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ORIGINAL ARTICLE

INHIBITION OF P38 MAP KINASE ATTENUATES LEFT VENTRICULAR HYPERTROPHY AND INHIBITS PROGRESSION OF SYSTOLIC DYSFUNCTION ON PRESSURE-OVERLOAD INDUCED PATHOLOGICAL CARDIAC HYPERTROPHY IN MICE

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Abstract ***Background:*** P38 mitogen-activated protein kinase (MAP kinase) plays on important role for progression of pathological cardiac hypertrophy. However, the role of p38 MAP kinase in cardiac hypertrophy induced by pressure overload remains unclear. We investigated the effect of chronic treatment with p38 MAP kinase inhibitor on the development of heart failure induced by transverse aortic constriction (TAC) in mice.
Methods and Results: TAC increased left ventricular septal wall thickness (LVSWT) and cross-sectional area (CSA) of cardiomyocyte, and decreased LV fractional shortening (FS) compared with sham operation after 6 weeks. TAC also increased phosphorylation of p38 MAP kinase, whereas other hypertrophic signals were unchanged. In another experiment, TAC mice and sham operated mice were treated with subcutaneous injection of p38 MAP kinase inhibitor SB202190 (5mg/kg/day) or placebo five times a week for six weeks. Treatment with p38 MAP kinase inhibitor attenuated the increase in LVSWT and CSA, and the decrease in FS in mice with TAC.
Conclusions: Inhibition of p38 MAP kinase attenuated left ventricular hypertrophy and inhibited progression of systolic dysfunction in pressure overload-induced cardiac hypertrophy. These results suggest that inhibition of p38 MAP kinase has a protective effect for development of heart failure induced by pressure overload.

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Key words: p38 MAP kinase; cardiac hypertrophy; transverse aortic constriction.

原　著

p38 MAP kinase の抑制はマウスの圧負荷による病的心肥大を軽減し心機能低下を抑制する

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抄録 ***背景:*** P38 mitogen-activated protein kinase (MAP kinase) は病的心肥大の形成に重要な役割を持つが，圧負荷肥大心における役割は不明である．そこで，大動脈縮窄術(TAC)による圧負荷心不全モデルに対して p38 MAP kinase の阻害薬を投与しその効果を検討した．
方法と結果: TAC6 週間後には，マウスの左室中隔壁厚および心筋細胞断面積は増加し，左室短縮率は減少していた．P38 MAP kinase のリン酸化が亢進していた一方で，他の心肥大促進シグナルの発現には変化は見られなかった．次に p38 MAP kinase 阻害薬 SB202190 を TAC 翌日より 6 週間皮下投与を行った(5 mg/kg/day)．左室中隔壁厚および心筋細胞断面積の増大は阻害薬非投与群に比べて軽減し，左室短縮率の低下も軽減した．
結語: 圧負荷肥大心に対して p38 MAP kinase 阻害薬を投与することにより，左室肥大を軽減し心機能低下を抑制した．圧負荷による心不全において p38 MAP kinase 阻害薬は，心不全の進行に対して保護的な効果を有することが示唆された．

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キーワード：p38 MAP kinase；心肥大；大動脈縮窄術．

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INTRODUCTION

Congestive heart failure has become an increasingly frequent reason for hospital admission and clearly represents a major health problem.[1] Cardiac hypertrophy is defined as an abnormal increase in the heart muscle mass with an increase in cardiomyocyte cell volume. Hypertrophic growth of the adult myocardium can occur in response to diverse pathophysiologic stimuli such as hypertension, ischemic heart disease, valvular insufficiency, and cardiomyopathy. Initially, it seems a supportive adaptation of the myocardium to maintain cardiac output characterized by cardiac hypertrophy with preserved systolic function. However, sustained myocardial strain induces contractile depression and myocardial fibrosis, and eventually leads to development of congestive heart failure and early lethality.[2),3)] The underlying mechanism for disease progression, including its initiation and transition to late-stage failure has been extensively investigated,[4)] but still remains unclear.

The mitogen-activated protein (MAP) kinase family plays critical role in intracellular signal transduction and regulation.[5)] The p38 MAP kinase is subfamily of stress-activated protein kinases that were originally discovered as an essential molecule in lipopolysaccharide-induced tumor necrosis factor-alpha expression.[6)] P38 MAP kinase is activated by diverse hypertrophic stimuli, catecholamines, angiotensin II, endothelin-1, hypoxia, and vascular wall stress. In the heart, p38 MAP kinase is activated during development of hypertrophy and heart failure in response to pressure overload[7)] and induced by ischemia-reperfusion.[8)] P38 MAP kinase pathway activation results in phosphorylation of transcription factors affecting cell division, myocyte hypertrophy, contractile function, extracellular matrix remodeling, and cell death regulations.[9),10)] These findings indicate that p38 MAP kinase seems to be a signaling molecule mediating pathological stimuli to cardiac hypertrophy and heart failure. Therefore, inhibition of p38 MAP kinase may be possible to have protective effect for cardiovascular diseases; however, how p38 activation leads to specific aspects of cardiac pathologies in the diseased heart and whether pharmacological inhibition of p38 MAP kinase is a valid approach to treat heart failure are still unknown.

We investigated the effect of chronic treatment with p38 MAP kinase inhibitor on the development of heart failure induced by TAC when the drug administration was started before the emergence of heart failure. The results of the present study suggest that chronic treatment with p38 MAP kinase inhibitor has protective effect for the development of cardiac hypertrophy and heart failure.

MATERIALS AND METHODS

These experiments were performed in accordance with Guidelines for Animal Experimentation, Hirosaki University.

Transverse Aortic Constriction

C57/BL6J mice were anesthetized with intraperitoneal injection of a mixture of ketamine (75mg/kg) and xylazine (7.5mg/kg), and were intubated with a 22 gauge blunt-tipped needle connected to a mechanical ventilator. A midline cervical incision was made and the transverse aorta was ligated with 7-0 nylon string between innominate and left common carotid arteries over 27-gauge needle. After ligation, the needle was removed promptly. Sham mice underwent a comparable operation that a string was twined around the aorta but it was not ligated and subsequently removed. Mice were allowed to recover and were used for experiments.

Transthoracic Echocardiography

Transthoracic echocardiography was performed using a Philips HD11 XE and 15-MHz

probe under light anesthesia with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). Left ventricular septal wall thickness (LVSWT), left ventricular end-diastolic diameter (LVEDD), and left ventricular fractional shortening (FS) were measured.

Experimental Protocols

The p38 MAP kinase inhibitor, SB202190 hydrochloride [FHPI . HCl, 4-(4-Fluorophenyl) -2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole. HCl], was purchased from Enzo Life Sciences (Farmingdale, NY). To evaluate the effect of chronic treatment with p38 MAP kinase on cardiac morphology and function, C57/BL6J mice were assigned to four groups (n =8 in each group): vehicle injection with sham operation (vehicle with sham), SB202190 injection with sham operation (inhibitor with sham), vehicle injection with TAC (vehicle with TAC), and SB202190 injection with TAC (inhibitor with TAC). Subcutaneous injection of SB202190 and the same amount of the vehicle were started at the next day after TAC and continued five times a week for 6 weeks. We used SB202190 at a dose of 5 mg/ kg/day, because it was reported that the same dose was effective to inhibit p38 MAP kinase in the mice heart.[11] In addition, analysis of protein lysates demonstrated that SB202190 treatment blocked cardiac phosphorylation of heart shock protein-27, a well-defined substrate of p38 MAP kinase.[11] SB202190 was dissolved in distilled water to give a final concentration of 0.5 mg/ml.

Body weight was measured before TAC and 6 weeks after TAC. LVSWT, LVEDD, and FS were measured by transthoracic echocardiography before TAC and 6 weeks after TAC. Six weeks after TAC, mice were euthanized by inhalation of ether after echocardiography, and the heart and lung were quickly removed.

Western Blot Analysis

Tissue samples of the heart were homogenized in RIPA lysis buffer (20 $mmol^{-1}$ Tris-HCl, pH 7.5; 150 $mmol^{-1}$ NaCl; 1 $mmol^{-1}$ EDTA; 1 mol^{-1} EGTA; 1% Triton X-100; 1% glycerol; 1 mol^{-1} dithiothretol; and 0.5 $mmol^{-1}$ phenylmethylsulfonyl fluoride). After homogenization, samples were centrifuged at 11,000×g for 10 minutes at 4℃. Protein concentrations of tissue lysates were determined by the Bradford method with bovine serum albumin as a standard. Samples were mixed with Laemmlli buffer and 5% β-mercaptoethanol, and then evenly loaded onto SDS-polyacrylamice gel electrophoresis. The protein was transferred electrophoretically to a polyvinilidene difluride membrane. After blocking for 1 hour, the membranes were incubated with the primary antibodies for phospho-Akt, Akt, phospho-p38 MAP kinase, p38 MAP kinase, phospho-c-Jun N-terminal kinase (JNK), JNK, phospho-extracellular-signal related kinase (Erk) 1/2, Erk1/2, pan-calcineurin A, phospho-p70S6 kinase, p70S6 kinase (all purchased from Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (purchased from Santa Cruz Biotechnology). All primary antibodies were incubated overnight at 4℃ in a 1:1000 dilution. Secondary antibody conjugated to horseradish peroxidase (anti-rabbit, Cell Signaling Technology) was incubated for 1 hour at 1:1000 dilution. Bands were visualized with the ECL system (GE Healthcare). Densitometric analysis was performed using image J software, and the ratio relative to the protein bands was calculated for each sample.

Histological Analysis

Histological analysis was performed by fixing hearts overnight in 10% phosphate-buffered formalin and processed into paraffin blocks for section. Serial-5μm sections were stained with hematoxylin and eosin. For mean cardiomyocyte size, the cross sectional area (100 cells/sample) was averaged.

Statistical Analysis

All data are shown as mean±1SD. One-way analysis of variance for multiple comparisons

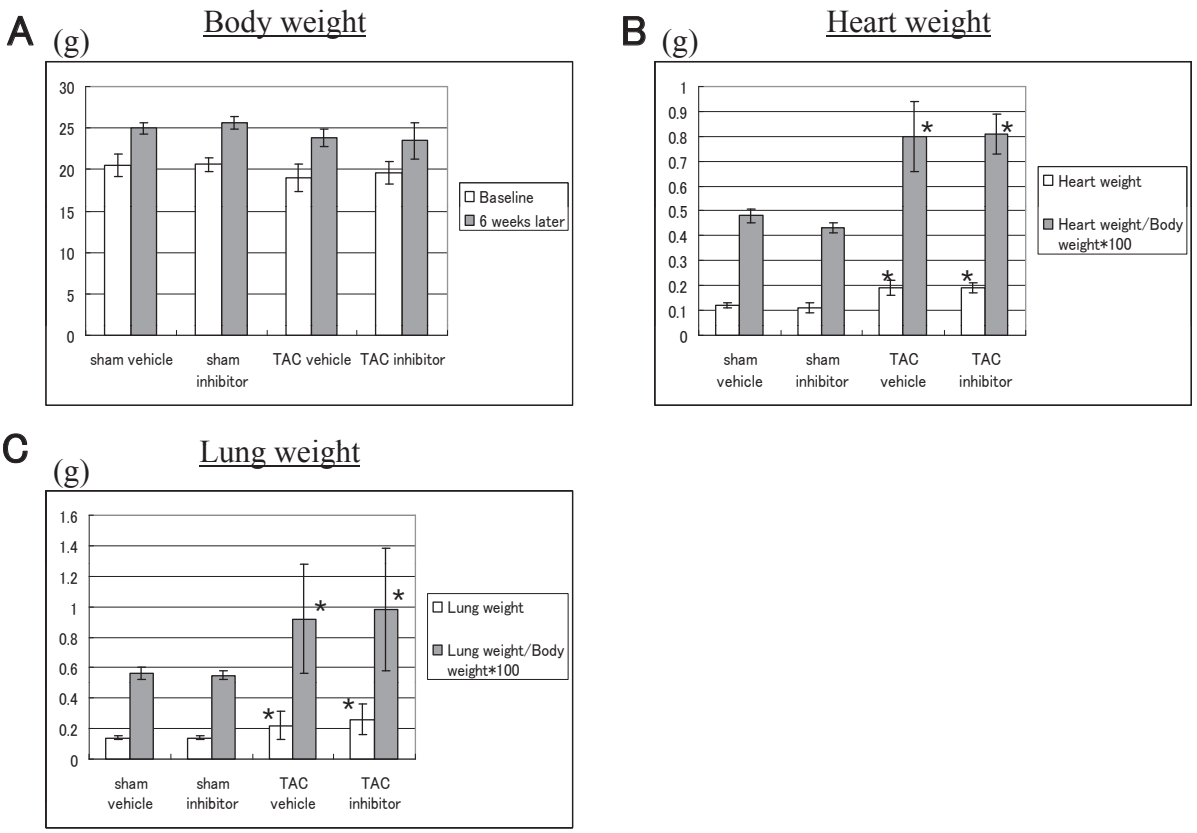

Fig. 1 Body, heart, and lung weight in mice with or without TAC. (A) Body weight did not differ among vehicle with sham, inhibitor with sham, vehicle with TAC, and inhibitor with TAC at baseline and 6 weeks after TAC or sham operation. (B, C) The ratio of heart weight to body weight (x100) was greater in vehicle TAC and inhibitor with TAC compared with vehicle with sham. The ratio of lung weight to body weight (x100) were also greater in vehicle with TAC and inhibitor with TAC compared with sham with vehicle. Both the ratio of heart weight and lung weight to body weight were similar between vehicle with sham and inhibitor with sham. *: $P<0.05$ vs sham with vehicle, 6 weeks later.

followed by Bonferroni's test was used for statistical analysis. The level of significance was less than 0.05.

RESULTS

Body, heart, and lung weights

As shown in Figure 1A, body weight did not differ among vehicle with sham, inhibitor with sham, vehicle with TAC, and inhibitor with TAC at baseline and 6 weeks after TAC or sham operation. Figure 1B and C illustrate the heart or lung weight and their ratio to body weight (x100) at 6 weeks after TAC or sham operation. The ratio of heart weight to body weight (x100) was greater in vehicle with TAC (0.80 ± 0.14) and inhibitor with TAC (0.81 ± 0.08) than vehicle with sham (0.48 ± 0.03, both $p<0.05$). The ratio of lung weight to body weight (x100) was also greater in vehicle with TAC (0.92 ± 0.36) and inhibitor with TAC (0.98 ± 0.40) than sham with vehicle (0.56 ± 0.04, both $p<0.05$). Both the ratio of heart weight and lung weight to body weight were similar between vehicle with sham and inhibitor with sham.

Analysis by transthoracic echocardiography

Mesurements of M-mode echocardiography at baseline and 6 weeks after operation are shown in Figure 2. As shown in Figure 2A, LVSWT was increased in vehicle with TAC

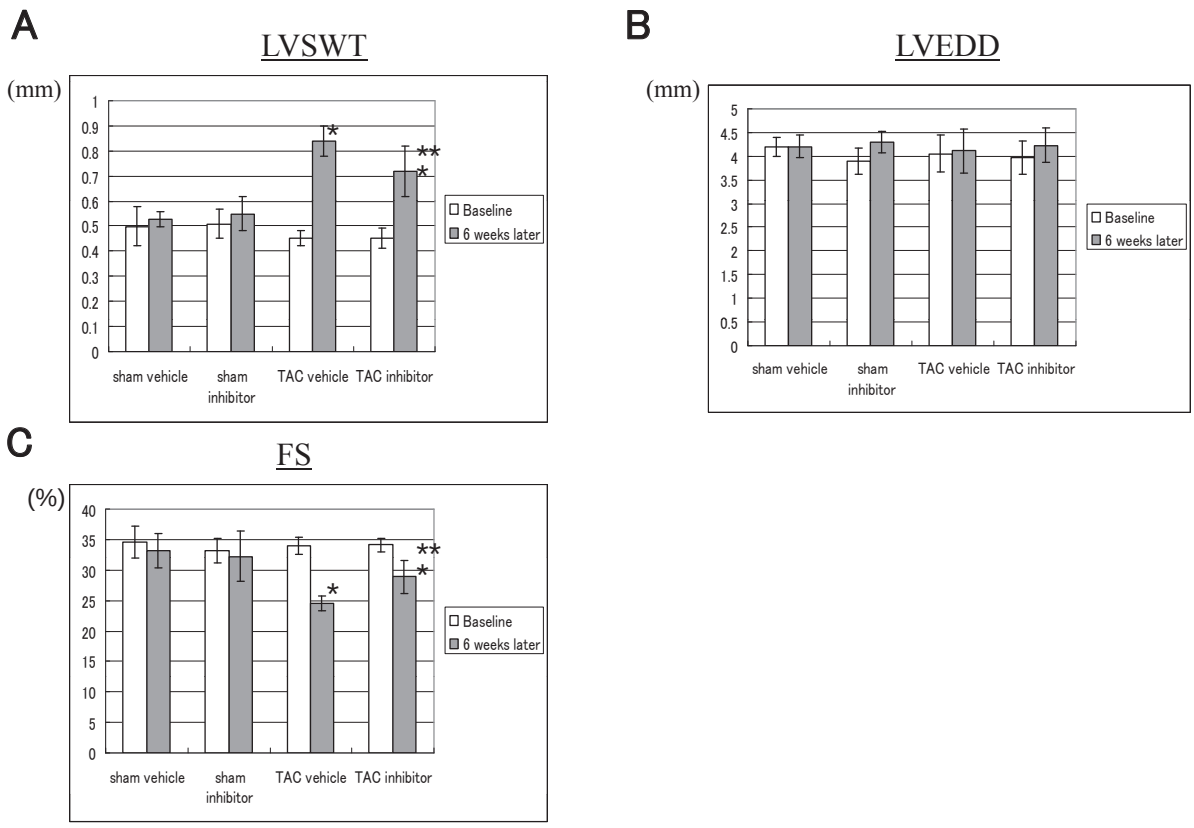

Fig. 2 Mesurements of M-mode echocardiography at baseline and 6 weeks after operation are shown. (A) Left ventricular septal wall thickness (LVSWT) was increased in vehicle with TAC and inhibitor with TAC compared with vehicle with sham 6 weeks later. When compared between vehicle with TAC and inhibitor with TAC, LVSWT in inhibitor with TAC was lower than in vehicle with TAC. LVSWT was similar between vehicle with sham and inhibitor with sham. (B) Left ventricular end-diastolic diameter (LVEDD) was unchanged after procedure in all four groups. (C) Fractional shortening (FS) was decreased in vehicle with TAC and inhibitor with TAC compared with vehicle with sham 6 weeks later. When compared between vehicle with TAC and inhibitor with TAC, it was greater in inhibitor with TAC than in vehicle with TAC. FS was similar between vehicle with sham and inhibitor with sham. *: $P<0.05$ vs vehicle with sham, 6 weeks later. **: $P<0.05$ vs vehicle with TAC, 6 weeks later.

(0.84±0.06mm) and inhibitor with TAC (0.72 ±0.10mm) compared with vehicle with sham (0.53±0.03 mm, both $p<0.05$) 6 weeks later. When compared between vehicle with TAC and inhibitor with TAC, LVSWT in inhibitor with TAC was lower than in vehicle with TAC ($p<0.05$). LVSWT was similar between vehicle with sham and inhibitor with sham. LVEDD was unchanged after procedure in all four groups (Figure 2B). As shown in Figure 2C, FS was decreased in vehicle with TAC (24.6 ±1.2%) and inhibitor with TAC (28.9±2.7%) compared with vehicle with sham (34.2±3.3%, both $p<0.05$) 6 weeks later. When compared between vehicle with TAC and inhibitor with TAC, it was greater in inhibitor with TAC than in vehicle with TAC ($p<0.05$). FS was similar between vehicle with sham and inhibitor with sham. These results suggested that inhibition of p38 MAP kinase attenuated cardiac hypertrophy and inhibited progression of systolic dysfunction in mice exposed to persistent pressure overload.

Expressions of hypertrophic signaling molecules

Figure 3 illustrates expressions of signaling molecules related to cardiac hypertrophy at 6 weeks after TAC or sham operation. The

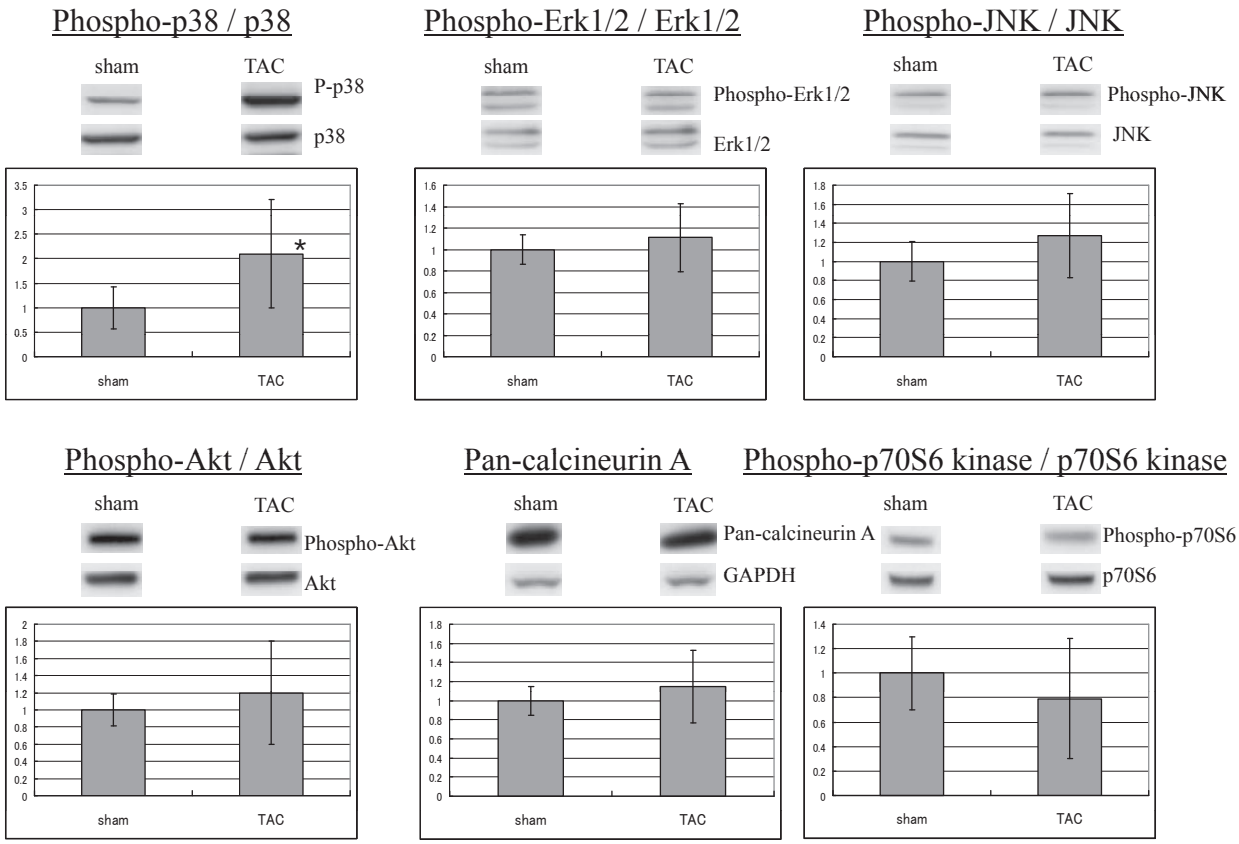

Fig. 3 Expressions of signaling molecules related to cardiac hypertrophy at 6 weeks after TAC or sham operation. The ratio of phospho-p38 MAP kinase to p38 MAP kinase was increased by 2.1 times in TAC compared with sham operation. The protein expression of phospho-JNK and phospho-Erk1/2 (other MAP kinases), phospho-Akt, pan-calcineurin A, and phospho-p70S6 kinase all did not differ between TAC and sham operation. *: P<0.05 vs vehicle with sham, 6 weeks later.

ratio of phospho-p38 MAP kinase to p38 MAP kinase was increased by 2.1 times in TAC compared with sham operation ($p<0.05$). The protein expression of phospho-JNK and phospho-Erk1/2 (other MAP kinases), phospho-Akt, pan-calcineurin A, and phospho-p70S6 kinase all did not differ between TAC and sham operation.

***Histological analysis for cell size* (*Figure 4A and B*)**

Cardiomyocyte cross-sectional area (CSA) was increased in vehicle with TAC ($426 \pm 55 \mu m^2$) and inhibitor with TAC ($322 \pm 70 \mu m^2$) compared with vehicle with sham ($230 \pm 21 \mu m^2$, both $p<0.05$). When compared between vehicle with TAC and inhibitor with TAC, CSA in inhibitor with TAC was smaller than in vehicle with TAC ($p<0.05$).

DISCUSSION

Chronic pressure overload to the heart, which occurs with arterial hypertension, is a common cause of left ventricular remodeling and leads to the development of heart failure.[12] This study showed that TAC caused left ventricular hypertrophy and systolic dysfunction, and increased phosphorylation of p38 MAP kinase, whereas no changes were found in other hypertrophic signals. Inhibition of p38 MAP kinase on persistent pressure-overloaded heart attenuated hypertrophy and inhibited progression of systolic dysfunction compared with vehicle-treated mice. It is noted that inhibition of p38

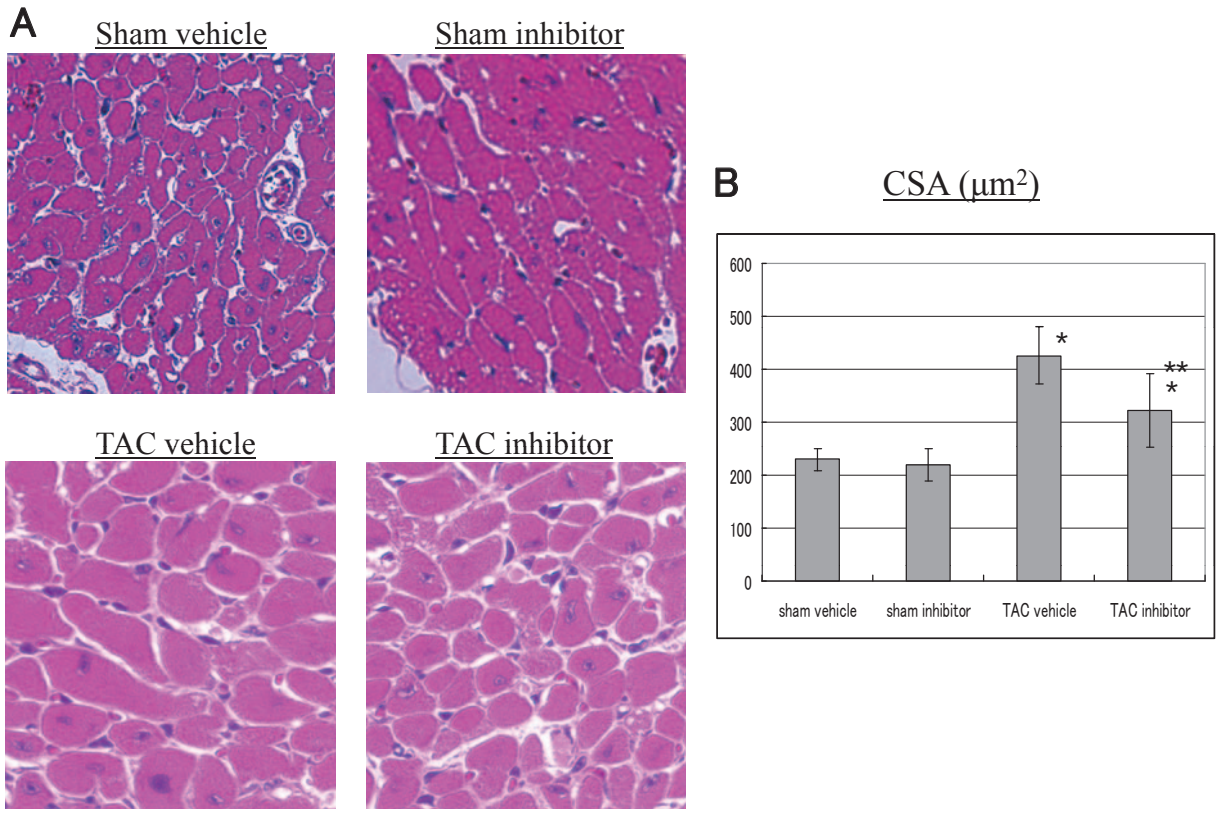

Fig. 4 Cardiomyocyte cross-sectional area (CSA) was increased in vehicle with TAC and inhibitor with TAC compared vehicle with sham. When compared between vehicle with TAC and inhibitor with TAC, CSA in inhibitor with TAC was smaller than in vehicle with TAC. *: P<0.05 vs vehicle with sham, 6 weeks later. **: P<0.05 vs vehicle with TAC, 6 weeks later.

MAP kinase with sham operation manifested no significant influence in left ventricular hypertrophy and systolic function.

There are growing evidences showing that p38 MAP kinase contribute to the regulation of the hypertrophic responses and contractile function. In cultured neonatal cardiomyocytes, activation of the p38 MAP kinase by MAP kinase kinase 3b(E) (MKK3bE) and MAP kinase kinase 6b(E) (MKK6bE) led to an increase in cell size, enhanced sarcomeric organization, and elevated atrial natriuretic factor expression, and this hypertrophic response was suppressed by the p38 beta dominant negative mutant.[7] Similarly, in cultured cardiomyocytes, activation of the p38 MAP kinase augmented cell size and induced natriuretic peptides, and these effects were inhibited by p38 MAP kinase inhibitor.[13] Activation of p38 MAP kinase by MKK3bE, led to a significant reduction in baseline contractility, and this inhibitory effect was largely prevented by coexpressing a dominant-negative mutant of p38 MAP kinase or treating cells with a p38 MAP kinase inhibitor.[14]

In vivo study, a pharmacological inhibitor of p38 enhanced survival, reduced left ventricular hypertrophy, and improved cardiac dysfunction in spontaneously hypertensive rats.[15] Other report said inhibition of p38 improved survival and reduced arrhythmogenic potential in angiotensinogen and renin overexpressing rats.[16] In isolated perfused rat hearts, suppression of p38 MAP kinase activity augmented endothelin-1-induced contractility.[17] TAK1 (MAP kinase kinase kinase signaling factor) overexpressing

transgenic mice in the myocardium induced p38 MAP kinase phosphorylation, and induced cardiac hypertrophy, severe myocardial dysfunction, fetal gene induction, apoptosis and early lethality.[18] Overexpression of MKK6bE exhibited systolic contractile depression,[19] restrictive diastolic abnormalities,[20] and impaired systolic and diastolic function, whereas p38 inhibition improved the cardiac performance and prolonged the survival.[21] Treatment with the p38 MAP kinase inhibitor improved systolic function in diabetic mice.[22] Overall, these studies indicate that p38 MAP kinase is involved in not only the progression of left ventricular hypertrophy but also the occurrence of systolic dysfunction.

Recently, one report said cardiac specific dominant-negative mutants of MKK3, or MKK6, or p38α expressing mice induced systolic dysfunction, and calcineurin was required for this effect.[23] Other report said cardiac specific p38 conditional knockout mice induces cardiac systolic dysfunction, myocyte apoptosis, and interstitial fibrosis against pressure overload.[24] These studies suggest that native p38 MAP kinase signaling may exhibit an antihypertrophic effect in the heart, whereas exaggerated p38 MAP kinase activation is cardiotoxic.

Clinical implications

Collectively, these data suggest that sustained activation of myocardial p38 MAP kinase plays a critical role in the development of hypertensive cardiac hypertrophy and subsequent dysfunction. Long-term treatment with a selective p38 MAP kinase inhibitor attenuated cardiac hypertrophy and inhibited progression of cardiac dysfunction. The efficacy observed with long-term p38 MAP kinase inhibition may represent a common signaling pathway approach to the treatment of heart failure that differs fundamentally from the traditional concept of blocking specific neurohormone receptors.

Sources of Funding

None.

Disclosures

None.

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