MMP-2 KO mice. These results indicate that MMP-2 suppressed the development of inflammatory disease. Therefore, TNF-α overexpression and MMP-2 deletion might synergistically activate the cellular inflammatory response in the heart, which could further damage cardiac function.

The present study could not demonstrate the long-term beneficial effects of MMP-2 deletion on the decline in LV systolic function in TNF-α-induced cardiomyopathy. These findings are in contrast to those from a previous study, in which broad-spectrum MMP inhibition could ameliorate myocardial dysfunction in TNF-α TG mice (18). The MMP inhibitor batimastat was found to reduce myocardial hypertrophy and diastolic dysfunction. Even though it is difficult to compare the two studies directly because of the broad-spectrum action of the MMP inhibitor used by Li et al. (18), these studies suggest that MMP-2 has a complex role in maintaining the physiological function of the heart, and information about the action of individual MMPs with the use of such an inhibitor is limited. The most effective way to evaluate the contribution of the specific MMP and obtain direct evidence for a role of MMP in heart failure is through gene manipulation, as employed in the present study. Whatever the mechanisms are, MMP-2 could protect the heart by inhibiting acceleration of interstitial inflammatory infiltration in this model. The disparity between selective and nonselective inhibition of MMP-2 may have important implications in the development of pharmacological agents for the treatment of heart failure. These findings may further draw attention to treatment of heart failure by using the nonselective, broad-spectrum MMP-2 inhibitor.

There are several limitations to be acknowledged in this study. *1*) We examined only female mice for physiological, pathological, and biochemical analyses, because most male TG mice died before 12 wk of age, when these analyses were performed. As a result, we are not certain whether selective disruption of the MMP-2 gene exacerbates myocardial inflammation and dysfunction even in male TNF-α TG mice. *2*) Even though in vivo assessment of LV function with echocardiography is feasible and reproducible in the mouse, it might be difficult to interpret the indexes in dilated LV. However, our validation study has shown that intra- and interobserver variabilities of our echocardiographic measurements for LV cavity dimensions and fractional shortening were small and that measurements in the same animals on separate days were highly reproducible (24). Therefore, our technique could be considered to allow for a noninvasive assessment of LV structure. *3*) The heart rates in the present study (450–470 beats/min) were lower than those measured in conscious mice (600 beats/min). Therefore, the LV size and function results might be greatly influenced by differences in anesthetic regimens and experimental conditions, such as heart rate. *4*) Although the present study employed the conventional gelatin zymography method to analyze MMP activities, as in a previous study (8), two-dimensional zymography is essential, especially to ascertain the existence of plasmin in the 66-kDa MMP complex (28). *5*) We assessed TGF-β, which has been well established to be involved in ECM remodeling in heart failure, in the present study. Further studies on TGF-α are also needed, because it plays an important role in cell proliferation and differentiation.

In summary, selective disruption of the MMP-2 gene exacerbated myocardial inflammation and dysfunction in TNF-α-induced cardiomyopathy. Thus myocardial expression of MMP-2 may play a protective role in the development of congestive heart failure in cytokine-induced cardiomyopathy.

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Rho-Kinase Inhibitor Improves Increased Vascular Resistance and Impaired Vasodilation of the Forearm in Patients With Heart Failure

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Rho-Kinase Inhibitor Improves Increased Vascular Resistance and Impaired Vasodilation of the Forearm in Patients With Heart Failure

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Background—Rho-kinase is suggested to have an important role in enhanced vasoconstriction in animal models of heart failure (HF). Patients with HF are characterized by increased vasoconstriction and reduced vasodilator responses to reactive hyperemia and exercise. The aim of the present study was to examine whether Rho-kinase is involved in the peripheral circulation abnormalities of HF in humans with the Rho-kinase inhibitor fasudil.

Methods and Results—Studies were performed in patients with HF (HF group, n=26) and an age-matched control group (n=26). Forearm blood flow was measured with a strain-gauge plethysmograph during intra-arterial infusion of graded doses of fasudil or sodium nitroprusside. Resting forearm vascular resistance (FVR) was significantly higher in the HF group than in the control group. The increase in forearm blood flow evoked by fasudil was significantly greater in the HF group than in the control group. The increased FVR was decreased by fasudil in the HF group toward the level of the control group. By contrast, FVR evoked by sodium nitroprusside was comparable between the 2 groups. Fasudil significantly augmented the impaired ischemic vasodilation during reactive hyperemia after arterial occlusion of the forearm in the HF group but not in the control group. Fasudil did not augment the increased FVR evoked by phenylephrine in the control group significantly.

Conclusions—These results indicate that Rho-kinase is involved in increased FVR and impaired vasodilation of the forearm in patients with HF. (*Circulation*. 2005;111:2741-2747.)

Key Words: heart failure ■ blood flow ■ vasoconstriction ■ vasodilation

Increased peripheral vascular resistance and impaired vasodilation of the peripheral vasculature are characteristic in patients with heart failure (HF).[1,2] These characteristics cause fatigue and exercise intolerance in patients with HF and are considered to be mainly due to enhanced sympathetic drive and activation of the renin-angiotensin system.[1–3] The dysfunction of vasodilator factors such as nitric oxide and atrial natriuretic peptide is also involved.[4–10] Ischemia- and exercise-induced vasodilation of the extremities of patients with HF is markedly attenuated, and decreased exercise tolerance in patients with HF is not only due to impaired pump function of the heart but also to inadequate increases in muscle blood flow as a result of impaired vasodilation during exercise.[2,5] Increased peripheral resistance and impaired vasodilation are consistent findings in HF.[1,2,5–8] The molecular mechanisms underlying impaired vasodilation in patients with HF, however, remain to be elucidated.

Myosin light chain (MLC) phosphorylation is a crucial step for vascular smooth muscle cell (VSMC) contraction, which is regulated in a dual manner by MLC kinase and MLC phosphatase.[11] Inhibition of MLC phosphatase results in increased MLC phosphorylation and subsequent VSMC hypercontraction (Ca^{2+} sensitization).[11,12] This mechanism of Ca^{2+} sensitization in VSMCs is enhanced in animal models of HF.[13] Rho-kinase/ROKα/RockII, which is activated by the small GTPase Rho, inhibits MLC phosphatase activity and thus plays a key role in Ca^{2+} sensitization and hypercontraction of VSMCs.[11,12,14] Y-27632, a Rho-kinase inhibitor, preferentially lowers arterial pressure in rat models of hypertension in vivo, which indirectly suggests an involvement of Rho-kinase in hypertension.[15] We recently demonstrated that Rho-kinase is upregulated and plays a key role in VSMC contraction in a porcine model of coronary artery spasm[16,17] and in spontaneously hypertensive rats[18] and that Rho-kinase might be involved in the pathogenesis of increased peripheral vascular resistance in hypertension in humans.[19] Previous reports suggest that the Rho/Rho-kinase pathway plays a critical role in Dahl salt-sensitive hypertensive rats with congestive HF[20] and might be involved in the enhanced arterial vasoconstriction in tachycardia-induced HF in dogs.[13]

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It is unknown, however, whether this is also the case in human patients with HF.

The present study was designed to determine whether Rho-kinase is involved in the increased peripheral vascular resistance in patients with HF by examining the vasodilator effect of a specific Rho-kinase inhibitor, fasudil. Fasudil is currently used in Japan for the treatment of cerebral vasospasm after subarachnoid hemorrhage.[21] Fasudil is a specific Rho-kinase inhibitor, as is Y-27632,[15,16,22,23] although the latter is not yet approved for human use. We recently used fasudil to examine forearm vascular resistance in patients with hypertension, and there were no complications.[19] Thus, fasudil is regarded as a specific Rho-kinase inhibitor that can be used safely in humans.

Methods

Patients

Twenty-six patients with HF (HF group; 17 men, 9 women; mean age 63±3 years) and 26 control subjects (control group; 15 men, 11 women; mean age 60±6 years) were enrolled in the present study. The HF group comprised 17 patients with ischemic heart disease, 8 patients with dilated cardiomyopathy, and 1 patient with valvular heart disease. All patients with HF were diagnosed according to the Framingham criteria.[24] Physical activity was determined on the basis of the New York Heart Association (NYHA) functional class. There were 14 patients with NYHA class 3 and 12 patients with NYHA class 2 in the HF group. Systemic hypertension was defined as systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg. Hyperlipidemia was defined as serum total cholesterol >220 mg/dL or serum triglyceride levels >150 mg/dL. Diabetes mellitus was defined as fasting blood sugar >110 mg/dL. Left ventricular ejection fraction was determined by the modified Simpson method or single-plane area-length method on echocardiogram. We defined the control group as a non-HF/nonhypertensive group. Subjects in the control group were admitted to our hospital because of atypical chest pain, fatigue, or palpitations. Careful examination was performed to rule out coronary artery disease (by coronary angiography) and other organic heart diseases (echocardiogram) or arrhythmia (Holter ECG or ECG monitoring during hospitalization and/or electrophysiological study). In the control group, 5 subjects had serum total cholesterol levels >220 mg/dL, and 4 had serum triglyceride levels >150 mg/dL. Serum total cholesterol levels were <250 mg/dL, and serum triglyceride levels were <180 mg/dL, even in the HF group. Six subjects had a history of smoking; however, they had quit smoking before admission. This was also true in the HF group. Some subjects in the control group transiently received some medications, such as ACE inhibitors, an angiotensin II (Ang II) receptor blocker, or a β-blocker, from general practitioners because of mild high blood pressure and/or palpitations after our careful interviews. The subjects, however, did not take those medications continuously, and we confirmed that they did not have HF, hypertension, or arrhythmias on admission. In contrast, patients in the HF group were given those medications because of HF. It was ethically difficult to discontinue those medications for the purpose of the study. Therefore, the medications were discontinued only on the day of the study, and restarted just after the study. The present study was approved by the ethics committee for human research in our institute, and written informed consent was obtained from each subject.

General Procedures

The present study was performed with subjects in a supine position and in a postabsorptive state with the room temperature at 25°C to 27°C. All medications were withheld on the day of the study. With the subjects under local anesthesia, the left brachial artery was cannulated with a 20-gauge intravascular cannula for drug infusion, and the cannula was connected to a pressure transducer for direct measurement of arterial pressure. The antecubital vein was cannulated, and blood samples were obtained for measurements of serum or plasma chemistry, including plasma brain natriuretic peptide (BNP), Ang II, and norepinephrine (NE).

Measurement of Forearm Blood Flow

Forearm blood flow (FBF) was measured with a strain-gauge plethysmograph with the venous-occlusion technique, as reported previously.[5,19,25–29] FBF (mL · min^{-1} · 100 mL^{-1} of forearm volume) was calculated from the rate of increase in forearm volume while venous return from the forearm was prevented by inflation of a cuff on the upper arm. The pressure in the venous-occlusion or congesting cuff was 40 mm Hg. Circulation to the hand was arrested by inflation of a cuff around the wrist. An average of 4 measurements made at 15-second intervals was used for later analysis. Forearm vascular resistance (FVR) was calculated by dividing mean arterial pressure (diastolic pressure plus one third of pulse pressure in mm Hg) by FBF. FVR was expressed as units.

Study Protocol 1: Effect of Fasudil on FBF and FVR in Patients With HF and in Control Subjects

FBF, arterial pressure, and heart rate were measured at rest and during administration of graded doses of fasudil (3.2, 6.4, 12.8, and 25.6 μg/min) or sodium nitroprusside (SNP; 0.4, 0.8, 1.6, and 3.2 μg/min).[19] Each dose of fasudil was infused for 15 minutes, and FBF was measured during the last minute of each infusion. Each dose of SNP was infused for 5 minutes, and FBF was measured after each infusion. Venous blood samples were drawn from the antecubital vein before and immediately after infusion of the peak dose of fasudil (25.6 μg/min) and at the end of the study for determination of the plasma fasudil concentration.

Study Protocol 2: Effects of Fasudil on FBF and FVR Responses to Reactive Hyperemia in Patients With HF and Control Subjects

We studied 8 patients with HF and 9 control subjects. FBF and FVR were measured as described in study protocol 1. To induce reactive hyperemia, FBF was occluded by inflation of a cuff placed over the left upper arm to a pressure of 200 mm Hg for 5 minutes. After the ischemic cuff occlusion was released, FBF was measured every 15 seconds for 3 minutes. Ten minutes after FBF returned to baseline values, fasudil (25.6 μg/min) was infused for 15 minutes, and the FBF responses to reactive hyperemia were measured by the same procedures described above.

Study Protocol 3: Effects of Fasudil on FBF and FVR During Infusion of Phenylephrine in Control Subjects

To exclude the possibility that baseline differences in FBF might affect the results, we examined the FBF responses evoked by fasudil before and after infusion of phenylephrine (400 ng/min) in control subjects (n=5). FBF, arterial pressure, and heart rate were measured at rest and during administration of graded doses of fasudil (3.2, 6.4, 12.8, and 25.6 μg/min) or SNP (0.4, 0.8, 1.6, and 3.2 μg/min) as described in study protocol 1.

Drugs

The following drugs were used: fasudil hydrochloride hydrate (Eril; Asahi Kasei Pharmaceutical Corporation), SNP (Nitpro Inj; Maruishi Pharmaceutical Co), and phenylephrine hydrochloride (Neosynesin; Kowa Company, Ltd). All drugs were dissolved in physiological saline immediately before use.

Statistical Analysis

All results are expressed as mean±SEM. Values at rest were compared by unpaired *t* test. Responses to graded doses of drugs in each group were examined by ANOVA for repeated measures. Two-way ANOVA was used to compare FBF, FVR, and reactive hyperemia responses in the 2 groups. The relationship between

Clinical Profiles and Baseline Characteristics of HF Group and Control Group

	HF Group (n=26)	Control Group (n=26)
Age, y	63±3	60±6
Male/female, n	17/9	15/11
BMI, kg/m^2	24±3	25±2
HT/DM/HL, n	0/0/12	0/0/9
LVEF, %	37±4*	62±3
BNP, pg/mL	364±120*	30±13
Ang II, pg/mL	39±6*	18±6
NE, pg/mL	492±193*	190±84
Total cholesterol, mg/dL	208±33	190±29
LDL cholesterol, mg/dL	142±14	130±22
FBS, mg/dL	98±15	89±14
MBP, mm Hg	85±13	86±14
HR, bpm	89±6*	68±8
FBF, mL · min^{-1} · 100 mL^{-1}	3.8±0.4*	5.2±0.6
FVR, U	39±11*	20±4
Fasudil, nmol/L	560±60	526±86

n indicates number of patients or subjects; BMI, body mass index; HT, hypertension; DM, diabetes mellitus; HL, hyperlipidemia; LVEF, left ventricular ejection fraction; FBS, fasting blood sugar; MBP, mean blood pressure; and HR, heart rate.

Data are presented as mean±SEM.

*P<0.05 vs control group.

plasma BNP or Ang II and FBF or FVR on the maximum dose of fasudil was examined with a linear regression analysis with Pearson correlation coefficients. A P value of <0.05 was considered to be statistically significant.

Results

Baseline Characteristics

There was no significant difference in mean blood pressure at rest between the HF and control group (Table). Resting heart rate was significantly higher in the HF group than in the control group (89±6 versus 68±8 bpm, P<0.01; Table). Basal FBF was significantly lower in the HF group than in the control group (3.8±0.4 versus 5.2±0.6 mL · 100 mL^{-1} · min^{-1}, P<0.05; Table). Basal FVR was significantly higher in the HF group than in the control group (39±11 versus 20±4 U, P<0.05; Table). The 2 groups were comparable in age, gender, body mass index, serum total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, fasting blood sugar, and previous smoking habit (Table). Compared with the control group, the average left ventricular ejection fraction was significantly lower and the average plasma BNP level and plasma concentrations of Ang II and NE were significantly higher in the HF group (Table).

Plasma Concentrations of Fasudil

Just after the peak dose of fasudil, plasma fasudil concentrations significantly increased in both groups; the levels were comparable between groups (560±60 versus 526±86 nmol/L, respectively; Table).

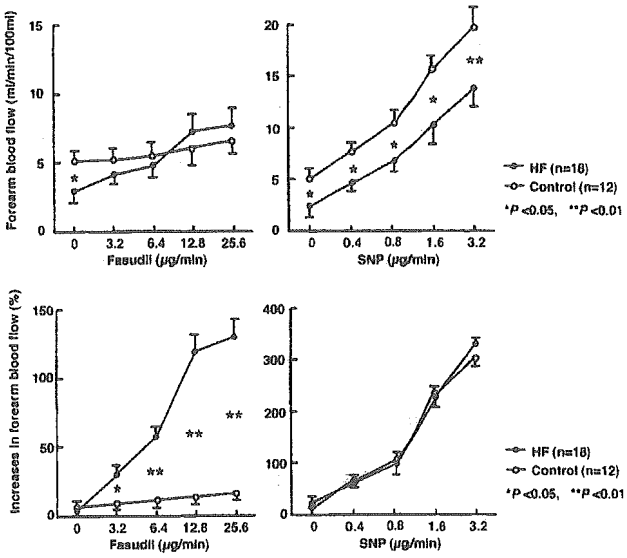

Figure 1. Plots showing responses of FBF to fasudil and SNP as expressed in absolute values (top) and percent change (bottom) in HF group and control group. Results are expressed as mean±SEM.

Forearm Vascular Responses to Fasudil and SNP

Fasudil evoked significant dose-dependent increases in FBF in the HF group but not in the control group (Figure 1). The increase in both the absolute FBF and percent change were apparent only in the HF group (Figure 1). In contrast, SNP induced comparable increases in FBF in the 2 groups (Figure 1). Fasudil evoked significantly greater decreases in both the absolute FVR value and percent change in the HF group compared with the control group, thus normalizing FVR in those patients (Figure 2). In contrast, SNP induced comparable decreases in FVR in both groups (Figure 2). Systemic arterial blood pressure and heart rate did not change signifi-

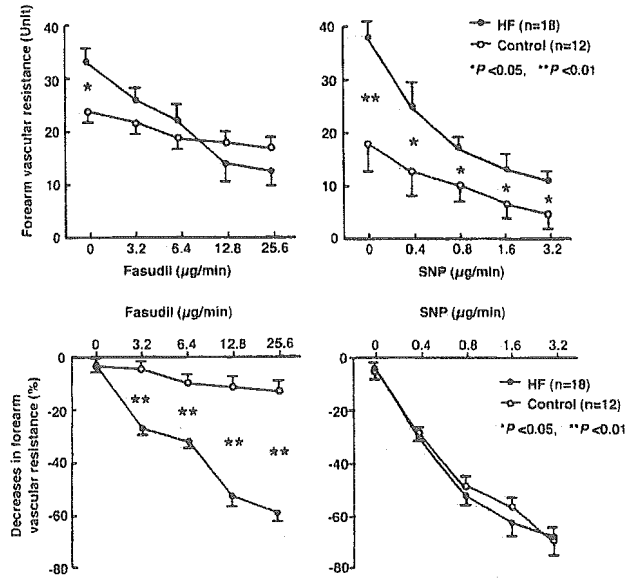

Figure 2. Plots showing responses of FVR to fasudil and SNP as expressed in absolute values (top) and percent change (bottom) in HF group and control group. Results are expressed as mean±SEM.

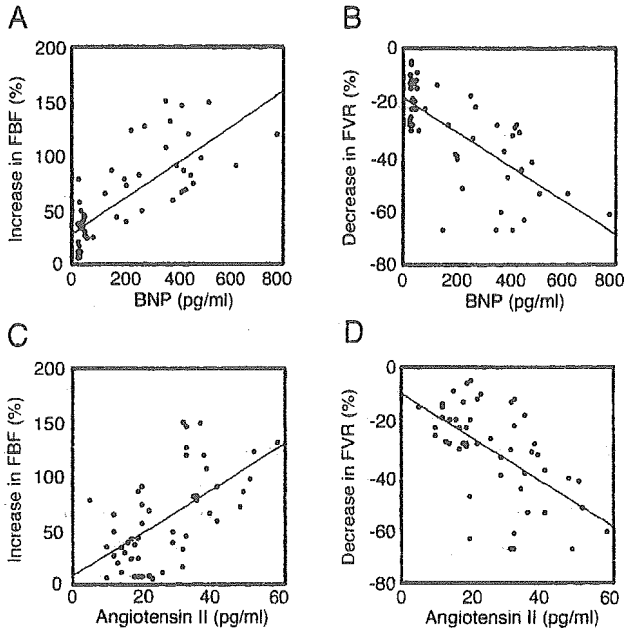

Figure 3. Relation between plasma levels of BNP and increase in FBF (A), plasma levels of BNP and decrease in FVR (B), plasma levels of Ang II and increase in FBF (C), and plasma levels of Ang II and decrease in FVR (D).

cantly during intra-arterial infusion of fasudil or SNP in either group. With the maximum dose of fasudil, there was a significant positive correlation between BNP and the increase in FBF (r=0.6, P=0.002; Figure 3A), and there was a significant negative correlation between BNP and the decrease in FVR (r=−0.45, P=0.006; Figure 3B). Moreover, with the maximum dose of fasudil, there was a significant positive correlation between plasma levels of Ang II and the increase in FBF (r=0.54, P=0.001; Figure 3C), and there was a significant negative correlation between plasma levels of Ang II and the decrease in FVR (r=−0.58, P=0.005; Figure 3D). There was, however, no significant correlation between baseline FBF and maximal forearm vasodilation in HF patients (r=0.15, P=0.548).

Effects of Fasudil on Response of FBF to Reactive Hyperemia

In the HF group, the response to reactive hyperemia was significantly lower than in the control group before the infusion of fasudil. After the infusion of fasudil, in the HF group, the response to reactive hyperemia was augmented to the levels of the control group (Figures 4A and 4C). In the control group, fasudil did not change the response to reactive hyperemia (Figures 4B and 4D).

Effects of Fasudil on FBF and FVR During Infusion of Phenylephrine in Control Group

Phenylephrine (400 ng/min) decreased FBF and increased FVR in the control group to levels comparable to those of the HF group. Fasudil did not significantly change FBF and FVR in the control subjects before or after infusion of phenylephrine (Figure 5). SNP induced comparable increases in FBF and decreases in FVR before and after infusion of phenylephrine (Figure 5).

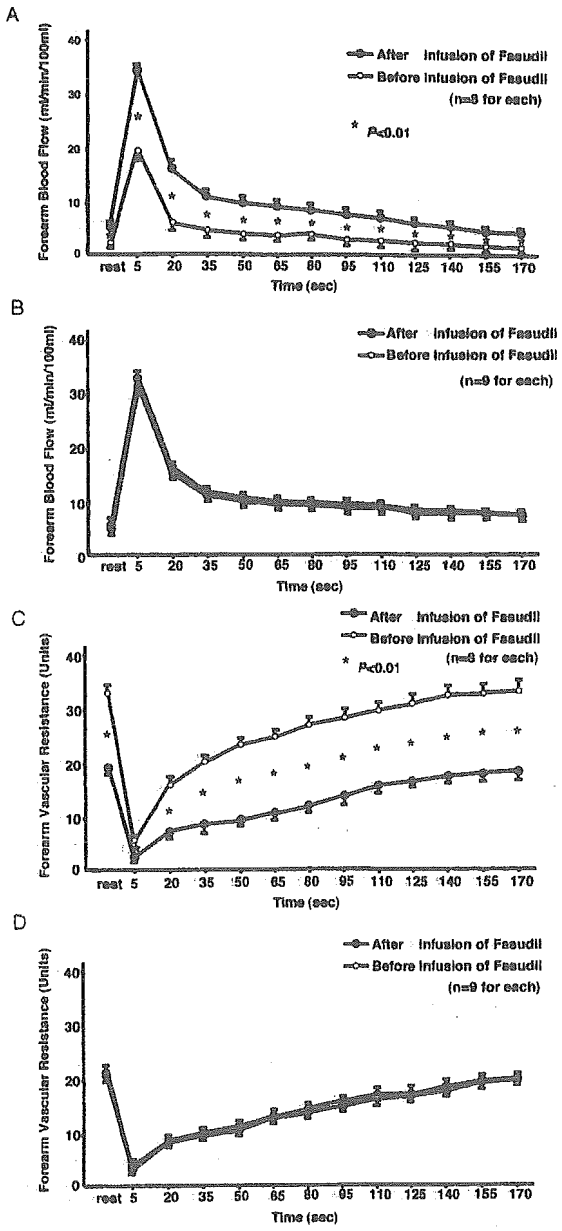

Figure 4. FBF at rest and during reactive hyperemia in HF group (A) and in control group (B) before and after infusion of fasudil. FVR at rest and during reactive hyperemia in HF group (C) and control group (D) before and after infusion of fasudil. Results are expressed as mean±SEM. Probability value refers to comparison of time-course curves with ANOVA for repeated measurements.

Discussion

The major findings of the present study were that (1) the forearm vasodilator response to fasudil was significantly greater in the HF group than in the control group, whereas SNP induced a similar forearm vasodilator response in the 2 groups, and (2) fasudil augmented the impaired response to reactive hyperemia in the HF group. In a similar hypercontractile condition evoked by phenylephrine in the control group, fasudil did not induce the vasodilator response. These results suggest that activation of Rho-kinase is involved in the increased peripheral vascular resistance in patients with HF.

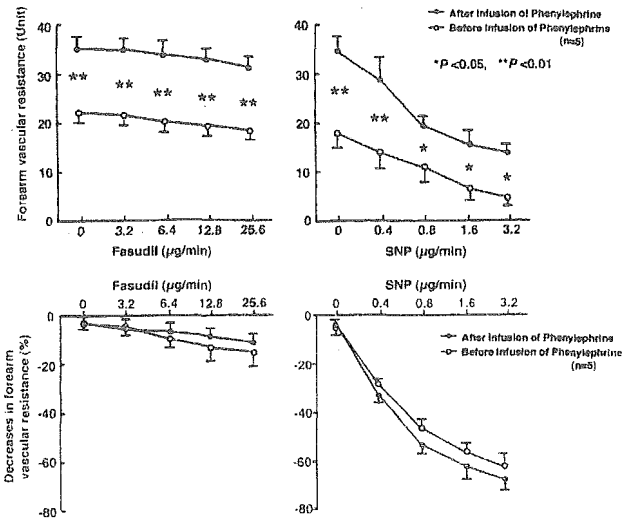

Figure 5. Plots showing responses of FVR in response to fasudil and SNP during infusion of phenylephrine or saline in control group as expressed in absolute values (top) and percent change (bottom). Results are expressed as mean±SEM.

In the present study, basal FVR was significantly higher in the HF group than in the control group, and administration of fasudil into the forearm improved vasodilation and decreased FVR to the levels of the control group. These results suggest that activation of the Rho/Rho-kinase pathway is involved in the hyperconstriction of peripheral arteries in patients with HF. The preferential vasodilator effect of fasudil in the HF group was not due to structural changes in the arterial wall, because the response to SNP was comparable between the 2 groups. Previously, we demonstrated that SNP-induced vasodilation was similar between patients with HF and normal subjects,[5,29] and the response to SNP in the present study was comparable. In the control group, the Rho/Rho-kinase pathway was not significantly activated, because fasudil did not significantly change FBF or FVR. These results are consistent with our previous report.[19] Furthermore, after infusion of phenylephrine in the control group, which decreased FBF to levels similar to those in patients with HF, fasudil did not significantly change FBF and FVR. Thus, the difference in basal FBF does not account for our observation. We cannot exclude the possibility, however, that long-term adrenergic stimulation activates the Rho/Rho-kinase pathway, because NE might activate Rho-kinase.[30] Together with the results of the effects of SNP, these findings suggest that the vasodilator effect of fasudil in the HF group was not due to the structural changes of the arterial wall and that Rho-kinase is involved in the pathogenesis of increased peripheral vascular resistance in patients with HF. Furthermore, we consider that the vascular effects of fasudil are independent of the basal FBF.

Previous studies suggested that maximal vasodilation is impaired in patients with HF.[1,2,5–8] The findings of the present study are consistent with this suggestion. In the present study, submaximal vasodilation induced by ischemia (reactive hyperemia) was significantly impaired in the HF group, and fasudil increased the maximal FBF and decreased the minimal FVR. These responses were not observed in the control group. We evaluated forearm vasodilating responses induced by reactive hyperemia during infusion of the maximum dose of fasudil, which increased FBF and decreased FVR in the HF group, by examining the effect of fasudil-induced Rho-kinase inhibition. At this dose of fasudil, baseline FBF and FVR were comparable between the 2 groups (Figure 4). These results indicate that activation of the Rho/Rho-kinase pathway is involved in impaired vasodilation induced by metabolic stimulation in patients with HF.

In the present study, the plasma concentrations of fasudil were not significantly different between the 2 groups. We and others previously demonstrated that the IC_{50} value of fasudil is <1.9 μmol/L when tested in vitro,[16,22] and the achieved concentration in patients in the present study was high enough to inhibit Rho-kinase activity. We previously demonstrated that fasudil augmented the impaired vasodilation in patients with hypertension,[19] and we used the same dose in the present study. Fasudil prevents acetylcholine-induced coronary artery spasm and the resultant myocardial ischemia in patients with vasospastic angina[31] and coronary microvascular spasm.[32] Noma et al[33] demonstrated that smoking activates Rho-kinase in forearm VSMCs but does not alter the vasodilating effect induced by exogenous nitric oxide in forearm VSMCs in healthy young men. In that study, graded doses of fasudil (3, 10, and 30 μg/min) were infused for 5 minutes, but the plasma fasudil concentration was not measured. We used a similar dose of fasudil administered for a longer period of time. These results suggest that the dose of fasudil selectively and specifically inhibited Rho-kinase.

Vasodilation evoked by fasudil correlated to the plasma BNP levels, which suggests that activation of the Rho/Rho-kinase pathway is related to the severity of HF.[34] Plasma BNP levels are now widely accepted as a prognostic marker of HF. In contrast, the basic disorder of HF did not correlate to the extent of vasodilation evoked by fasudil, which suggests that activation of the Rho/Rho-kinase pathway in the forearm vasculature is associated with an HF state rather than basic disorders.

In the present study, 6 control subjects had a "previous" smoking habit. Chronic smoking activates Rho-kinase in forearm VSMCs in healthy young men.[33] In the present study, there was no vasodilator response to fasudil in the control group, even in previous smokers. There was also no significant difference in the responses of FBF and FVR to fasudil between nonsmokers and the previous smokers in the HF and control groups. From these results, we consider that the previous smoking habit had no effects on activation of Rho-kinase in these patients.

Previous studies suggest that hypercholesterolemia impairs endothelial function.[35,36] Creager et al[35] reported that in humans with hypercholesterolemia, whose average serum cholesterol was 275 mg/dL, there is a decreased effect of nitrovasodilators, including endothelium-derived relaxing factor, on the vascular smooth muscle of resistance vessels. Casino et al[36] reported that hypercholesterolemic patients, whose average serum cholesterol levels were 292 mg/dL, have impaired endothelium-dependent vascular relaxation. In the present study, in the control group, 5 subjects had serum total cholesterol levels >220 mg/dL, and 4 had serum triglyceride levels >150 mg/dL. Serum total cholesterol

levels were <250 mg/dL and serum triglyceride levels were <180 mg/dL, however, even in the HF group. Therefore, we consider that these levels of hypercholesterolemia are not enough to activate Rho-kinase.

The present study did not address the precise mechanism(s) by which the Rho/Rho-kinase pathway is activated in patients with HF. In the animal models of HF, however, the Rho/Rho-kinase pathway is reported to be involved in the pathogenesis of HF.[13,20,37] Rho-kinase is substantially involved in the pathogenesis of left ventricular remodeling after myocardial infarction associated with upregulation of proinflammatory cytokines.[37] Differential activation of the Rho/Rho-kinase pathway plays a critical role in HF, and the Rho/Rho-kinase pathway is involved in the pathogenesis of cardiac dysfunction and cardiovascular remodeling.[20] Hisaoka et al[13] demonstrated that the Rho/Rho-kinase system is critically involved in the enhanced arterial vasoconstriction observed in HF. In that study, enhanced vasoconstriction was induced by a marked increase in Ca^{2+} sensitivity mediated through activation of the Rho/Rho-kinase pathway.[13] Activation of the sympathetic nervous system and renin-angiotensin system occurs in HF, and this neurohumoral activation causes HF to deteriorate.[2,38] This mechanism is also related to abnormal peripheral circulation in HF.[2,3,39] Previous in vitro studies suggest that Rho-kinase is deeply involved in the Ang II–induced signaling pathway.[40–42] Therefore, those studies suggest that activation of the renin-angiotensin system is upstream of the Rho/Rho-kinase pathway and that Ang II upregulates the Rho-kinase pathway in patients with HF. In fact, several neurohumoral factors such as Ang II, NE, and endothelin-1, which activate the Rho/Rho-kinase pathway, are increased in patients with HF.[39,43] In the present study, we evaluated the correlation between plasma Ang II levels and the increases in FBF or the decreases in FVR caused by the infusion of fasudil. These results suggest that there is a correlation between plasma Ang II levels and Rho-kinase activity.

Furthermore, fasudil likely influences endothelial function. In fact, recent studies suggest an interaction between endothelial NO synthase activity and Rho-kinase.[44] For example, it is suggested that NO induces vasodilation through inhibition of the Rho/Rho-kinase signaling pathway and that the Rho/Rho-kinase activity negatively regulates endothelial NO synthase phosphorylation.[45] It is well established that NO activity is decreased in HF.[6–10] In addition, we previously demonstrated that L-arginine supplementation improves both acetylcholine-induced and reactive hyperemia–induced forearm vasodilation in patients with HF, which suggests that the impaired vasodilation is caused by reduced NO activity in HF.[5] Therefore, it is possible that inhibition of Rho-kinase improves NO activity in patients with HF. We did not address this issue in the present study. We do consider, however, that inhibition of Rho-kinase has multiple actions, including modulation of both endothelial and VSMC function. Because Rho-kinase causes VSMC hypercontraction via a Ca^{2+}-sensitizing mechanism, it is difficult to exclude the possibility that fasudil acts directly on VSMCs. Also, in an in vitro study, activation of the Rho/Rho-kinase pathway was involved in arterial hypercontraction in an HF model through a Ca^{2+}-sensitization mechanism.[13] Thus, we suggest that abnormalities of both endothelial and VSMC function are involved in the effects of fasudil on the increased vascular resistance in patients with HF. Further studies are needed to examine this issue.

In conclusion, the present study indicates that fasudil, a Rho-kinase inhibitor, improves the increased FVR and impaired vascular response to reactive hyperemia in the forearm of patients with HF and that these effects are not due to changes in the vascular structures.

Clinical Implications

We demonstrated that the Rho/Rho-kinase pathway is involved in the pathogenesis of HF. Thus, inhibition of the Rho-kinase pathway might be a potential therapeutic strategy for HF. Our results suggest that inhibition of Rho-kinase might improve abnormal peripheral circulation, thereby augmenting exercise tolerance in patients with HF.[2,3,39] Furthermore, Rho-kinase is substantially involved in the pathogenesis of left ventricular remodeling after myocardial infarction associated with upregulation of proinflammatory cytokines,[37] which suggests that these molecular mechanisms might be important targets for the prevention of post–myocardial infarction HF.

Acknowledgments

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Blockade of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II

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Abstract

Objective: Nuclear factor (NF)-κB is a key transcription factor that regulates inflammatory processes. In the present study, we assessed the hypothesis that blockade of NF-κB may ameliorate ventricular hypertrophy in response to chronic infusion of angiotensin II.
Methods: Mice with targeted disruption of the p50 subunit of NF-κB (KO) were used to block the activation of NF-κB. Male KO and age-matched wild-type (WT) mice were chronically infused with angiotensin II at the rate of 0.2 (low dose) or 2 μg/kg/min (high dose) for 4 weeks.
Results: High- but not low-dose angiotensin II significantly increased systemic blood pressure and left ventricular weight in WT mice. In contrast, although the pressor response was slightly but significantly augmented, the hypertrophic effect of angiotensin II was significantly attenuated in KO mice. The attenuated hypertrophic responsiveness was confirmed histologically (cross-sectional area) and transcriptionally (atrial natriuretic peptide). Echocardiography revealed no evidence of cardiac dysfunction in angiotensin II-treated KO mice. Although phosphorylation of MAPKs, including ERK, JNK, or p38-MAPK, was not affected after 4 weeks of angiotensin II treatment in WT mice, phosphorylation of JNK was specifically abrogated in KO mice. Angiotensin II increased myocardial expression of proinflammatory cytokines and chemokines in WT mice, while expression of TNF-α and RANTES was paradoxically augmented in KO mice.
Conclusion: Blockade of NF-κB activation attenuated myocardial hypertrophy without deteriorating cardiac function. NF-κB may play an important role in cardiac hypertrophy and remodeling besides promoting inflammation.

Keywords: Angiotensin; Cytokines; Hypertrophy; MAP kinase; Nuclear factor-κB; Cardiac remodeling

1. Introduction

Nuclear factor-kappa B (NF-κB) is a key transcription factor that regulates inflammatory processes (see Ref. [1] for review). Many stimuli activate NF-κB, including proinflammatory cytokines, lipopolysaccharide, and reactive oxygen species. Activation of NF-κB involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB (typically, a heterodimer of p50 and p65) then passes into the nucleus, where it binds to κB sites in the promoter regions of genes for inflammatory proteins such as tumor necrosis factor (TNF)-α, inducible nitric oxide synthase, and adhesion molecules. Thus the activation of NF-κB leads to a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses [1].

Recent studies have suggested that the activation of NF-κB may also play an important role in the pathogenesis of cardiac remodeling and heart failure [2]. First, NF-κB is activated in the failing human heart [3]. Second, plasma levels as well as myocardial expression of proinflammatory cytokines, including TNF-α, are increased in patients with heart failure [4,5]. Third, cardiac-specific overexpression of TNF-α causes myocardial inflammation and remodeling, resulting in the development of congestive heart failure [6].

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Fourth, angiotensin II has been shown to activate NF-κB and induce TNF-α in myocardium [7]. Finally, recent in vitro studies demonstrate that activation of NF-κB is also required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes in response to angiotensin II, phenylephrine, and endothelin-1 [8,9]. Therefore, blockade of NF-κB activation may be a new therapeutic strategy for heart failure by attenuating myocardial inflammation and hypertrophy. However, little is known about in vivo effects of NF-κB inhibition in patients with heart failure or any animal models of cardiac hypertrophy and heart failure.

The purpose of the present study therefore was to assess the hypothesis that blockade of NF-κB activation might ameliorate ventricular hypertrophy and inflammation in response to chronic infusion of angiotensin II. Mice with targeted disruption of the p50 subunit of NF-κB (KO) [10] were used to block the activation of NF-κB chronically.

2. Methods

2.1. Animal models

Mice with targeted disruption of the p50 subunit of NF-κB [10], backcrossed into the FVB background more than six generations, were used to block the activation of NF-κB. These mice were born normally without any major defects. Homo-knockout mice (KO) were compared with age- and gender-matched wild type littermates (WT) in each analysis to minimize the effect of genetic background variation. Male mice at the age of 8 weeks were used unless mentioned otherwise. First, to confirm that angiotensin II induces activation of NF-κB in WT hearts but not in NF-κB KO hearts, 30 g/kg of angiotensin II was injected intraperitoneally and the heart was harvested 30 min after. Secondly, to investigate whether blockade of NF-κB activation might ameliorate ventricular hypertrophy and inflammation in response to chronic infusion of angiotensin II, an osmotic minipump (Alzet Model 2004) was implanted into the peritoneal cavity under pentobarbital anesthesia (50 mg/kg i.p.) to infuse angiotensin II continuously at a rate of 0.2 or 2 μg/kg/min (low or high) for 4 weeks. Saline with 0.01 mol/L acetic acid was used as vehicle. Since the rate of infusion was fixed as 0.25 μL/h, the concentration of angiotensin II was carefully titrated in each mouse in accordance with the body weight and the dosage. Systemic blood pressure was measured twice a week after the implantation of the minipump using tail-cuff method without anesthesia (model UR5000; Ueda, Tokyo, Japan). This experiment was reviewed by the Committee of the Ethics on Animal Experiment, Kyushu University Graduate School of Medical Sciences and carried out under the control of the Guideline for Animal Experiment, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer's instructions (Gel Shift Assay System E3300, Promega, Madison, Wisconsin, USA) as previously described [11]. Nuclear proteins were isolated using the method of Haudek et al. [12]. Protein concentrations were measured by BCA Protein Assay Reagents (PIERCE, Rockford, Illinois, USA) using bovine serum albumin (BSA) as a standard. Protein–DNA binding was carried out in a final volume of 40 μL. To each tube, 4 μL of 10× binding buffer (100 mM Tris pH 8.0, 10 mM EDTA, 40% glycerol, 1 M NaCl), 100 ng of 1,4-dithiothreitol (DTT), 4 μg of BSA, 2 μg of dIdC and 30 μg nuclear proteins was added. After the samples were incubated at room temperature for 10 min, 1 μL of ^{32}P-labeled NF-κB probe (a double-stranded oligonucleotide corresponding to the consensus NF-κB binding site of the κ light-chain enhancer: 5′-AGTTGAGGGGACTTTCC-CAGGC-3′, approximately 20000 cpm/ng) was added to each reaction and incubate for 20 min at room temperature. Samples were resolved on a 5% acrylamide gel in 0.25% TBE buffer.

2.3. Echocardiography

After 4 weeks of the treatment, echocardiography was performed using an ultrasonographic system (SSD-5500 ALOKA) as previously reported [13,14]. After anesthetization with 2.5% avertin (14 μL/g body weight, i.p., Aldrich Chemical Co.), mice were placed in a supine position. A 7.5-MHz transducer was applied to the left hemithorax. Two-dimensional targeted M-mode imaging was obtained from the short-axis view at the level of the greatest left ventricular dimension. M-mode measurements of left ventricular end-diastolic and end-systolic diameter and left ventricular anterior and posterior wall thickness were made using the leading-edge convention of the American Society of Echocardiography. End-diastole was determined at the maximal left ventricular diastolic dimension, and end-systole was taken at the peak of posterior wall motion. The percentage of left ventricular fractional shortening (FS) was calculated as FS (%)=(LVDd − LVDs)/LVDd × 100, where LVDd and LVDs indicate left ventricular end-diastolic and end-systolic diameter, respectively.

2.4. Tissue preparation and morphometric analysis

After echocardiography, the heart was excised, weighed, and fixed in 4% paraformaldehyde for histology or immediately frozen and stored at −80 °C for RNA or protein analysis. After hematoxylin–eosin staining, cross-sectional area of cardiomyocytes in the left ventricle was

evaluated as previously reported [14]. The outline of 100–200 myocytes was traced in each section and NIH image system software was used to determine myocyte cross-sectional area.

2.5. Northern blot analysis

Total RNA was extracted from the left ventricle by an acid guanidium thiocyanate–phenol chloroform method (ISOGEN, Nippon Gene). RNA samples (5 μg) were electrophoresed in a formaldehyde-agarose gel, and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). The membrane was then hybridized with a ^{32}P-labeled probes for murine atrial natriuretic peptide (ANP, nucleotides 592 to 1094, GenBank K02781) or 18S rRNA as previously reported [6]. Result of the cDNA hybridization was normalized to that of the 18S probe to correct for differences in RNA mass and efficiency of transfer. Data were in turn normalized to the mean of WT samples, arbitrarily set at 1.

2.6. Activity of mitogen-activated protein kinase (MAPK)

The left ventricle was homogenized with a lysis buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 1% (vol/vol) Triton X-100, 1% (vol/vol) glycerol. Equal amounts of the heart homogenate (30 μg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred onto a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Lab), and blocked with 5% skimmed milk at room temperature for 60 min. The filters were subjected to immunoblot analyses with anti-phospho-extracellular signal-regulated kinase (ERK) antibody (no. 9106; Cell Signaling Technology Inc.), anti-phospho-c-Jun NH_2-terminal kinase (JNK) antibody (no. 9255; Cell Signaling Technology Inc.) or anti-phospho-p38 antibody (no. 9211; Cell Signaling Technology Inc.). Duplicate samples were subjected to immunoblot analyses with anti-ERK antibody (no. 9102; Cell Signaling Technology Inc.), anti-JNK1 antibody (sc-474; Santa Cruz Biotechnology Inc.) or anti-p38 antibody (no. 9212; Cell Signaling Technology Inc.). Immunodetection was accomplished with a horseradish anti-rabbit or anti-mouse secondary antibody (1:2000 dilution, Amersham) using an enhanced chemiluminescence kit (Amersham). The data were quantified by the densitometry using NIH Image.

2.7. RNase protection assay

Multi-probe RNase protection assay (RPA) was performed according to the manufacturer's protocol (RiboQuant, PharMingen) with 5 μg of total RNA [13]. A custom template set containing murine TNF-α, interleukin (IL)-1β, transforming growth factor (TGF)-β1, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein (MCP)-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied. After RNAase digestion, protected probes were resolved on denaturing polyacrylamide gels and quantified by NIH image. The value of each hybridized probe was normalized to that of GAPDH included in each template set as an internal control.

2.8. Immunohistochemistry

Sections fixed in paraformaldehyde were incubated with a goat polyclonal anti-mouse TNF-α antibody (L-19; Santa Cruz Biochemistry) for overnight at 4 °C, washed three times, and incubated with affinity-purified biotinylated rabbit anti-goat IgG for 1 h at room temperature. They were washed again and overlaid with streptavidin–biotin–peroxidase complex for 1 h at room temperature (Nichirei; Tokyo, Japan). After a final wash, the labeling was visualized with aminoethyl carbazole (Nichirei; Tokyo, Japan). Counterstaining was then performed with Mayer's hematoxylin. The heart sections of transgenic mice with cardiac-specific overexpression of TNF-α [6] were used as positive controls.

2.9. RT-PCR

First-strand cDNA was synthesized using reverse transcriptase with oligo(dT) from 1 μg of total RNA in 20 μL reaction volume according to the manufacturer's protocol (ReverTra Ace, TOYOBO), then 5 μL of the resulting reverse transcription (RT) product was applied to each 20 μL PCR. The PCR reactions contained 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 U Taq DNA polymerase (rTaq DNA polymerase; TOYOBO) and 0.1 mM PCR primers for one of angiotensin type 1 (AT1) or type 2 (AT2) receptor, or GAPDH (Table 1). PCR conditions were 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C (AT1, GAPDH) or 60 °C (AT2), and 1 min of extension at 72 °C. PCR cycles were limited to 26 for AT1, 32 for AT2 and 22 for GAPDH after a pilot study to avoid over-amplification. PCR products were separated by 2% agarose gel electrophoresis.

Table 1
Primer sequences for RT-PCR

cDNA and primer	Sequence of primer	Product size
AT1 receptor		
Forward	5′-GGAACAGCTTGGTGGTG-3′	555 bp
Reverse	5′-CTGAATTTCATAAGCCTTCTT-3′	
AT2 receptor		
Forward	5′-GCCTGCATTTTAAGGAGTGC-3′	164 bp
Reverse	5′-ACGGCTGCTGGTAATGTTTC-3′	
GAPDH		
Forward	5′-CTTGCTCAGTGTCCTTGCTG-3′	1064 bp
Reverse	5′-AGCCTCGTCCCGTAGACAA-3′	

AT1 and AT2 indicate angiotensin type 1 and type 2; GAPDH, glyceraldehyde-3-phoshate dehydrogenase.

2.10. Statistics

Results are presented as mean ± S.D. Statistical comparisons were performed by ANOVA with Student–Newman–Keuls post hoc test or two-way ANOVA where appropriate (Fig. 6). Differences were considered significant at a value of $P<0.05$.

3. Results

3.1. Absence of NF-κB activation in NF-κB KO mice

As shown in Fig. 1, intraperitoneal injection of angiotensin II increased activation of NF-κB in the myocardium of WT mice, while it was completely abolished in NF-κB KO mice irrespective of angiotensin II treatment.

3.2. Augmented pressor response in NF-κB KO mice

Systemic blood pressure was measured twice a week after implantation of an osmotic minipump with a low or high dose of angiotensin II (0.2 or 2 mg/kg/min) or vehicle alone. There were no significant differences in systemic blood pressure between WT and NF-κB KO mice before the implantation of the osmotic minipumps (Fig. 2A,B). Although male NF-κB KO mice at the age of 8 weeks were significantly smaller (26.6 ± 1.6 g, $n=34$) than age- and gender-matched WT littermates (28.9 ± 2.7 g, $n=34$, $P<0.05$), the concentration of angiotensin II was titrated carefully in accordance with the body weight in each mouse. Infusion of vehicle alone did not change systemic blood pressure either in WT or in NF-κB KO mice during 4 weeks

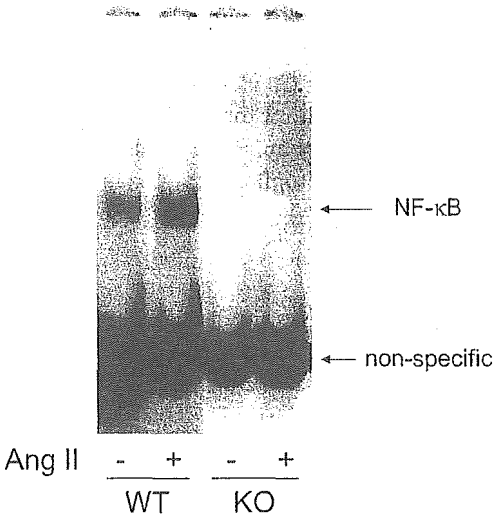

Fig. 1. Electrophoretic mobility shift assay for activation of NF-κB in myocardium. Nuclear proteins were isolated from the left ventricle of wild-type (WT) or NF-κB knockout mice (KO) 30 min after injection of angiotensin II (Ang II) or saline. The activation of NF-κB was completely abolished in NF-κB KO mice.

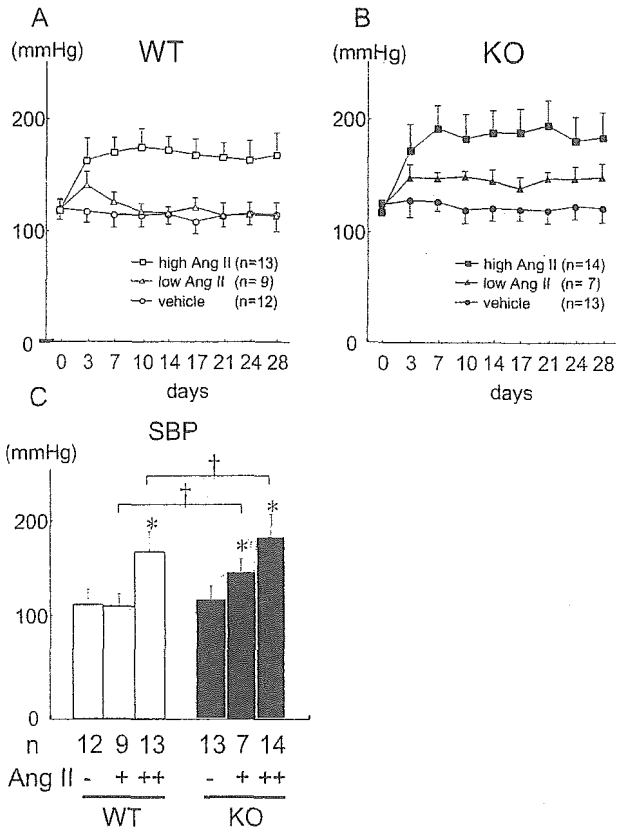

Fig. 2. Effects of angiotensin II (Ang II) on systolic blood pressure (SBP). SBP was measured twice a week after implantation of osmotic minipumps in wild type (WT, A) and NF-κB knockout mice (KO, B). SBP after 4 weeks of the treatment with vehicle (−), low dose (+) or high dose (++) of angiotensin II was summarized in (C). Values are mean ± S.D. *$P<0.05$ vs. vehicle, †$P<0.05$ vs. WT.

of the treatment. In contrast, although the low dose of angiotensin II did not significantly affect systemic blood pressure in WT mice, it significantly increased it in NF-κB KO mice even after 4 weeks of the treatment (Fig. 2C). The high dose of angiotensin II significantly increased systemic blood pressure both in WT and in NF-κB KO mice (Fig. 2A,B). However, the systemic blood pressure was significantly higher in NF-κB KO mice than WT mice after 4 weeks of the treatment (Fig. 2C). These results indicated that the pressor effects of angiotensin II were significantly augmented in NF-κB KO mice.

3.3. Attenuated hypertrophic response by NF-κB KO

After 4 weeks of the treatment, the heart was harvested and weighed. Since NF-κB KO mice were slightly but significantly smaller than WT littermates, left ventricular weight of NF-κB KO mice treated with vehicle alone (76.5 ± 5.9 mg) was significantly smaller than that of WT mice (88.4 ± 7.6 mg, $P<0.05$). However, after normalization to the body weight, there were no differences between vehicle-treated NF-κB KO and WT mice (Fig. 3A). The low dose of angiotensin II did not affect left ventricular weight

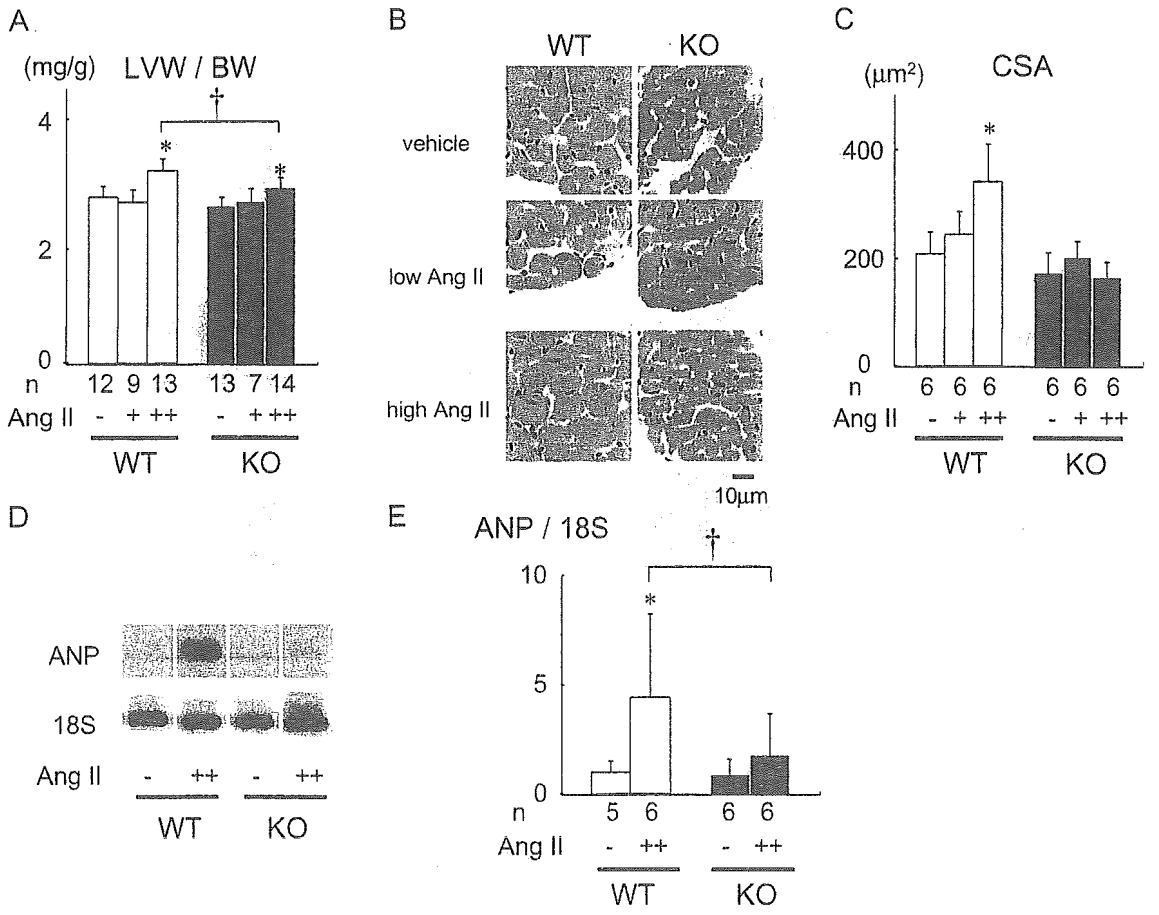

Fig. 3. Effects of angiotensin II (Ang II) on ventricular hypertrophy. (A) Left ventricular weight normalized to body weight (LVW/BW) in wild type (WT) and NF-κB knockout mice (KO). Values are normalized to body weight (BW) in each mice. (B) Representative images of hematoxylin–eosin staining of myocardium. (C) Cross-sectional area (CSA) of cardiomyocytes. (D) Representative images of Northern blot analysis for atrial natriuretic peptide (ANP) and 18S RNA. (E) Summarized data of ANP expression. Normalized values in vehicle-treated WT mice are arbitrarily expressed as 1. $*P<0.05$ vs. vehicle, $\dagger P<0.05$ vs. WT.

either in WT or in NF-κB KO mice. In contrast, the high dose of angiotensin II significantly increased left ventricular weight both in WT and in NF-κB KO mice. However, left ventricular hypertrophy observed in NF-κB KO mice was significantly less than that in WT mice (Fig. 3A). These results indicated that, despite the augmented pressor response, the hypertrophic effects of angiotensin II were significantly attenuated in NF-κB KO mice.

Cross-sectional area of cardiomyocytes was evaluated to confirm the attenuated ventricular hypertrophy in NF-κB KO mice (Fig. 3B,C). Cross-sectional area of NF-κB KO mice treated with vehicle alone was similar to that of WT mice. The low dose of angiotensin II did not affect cross-sectional area of either WT or NF-κB KO mice. In contrast, the high dose of angiotensin II significantly increased cross-sectional area in WT mice but not in NF-κB KO mice. Therefore, attenuated ventricular hypertrophy in NF-κB KO mice might be related to the reduced hypertrophic response in cardiomyocytes.

To further verify the attenuated hypertrophic response in NF-κB KO mice transcriptionally, gene expression of ANP in the left ventricle was evaluated by Northern blot analysis (Fig. 3D,E). While chronic infusion of the high dose angiotensin II significantly increased myocardial expression of ANP in WT mice, this up-regulation of ANP was significantly attenuated in NF-κB KO mice. Taken together, these results suggest that the targeted disruption of the p50 subunit of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II.

3.4. Preserved cardiac function in NF-κB KO mice

Echocardiography was performed to evaluate the effects of NF-κB KO on left ventricular function (Fig. 4). The high dose of angiotensin II significantly increased wall thickness, decreased end-diastolic dimension, and enhanced fractional shortening in WT mice. In contrast, in NF-κB KO mice, wall thickness, end-diastolic dimension, or fractional shortening was not affected by angiotensin II infusion. These results indicated that, despite the absence of ventricular hypertrophy, cardiac function was well preserved against increased afterload in NF-κB KO mice.

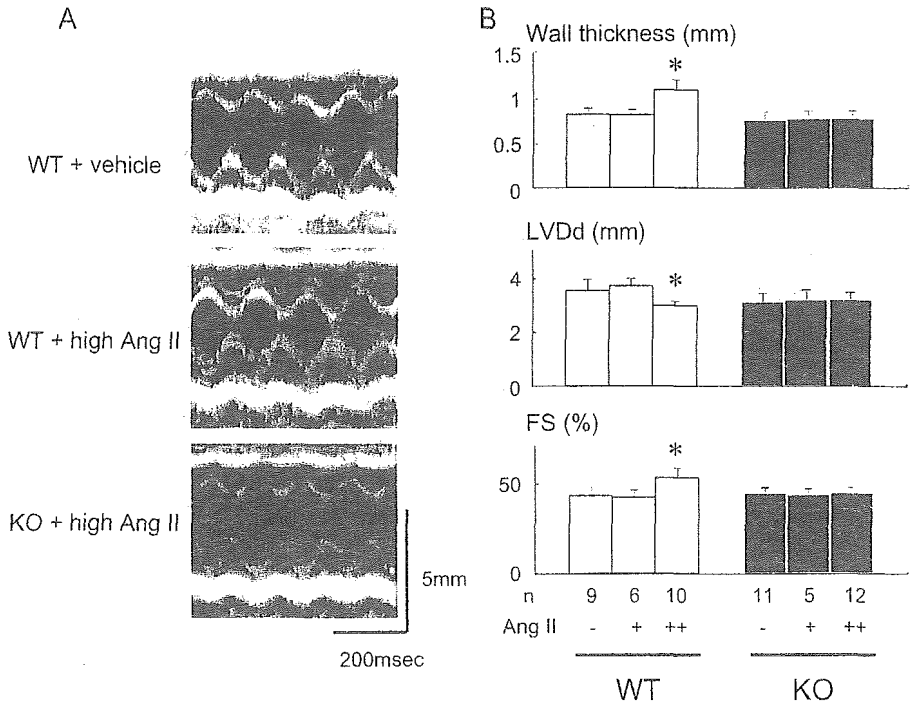

Fig. 4. Echocardiographic analysis of cardiac hypertrophy and function. (A) Representative images of M-mode echocardiography from wild type (WT) and NF-κB knockout mice (KO) treated with vehicle or the high dose of angiotensin II. (B) Summarized data of left ventricular wall thickness, end-diastolic dimension (LVDd), and fractional shortening (FS). Values are mean±S.D. $*P<0.05$ vs. vehicle.

3.5. Absence of JNK phosphorylation in NF-κB KO mice

To elucidate the changes in hypertrophic signaling pathways, phosphorylation of MAP kinases in the myocardium was evaluated after 3-day or 4-week treatment with the high dose of angiotensin II. As shown in Fig. 5, protein levels of ERK, JNK, or p38 were not different between WT and NF-κB KO mice. Although neither ERK nor p38 was phosphorylated by 3-day or 4-week treatment, JNK was further phosphorylated by the 3-day, but not 4-week, treatment with angiotensin II. It is notable that the phosphorylation of JNK was selectively abrogated in NF-κB KO mice regardless of the treatment. Specificity of this finding was confirmed by a phospho-Western analysis after immunoprecipitation with the anti-JNK1 antibody (data not shown).

3.6. Effects of NF-κB KO on myocardial expression of cytokines

Transcript levels of proinflammatory cytokines and chemokines were evaluated by multiprobe ribonuclease

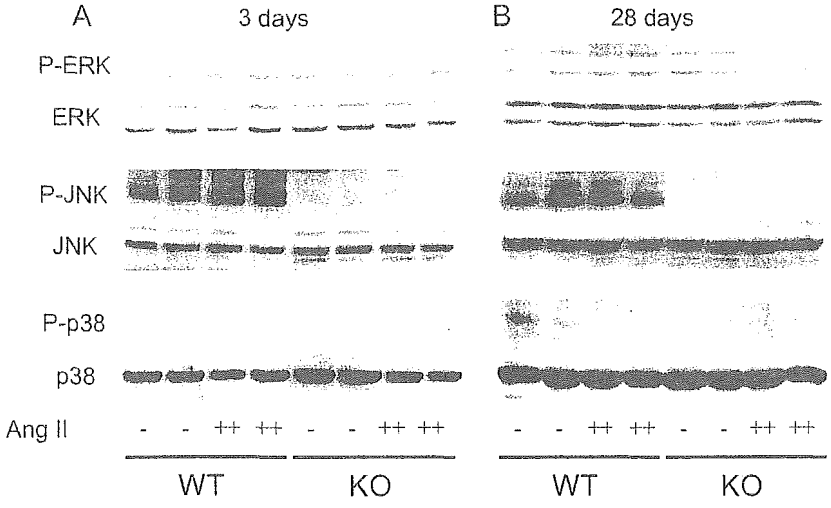

Fig. 5. Western blot analysis of ERK, JNK and p38 with anti-phosphospecific or non-phosphospecific antibodies after 3 days (A) or 4 weeks (B) treated with high dose (++) of angiotensin II (Ang II). WT indicates wild type mice; KO, NF-κB knockout mice.

protection assay after 4 weeks of the treatment with vehicle or the high dose of angiotensin II (Fig. 6A). The results were analyzed by two-way ANOVA and summarized in Fig. 6B. The high dose of angiotensin II significantly increased expression of RANTES, TNF-α, IL-1β, TGF-β, and MCP-1 in the myocardium. Although NF-κB KO did not affect myocardial expression of IL-1β, MCP-1, or TGF-β, it significantly increased that of TNF-α and RANTES. Since no interaction was found between NF-κB KO and angiotensin II treatment, it is suggested that induction of proinflammatory cytokines in response to chronic infusion of angiotensin II may not be mediated by NF-κB dependent pathways. To confirm the increased expression of TNF-α in NF-κB KO mice, immunohistochemical staining was performed (Fig. 7). Consistent with the transcript levels, while TNF-α staining was increased in response to angiotensin II treatment in WT mice, it was evident without angiotensin II treatment in NF-κB KO mice. Since staining was diffuse and not localized to interstitial cells, one of major sources of TNF-α was suggested to be cardiomyocytes.

3.7. Expression of angiotensin II receptors

Since targeted disruption of the p50 subunit of NF-κB may have affected expression of angiotensin II receptors in the heart, transcript levels of AT1 and AT2 receptors were evaluated by RT-PCR. As shown in Fig. 8, there was no difference in the expression of either AT1 or AT2 receptors in the heart between WT and NF-κB KO mice. Therefore, the attenuated hypertrophic response in NF-κB KO mice may not be attributable to the difference in the expression of angiotensin II receptors.

4. Discussion

We have evaluated in vivo effects of NF-κB inhibition on the development of angiotensin II-induced cardiac hypertrophy. Chronic infusion of angiotensin II, significantly increased systemic blood pressure, provoked ventricular hypertrophy, and enhanced expression of proinflammatory cytokines in the myocardium. Since the targeted disruption of the p50 subunit of NF-κB significantly attenuated ventricular hypertrophy in response to angiotensin II, it is suggested that the hypertrophic effect of angiotensin II is mediated by the activation of NF-κB. In contrast, elevation of systemic blood pressure and induction of proinflammatory cytokines were rather enhanced in NF-κB KO mice, suggesting that pressor and proinflammatory effects of angiotensin II may be mediated by pathways independent of NF-κB. Despite the absence of ventricular hypertrophy with higher blood pressure and enhanced expression of proinflammatory cytokines, echocardiography revealed no

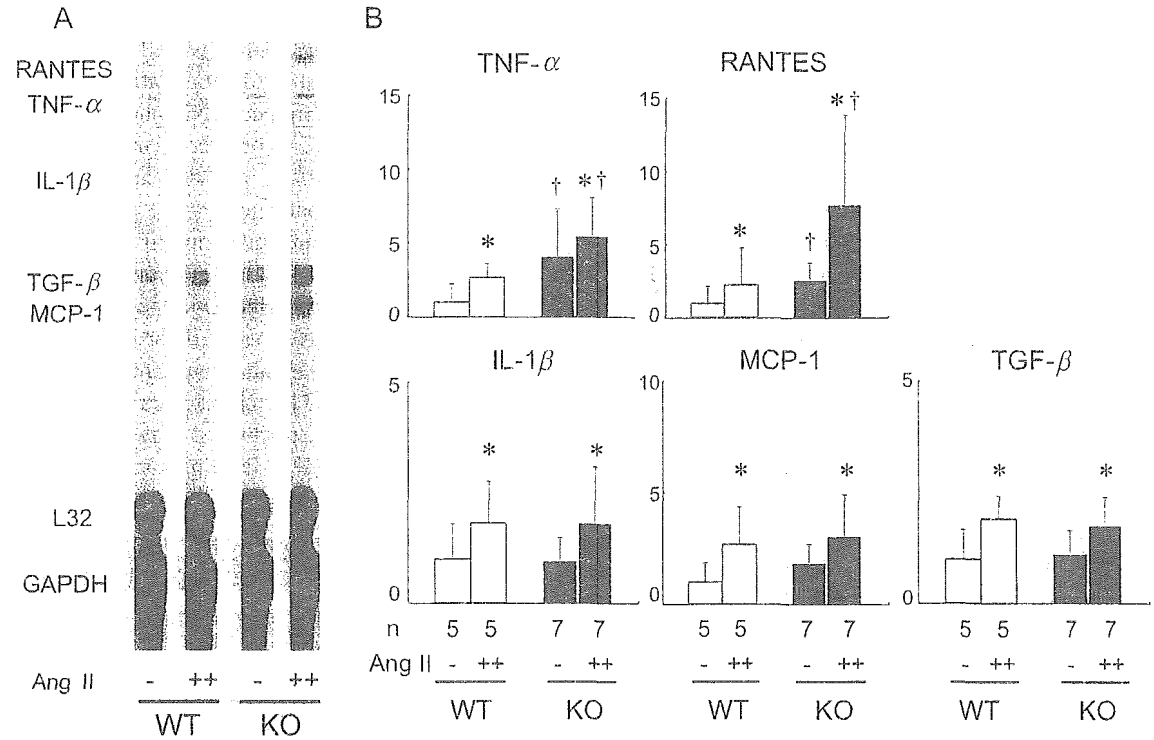

Fig. 6. Myocardial expression of proinflammatory cytokines and chemokines. (A) Representative images of multiprobe RPA. (B) Summarized data of cytokine expression: TNF-α, RANTES, IL-1β, MCP-1, and TGF-β. Normalized values in vehicle-treated wild type mice (WT) are arbitrarily expressed as 1. KO indicates NF-κB knockout mice. Based on two-way ANOVA, * indicates a significant effect of angiotensin II ($p<0.05$) and † demonstrates that of NF-κB KO ($p<0.05$). No interaction was found between angiotensin II and NF-κB.

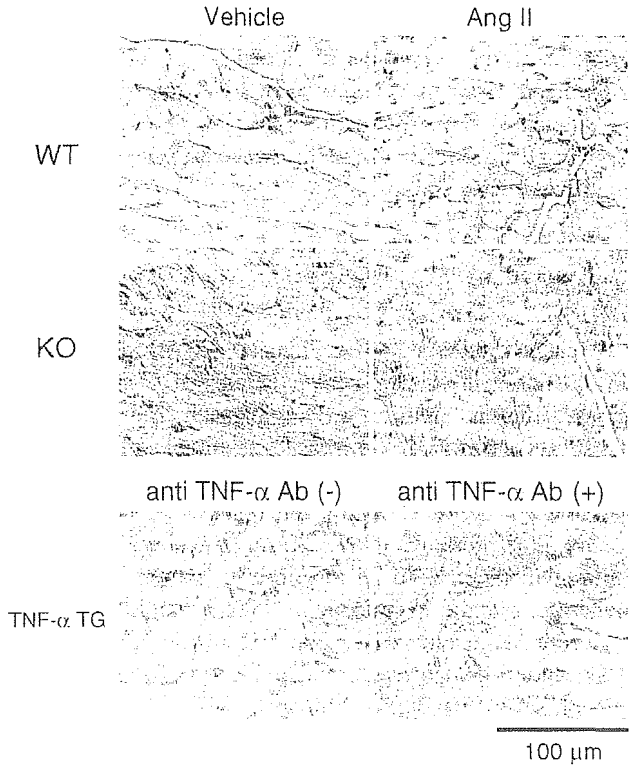

Fig. 7. Immunohistochemistry for TNF-α in the myocardium of wild type (WT) and NF-κB knockout (KO) mice with or without angiotensin II (Ang II) treatment. The heart sections of transgenic mice with cardiac-specific overexpression of TNF-α (TG) were shown as positive controls and those without the primary antibody (Ab) were included as negative controls. The scale bar indicates 100 μm.

evidence of cardiac dysfunction in NF-κB KO mice. Therefore, we conclude that NF-κB may be a new therapeutic target to attenuate ventricular hypertrophy in a setting in which angiotensin II is activated.

Recent in vitro studies [8,9] have demonstrated that the activation of NF-κB is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes in response to G-protein-coupled receptor agonists, including phenylephrine, endothelin-1, and angiotensin II. However, it has not been clear whether the activation of NF-κB may also play an important role in the development of ventricular hypertrophy in vivo. Therefore, we took advantage of knockout mice in which the p50 subunit of NF-κB was disrupted [10]. Chronic infusion of angiotensin II was adopted as a model of ventricular hypertrophy. Two different doses of angiotensin II were infused for 4 weeks intraperitoneally. The low dose of angiotensin II (0.2 μg/kg/min) was chosen based on previous reports demonstrating the hypertrophic effect of angiotensin II without affecting systemic blood pressure [15,16]. However, in the present study, the low dose of angiotensin II did not show any hypertrophic effects either in WT or in NF-κB KO mice. It might be of interest that the low dose of angiotensin II slightly but significantly increased systemic blood pressure not in WT but in NF-κB KO mice, suggesting enhanced pressor responsiveness in NF-κB KO mice. In contrast, the high dose of angiotensin II (2 μg/kg/min) significantly increased systemic blood pressure as well as left ventricular weight in WT mice. Although the systemic blood pressure was even higher in NF-κB KO mice, the hypertrophic response was significantly attenuated by targeted disruption of the p50 subunit of NF-κB. The attenuated hypertrophic responsiveness was confirmed histologically (cross-sectional area) and transcriptionally (ANP expression). These results indicate that targeted disruption of the p50 subunit of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II. Taking into account the previous study that indicates that pharmacological inhibition of NF-κB with pyrrolidine dithiocarbamate was able to prevent cardiac hypertrophy in rats harboring both human renin and angiotensinogen genes [17], it is suggested that NF-κB plays an important role in the development of hypertrophy by angiotensin II.

Although the precise mechanisms by which NF-κB mediates cardiac hypertrophy remain undetermined, it is of interest that phosphorylation of JNK was abrogated in p50 knockout mice. MAP kinase signaling pathways, including ERK, JNK and p38, are supposed to play an important role in cardiac hypertrophy and remodeling, since they are phosphorylated and activated by G-protein-coupled receptor agonists such as phenylephrine, endothelin-1, and angiotensin II [18,19]. JNK is especially called stress-activated protein kinase, since it is additionally activated by cellular stresses such as reactive oxygen species and proinflammatory cytokines, including IL-1β and TNF-α [18]. Although these cellular stresses activate NF-κB as well as JNK, as far as we know, this is the first report demonstrating that the blockade of NF-κB activation abrogates JNK phosphorylation. Since the protein level of JNK is not affected in p50 knockout mice, expression or activation of upstream kinases may be modulated by NF-κB pathways.

Substrates of JNK are transcription factors, including c-Jun, ATF2, and Elk1 [18]. Inhibition of JNK has been shown to abrogate ventricular hypertrophy in vivo in response to pressure overload [20] or Gαq overexpression [21]. Therefore, the attenuated hypertrophic responsiveness to angiotensin II in p50 knockout mice may be mediated by the abrogation of JNK pathways. However, a recent study suggests that the MEKK1-JNK pathway does not mediate

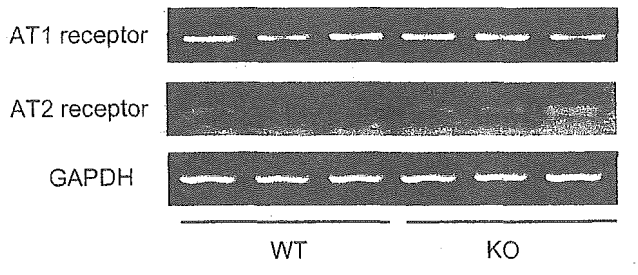

Fig. 8. RT-PCR for angiotensin type 1 (AT1) and type 2 (AT2) receptors. GAPDH indicates glyceraldehydes-3-phosphate dehydrogenase.

cardiac hypertrophy but rather plays a protective role in pressure overload [22]. Furthermore, JNK may antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling [23]. Taken together, these results indicate that further studies will be required to draw the final conclusion.

Angiotensin II has been shown to induce TNF-α biosynthesis in the adult mammalian heart with activation of NF-κB [7]. Since activation of NF-κB has been shown to induce various members of proinflammatory cytokines and chemokines, including TNF-α [1], we hypothesized that the blockade of NF-κB activation might attenuate myocardial expression of proinflammatory cytokines in response to chronic infusion of angiotensin II. However, the results did not support our hypothesis: although chronic infusion of angiotensin II increased myocardial expression of TNF-α, RANTES, IL-1β, MCP-1, and TGF-β, the targeted disruption of the p50 subunit of NF-κB rather enhanced myocardial expression of TNF-α and RANTES without affecting upregulation of IL-1β, MCP-1, and TGF-β. These results indicate that induction of proinflammatory cytokines in response to chronic infusion of angiotensin II was not mediated by NF-κB dependent pathways. Furthermore, myocardial expression of proinflammatory cytokines may not aggravate hypertrophy in this mouse model with chronic infusion of angiotensin II.

Paradoxical increase of TNF-α and RANTES expression by the blockade of NF-κB may be explained by the increased wall stress due to higher systemic blood pressure and attenuated hypertrophic responsiveness in angiotensin II-treated NF-κB KO mice. However, these cytokines were also up-regulated even without angiotensin II treatment. Therefore, it is suggested that factors other than increased wall stress should exist in NF-κB KO mice. The difference in transcriptional activity of p50–p65 heterodimers and p50–p50 homodimers [24] may be one of them. The NF-κB/Rel family consists of five subunit members, including p50, p52, c-Rel, RelA (p65), and RelB. In most cells, NF-κB is a heterodimer of p50 and p65 that is retained in the cytoplasm bound to the inhibitory protein IκB. Activation of NF-κB will occur when the specific IκB kinases phosphorylate the IκB. After chronic exposure to proinflammatory cytokines, including TNF-α, NF-κB has been shown to be converted from transcriptionally active p50–p65 heterodimers to transcriptionally inactive p50–p50 homodimers [12,24], which may act as a native negative feedback mechanism to prevent excessive inflammatory responses. The absence of p50–p50 homodimers in mice with targeted disruption of the p50 subunit therefore may account for enhanced expression of TNF-α and RANTES in response to chronic infusion of angiotensin II.

In the present study, to assure the complete and chronic inhibition of NF-κB in vivo, we used gene-manipulated mice lacking the p50 subunit of NF-κB [10], which show no developmental abnormalities, but exhibit multifocal defects in immune responses involving B lymphocytes and non-specific responses to infection; B cells do not proliferate in response to bacterial lipopolysaccharide and are defective in basal and specific antibody production. Although we did not detect any adverse effects or premature death as long as we observed, systemic inhibition of NF-κB may be deleterious in the long run. Therefore, it may be desirable to introduce cardiac-specific inhibition of NF-κB to minimize immunological detrimental effects. Furthermore, the pressor responsiveness to angiotensin II seems to be augmented in NF-κB KO mice. Although pressor responses to bolus injections of angiotensin II were similar between WT and NF-κB KO mice (data not shown), the differences in systemic blood pressure were evident after 7 days of the treatment, as shown in Fig. 2, suggesting that NF-κB KO might have augmented vascular remodeling in response to angiotensin II. Since inhibition of NF-κB activation in macrophages has been shown to increase atherosclerosis in LDL receptor-deficient mice [25], systemic inhibition of NF-κB may promote vascular injury and atherosclerosis. Finally, it remains to be determined whether inhibition of NF-κB may also attenuate myocardial hypertrophy caused by other stimuli besides angiotensin II. Although Li et al. [26] have recently reported that NF-κB is necessary for aortic banding-induced cardiac hypertrophy, further studies are required in other models of cardiac hypertrophy and heart failure, including volume overload and myocardial infarction.

In conclusion, targeted disruption of the p50 subunit of NF-κB ameliorated myocardial hypertrophy in response to chronic infusion of angiotensin II. The activation of NF-κB may play an important role in the pathogenesis of myocardial hypertrophy and remodeling besides promoting myocardial inflammation. Further studies will be warranted to verify that inhibition of NF-κB may be a promising therapeutic strategy for cardiac remodeling and heart failure.

Acknowledgements

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