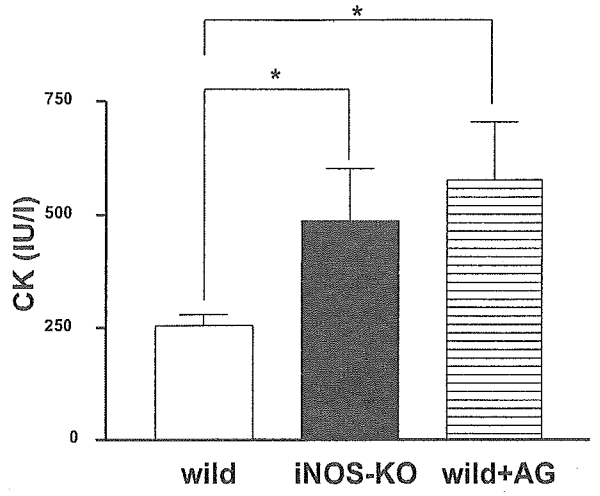


Fig. 2. Serum CK value in acute hindlimb ischemia. Open bar, control group; closed bar, iNOS-knockout (iNOS-KO) group; hatched bar, aminoguanidine-treated group (Wild + AG). The CK values in the iNOS-KO group and Wild + AG group were significantly higher than that in the control (Wild) group (*p<0.05).

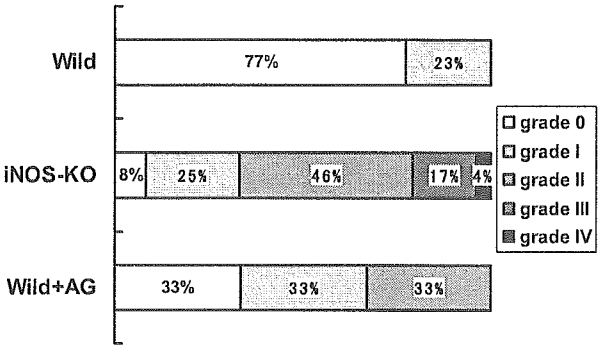


Fig. 3. Ischemic score in acute hindlimb ischemia. Open column, grade 0; dotted column, grade I; vertically lined column, grade II; hatched column, grade III; cross lined column, grade IV.

blood flow at the ischemic lesion was significantly reduced in all three groups on day 3 after surgery. However, at post-operative day 14, it was significantly higher in the iNOS-KO group (58.7 ± 8.7%) than in the Wild group (38.1 ± 5.2%) or the Wild + AG group (43.5 ± 6.4%) (Figure 4).

Sequential microangiography in in vivo—No vessels were apparent in hind limb angiography on day 0, and fine vessels were barely visible on day 14 in the Wild group (Figure 5 A and B). In contrast, many vessels were supplying the hind-limb on the injured side on day 14 in the iNOS-KO group. The angiogenic score on day 14 was significantly increased in the iNOS-KO group (0.29 ± 0.02) compared with that in

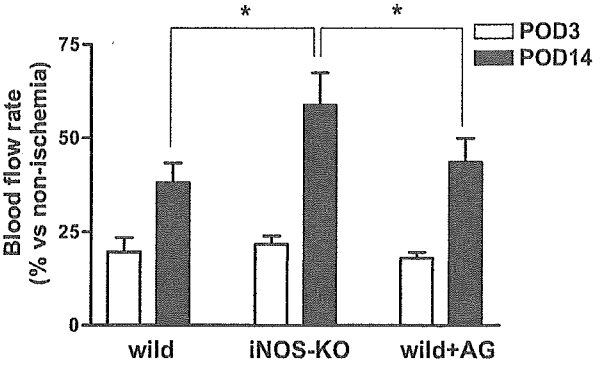


Fig. 4. Blood flow rate in non-contact laser-Doppler flowmetry. Open bar, blood flow ratio on day 3. Closed bar, blood flow ratio on day 14. The blood flow ratio on day 14 in the iNOS-KO group was significantly higher than in the Wild group or Wild + AG group (*p<0.05).

the Wild group or the Wild + AG group (0.12 ± 0.01 and 0.15 ± 0.02, respectively) (p<0.05) (Figure 5).

FITC angiography—The presence of fine vascular networks in the iNOS-KO group (Fig. 6B) implies that marked angiogenesis had occurred. There was a distinct difference in induction of vascular networks between the iNOS-KO group and the Wild and Wild + AG groups (Figure 6 A,B,C).

Discussion

In this experiment, ischemic injury was severe, but angiogenesis was markedly greater in the iNOS-KO group than in the Wild + AG or Wild group. The results indicate that NO generated by iNOS inhibited acute ischemic injury, but reduced angiogenesis in the chronic stage of ischemia.

Of the three NO synthese isoforms, nNOS is mainly localized in the central nervous system, postsynaptic density (PSD), and muscular sarcolemma (muscle fiber myelin) and participates in neural transmission [*39-41*]. iNOS is usually not expressed, but is induced in vascular smooth muscle cells and macrophages *via* cytokine stimulation during sepsis or ischemia with or without reperfusion, and produces large quantities of NO [*42*]. eNOS is mainly localized in vascular endothelial cells and produces NO continuously in response to shear stress, playing important roles in platelet aggregation and vasodilation [*12, 17*]. So, it appears reasonable that NO inhibits acute ischemic injury. In contrast, many studies have shown that NO production by iNOS aggravates injury in the hindlimb ischemia model. Nevertheless, we found that iNOS reduced ischemic injury in the acute stage in the present experiment. A key difference between our study and the others is the presence or absence of reperfusion following the ischemic period. We have

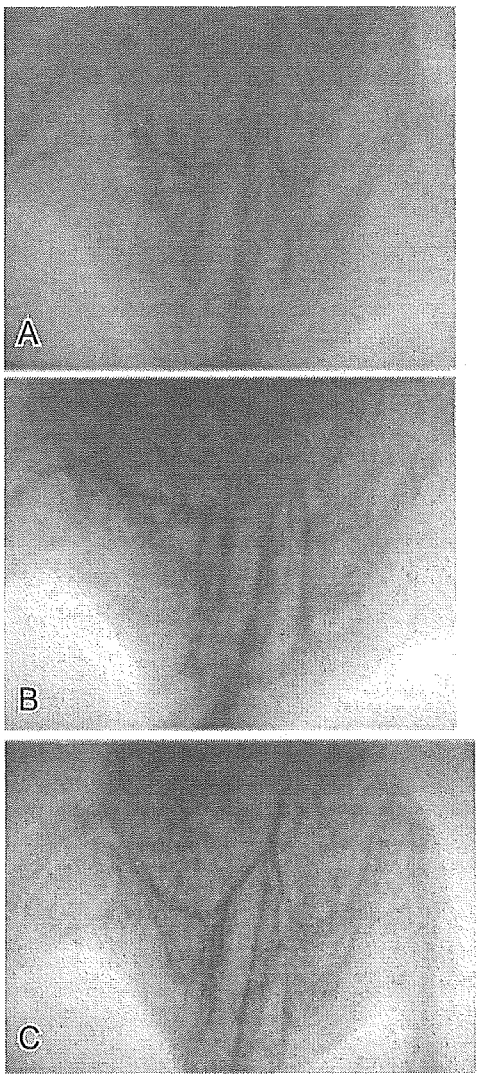


Fig. 5. Representative microangiograms. A : angiogram taken on day 0 in an animal of the Wild group ; B : angiogram taken on day 14 in an animal of the Wild group ; C : angiogram taken on day 14 in an animal of the iNOS-KO group.

already reported that superoxide ($O_2^{\cdot-}$) is produced in ischemic tissue at the time of reperfusion, and reacts with NO to form peroxynitrite [*43*]. Peroxynitrite is a potent oxidant that directly oxidizes sulfhydryl groups at a 1000-fold greater rate than hydrogen peroxide. It inhibits the function of various enzymes, including components of the mitochondrial electron transport chain. In our experiment, we examined ischemia without reperfusion, so that $O_2^{\cdot-}$ (and hence peroxynitrite) would not be produced, and only NO was present.

A second difference from previous experiments is that we used mice treated with iNOS inhibitor in the acute stage, as well as iNOS knockout mice, to examine the effect of iNOS [*44*]. It is noteworthy that one study in which iNOS knockout mice were used and reperfusion was not performed (similar to our protocol) found that injury was severe and angiogenesis in the chronic stage was augmented [*45*]. This is consistent with our results, and indicates that reperfusion plays a critical role in the outcome [*46*].

As the ischemic signal in the acute phase is a critical factor inducing angiogenesis [*34*], and angiogenesis increases in proportion to the degree of ischemia [*35, 36*], angiogenesis has to be compared among groups with comparable severity of acute ischemic injury. We therefore selected animals with grade I ischemic score in all cases for comparison among groups. Aminoguanidine was administered for only three days in the Wild + AG group in order to allow iNOS to function in the chronic stage. At corresponding levels of acute ischemia, angiogenesis in the chronic stage was obviously enhanced in the iNOS-KO group in comparison with the Wild + AG group, i.e., angiogenesis in the chronic stage was inhibited by the function of iNOS.

Many reports indicate that iNOS enhances angiogenesis in various neoplastic disease models [*44, 47–49*]. However, factors secreted by the cancer cells may play important roles in these models. It is important to note that our results showing a biphasic action of iNOS depended on the use of both an acutely iNOS inhibitor-treated wild-type group and an iNOS knockout group in an ischemic model. The mechanism underlying the inhibitory action of NO appears to be down-regulation of the VEGF receptor [*50*]. Possible compensatory roles of eNOS and nNOS in iNOS knockout mice have been ruled out by a previous study, in which their expression was shown to remain unchanged [*51*].

In summary, we have shown that iNOS reduces acute ischemia, but inhibits angiogenesis in a hindlimb ischemia model. Thus we suggest to use iNOS inducer or agents to increase NO production such as arginine with acute phase and supplement iNOS inhibitor in chronic stage. However these remained to be examined prior to clinical trial.

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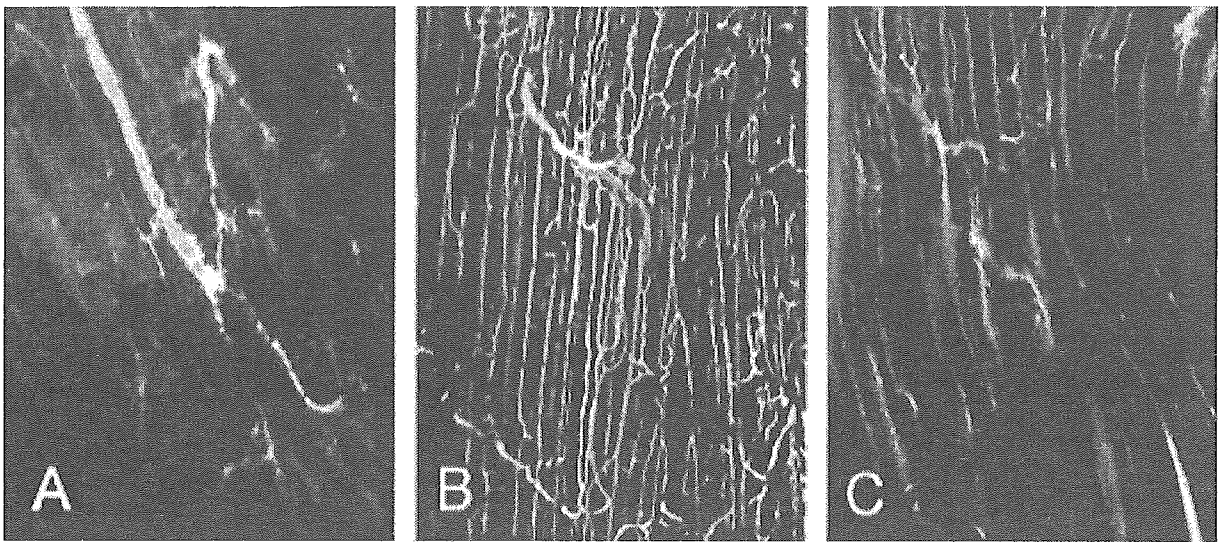

Fig. 6. FITC angiography. FITC angiograms were evaluated on day 14. A: in an animal of the Wild group; B: in an animal of the iNOS-KO group; C: in an animal of the Wild + AG group. Clear angiogenesis was visualized in the iNOS-KO group compared with the control group and aminoguanidine-treated group.

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