Cardiac-specific disruption of the c-raf-1 gene induces cardiac dysfunction and apoptosis

Osamu Yamaguchi,1 Tetsuya Watanabe,1 Kazuhiko Nishida,1,2 Kazunori Kashiwase,1 Yoshiharu Higuchi,1 Toshihiro Takeda,1 Shungo Hikoso,1 Shinichi Hirotani,1 Michio Asahi,1 Masayuki Tanike,1 Atsuko Nakai,1 Ikuko Tsujimoto,3 Yasushi Matsumura,4 Jun-ichi Miyazaki,5 Kenneth R. Chien,6 Atsushi Matsuizawa,7 Chiharu Sadamitsu,7 Hidenori Ichijo,7 Manuela Baccarini,8 Masatsugu Hori,1 and Kinya Otsu1

1Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka, Japan. 2Department of Dental Anesthesiology, First Department of Oral and Maxillofacial Surgery, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan. 3Department of Medical Information Science, Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine, Suita, Osaka, Japan. 4Department of Medicine, School of Medicine, University of California, San Diego, La Jolla, California, USA. 5Laboratory of Cell Signaling and Core Research for Evolutional Science and Technology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan. 6Department of Microbiology and Immunology, Vienna Biocenter, Institute of Microbiology and Genetics, Vienna, Austria.


The Raf/MEK/extracellular signal–regulated kinase (ERK) signaling pathway regulates diverse cellular processes such as proliferation, differentiation, and apoptosis and is implicated as an important contributor to the pathogenesis of cardiac hypertrophy and heart failure. To examine the in vivo role of Raf-1 in the heart, we generated cardiac muscle–specific Raf-1–knockout (Raf CKO) mice with Cre-loxP–mediated recombination. The mice demonstrated left ventricular systolic dysfunction and heart dilatation without cardiac hypertrophy or lethality. The Raf CKO mice showed a significant increase in the number of apoptotic cardiomyocytes. The expression level and activation of MEK1/2 or ERK showed no difference, but the kinase activity of apoptosis signal–regulating kinase 1 (ASK1), JNK, or p38 increased significantly compared with that in controls. The ablation of ASK1 rescued heart dysfunction and dilatation as well as cardiac fibrosis. These results indicate that Raf-1 promotes cardiomyocyte survival through a MEK/ERK–independent mechanism.

Introduction
Heart failure, one of the leading causes of both morbidity and mortality in developed countries, is the convergent phenotype of various diseases that cause loss and dysfunction of cardiomyocytes. Series of parallel and convergent signaling pathways are activated that lead to the common phenotype of heart failure, but molecular mechanisms underlying the pathogenesis of heart failure await clarification. Recent studies have indicated that apoptosis may be an important feature of heart failure (1).

Raf is a family of 3 serine/threonine–specific kinases (A-Raf, B-Raf, and Raf-1) ubiquitously expressed throughout embryonic development. The principal function of the Raf protein kinases appears to be participation in the highly conserved Ras/Raf/MEK/extracellular signal–regulated kinase (ERK) intracellular signaling pathway, which has been implicated in the transduction of signals directing cell proliferation and differentiation. In addition, Raf-1 activation of the MEK/ERK pathway has been associated with inhibition of apoptosis, leading to cell survival (2, 3).

To determine the in vivo role of Raf-1, Raf-1 knockout mice were generated (4, 5). The embryos of these mice are growth retarded and die progressively around midgestation with defects in the placenta and in the liver due to apoptosis (4). The phenotype observed appears to be the result of a lack of activation of Raf-1–specific effectors distinct from the ERK pathway (4–6). At present, the molecular mechanisms underlying the MEK/ERK–independent prosurvival function of Raf-1 are poorly understood. A recent study has demonstrated that Raf-1 physically interacts with the proapoptotic, stress-activated mitogen-activated protein kinase kinase kinase, apoptosis signal–regulating kinase 1 (ASK1) (7), which is a key element in the mechanism of stress- and cytokine-induced apoptosis (8, 9). A variety of stress-related stimuli activate ASK1, including TNF-α, reactive oxygen species, and Fas. ASK1 phosphorylates and activates mitogen-activated protein kinase kinase kinase 4/7 (MKK4/7) and MKK3/6, which in turn activate JNK and p38 MAPK, respectively.

In the heart, the MEK/ERK pathway has been implicated as the signaling cascade leading to hypertrophy of cardiomyocytes (10–12), although a number of additional studies have disputed the importance of the pathway in the regulation of cardiac hypertrophy (13–15). A recent study of MEK1 transgenic mice has suggested that the MEK/ERK signaling pathway stimulates a hypertrophic response associated with resistance to apoptosis (16). However, the in vivo role of Raf-1 in the heart has not been identified yet. In this study, we generated cardiac-specific Raf-1 knockout (Raf CKO) mice to clarify the role of Raf-1 in the heart. These mice showed left ventricular (LV) dysfunction and heart dilatation with an increased number of apoptotic cells. These phenotypes were rescued by inactivation of ASK1. To the best of our knowledge, this is the first study demonstrating that Raf-1 plays a role at the organ level.

Results
Generation of cardiac muscle–specific Raf-1 knockout mice. To obtain a cardiac muscle–specific knockout of the c-raf-1 gene, we used Cre-
Although there were no significant differences in septal wall thickness between Raf CKO mice and CTLs, posterior wall thickness significantly decreased in Raf CKO mice. Catheterization of the LV showed a marked reduction in the maximum or minimum first derivative of LV pressure in Raf CKO mice, thus demonstrating a reduction in myocardial contractility and relaxation (Table 2). There were no significant differences in heart rate, systolic and diastolic pressure, or LV end-diastolic pressure. The mice were followed up at 5, 10, and 15 weeks of age (Figure 1C). In Raf CKO mice, FS was significantly decreased at 10 weeks compared with that at 5 weeks but remained unchanged thereafter.

The Raf CKO mice developed enlarged hearts (Figure 2A) but displayed no differences in heart weight or the average ratio of heart weight to tibia length or to body weight (Table 2). In addition, there were no significant differences in the weight of the LV, RV, atrium, lung, or liver. Histological examination of the heart demonstrated LV enlargement in Raf CKO hearts (Figure 2B), while also demonstrating that the cross-sectional area of cardiomyocytes exhibited no significant differences between Raf CKO mice and CTLs (174.2 ± 6.8 μm² for Raf CKO mice and 184.6 ± 7.7 μm² for CTLs) (Figure 2C). These findings are consistent with LV pump dysfunction without ventricular pump failure. In addition, either endogenous Raf-1 does not play a role in the regulation of developmental cardiomyocyte hypertrophic growth, or its function in the growth process is offset by that of other MEK activators. Masson trichrome staining revealed significant fibrosis in intermuscular areas in Raf CKO mice compared with those in CTLs (Figure 2D).

Increased apoptosis in hearts with Raf-1 conditional depletion. Raf-1 activation of the MEK/ERK pathway is associated with inhibition of apoptosis, leading to cell survival (2, 3, 18). Therefore, it can be hypothesized that the cardiac dysfunction observed in Raf CKO mice was associated with an increase in myocyte apoptosis. TUNEL assay revealed a significant increase in apoptotic cells in Raf CKO hearts at 3, 4, and 5 weeks of age but did not at 2 weeks and after 6 weeks of age (Figure 3A). The appearance of apoptosis was further confirmed by morphological examination, which demonstrated condensed chromatin and fragmented nuclei in TUNEL-positive cells at 3, 4, and 5 weeks of age (Figure 3B). These characteristics of apoptosis. The TUNEL-positive cells were identified as cardiac myocytes by anti–α-sarcomeric actin staining (Figure 3B).

Analysis of apoptosis-related proteins showed an increased level of Bax in Raf CKO mice compared with CTLs. No significant difference was observed in the level of Bcl-2 (Figure 3C).

### Table 1

<table>
<thead>
<tr>
<th>Echocardiographic measurements of the knockout mice at 10 weeks of age</th>
<th>Raf CKO (n = 12)</th>
<th>CTLs (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD (mm)</td>
<td>4.79 ± 0.14*</td>
<td>4.16 ± 0.08</td>
</tr>
<tr>
<td>LVDS (mm)</td>
<td>3.89 ± 0.16*</td>
<td>2.57 ± 0.08</td>
</tr>
<tr>
<td>FS (%)</td>
<td>19.3 ± 1.4*</td>
<td>38.4 ± 1.0</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.76 ± 0.04</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.49 ± 0.02</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>473 ± 16</td>
<td>491 ± 15</td>
</tr>
</tbody>
</table>

LVDD, diastolic left ventricle internal dimension; LVDS, systolic left ventricle internal dimension; IVSd, diastolic interventricle septal wall thickness; LVPWd, diastolic left ventricle posterior wall thickness. Data are expressed as mean ± SEM. *P < 0.05 versus CTLs.
A – 5,957 ± 796
n – A 9,657 ± 835

Table 2
Physiological base-line parameters of Raf CKO mice at 10 weeks of age

<table>
<thead>
<tr>
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<th>Raf CKO (n = 9)</th>
<th>CTLs (n = 7)</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.1 ± 1.0</td>
<td>28.8 ± 1.2</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>130 ± 4</td>
<td>132 ± 7</td>
</tr>
<tr>
<td>LV weight (mg)</td>
<td>89.4 ± 3.6</td>
<td>89.6 ± 4.2</td>
</tr>
<tr>
<td>RV weight (mg)</td>
<td>24.1 ± 1.5</td>
<td>26.1 ± 1.6</td>
</tr>
<tr>
<td>Atrium weight (mg)</td>
<td>12.6 ± 0.6</td>
<td>13.2 ± 1.2</td>
</tr>
<tr>
<td>Lung weight (mg)</td>
<td>141 ± 1</td>
<td>149 ± 5</td>
</tr>
<tr>
<td>Tibia length (mm)</td>
<td>17.7 ± 0.2</td>
<td>17.9 ± 0.1</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>391 ± 33</td>
<td>427 ± 24</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>84.6 ± 4.2</td>
<td>86.8 ± 5.1</td>
</tr>
<tr>
<td>LV systolic pressure (mmHg)</td>
<td>82.4 ± 2.6</td>
<td>86.0 ± 4.1</td>
</tr>
<tr>
<td>LV dp/dt max (mmHg/s)</td>
<td>1.3 ± 0.3</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>LV dp/dt min (mmHg/s)</td>
<td>6,733 ± 411A</td>
<td>9,657 ± 835</td>
</tr>
<tr>
<td></td>
<td>–4,344 ± 287A</td>
<td>–5,957 ± 796</td>
</tr>
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LVEDP, left ventricular end-diastolic pressure. LV dp/dt max and LV dp/dt min are the maximum rates of pressure development during contraction and relaxation, respectively. Data are expressed as mean ± SEM.

MAPK activation in hearts with Raf-1 depletion. To test the activation profile of MEK1/2 or ERK in Raf CKO hearts, the protein level of phosphorylated MEK1/2 or ERK was measured after 3 minutes of intra-arterial infusion of endothelin-1 (Figure 4A). A similar level of MEK1/2 or ERK phosphorylation was observed in Raf CKO and CTL hearts in response to endothelin-1. This indicates that the MEK/ERK pathway was not impaired in Raf CKO hearts. To examine whether the MEK/ERK pathway is involved in cardiac dysfunction observed in Raf CKO mice, we assessed activation of MEK1/2 or ERK in the hearts. There were no differences in the basal protein levels of ERK or MEK1/2 in Raf CKO and wild-type (9), and displayed no differences in either physiological parameters or cardiac performance as analyzed by means of echocardiography and hemodynamic measurement (21). The Raf-1/ASK1 double-knockout mouse was born normally and were fertile. The increase in heart size appeared to be reduced in double-knockout mice compared with that in Raf CKO:ASK+/+ mice (Figure 6A). The protein level of Raf-1 in the double-knockout mice was significantly reduced, as in Raf CKO:ASK+/+ mice (Figure 6B). LV end-diastolic diameter was significantly reduced and FS increased in the Raf-1/ASK1 double-knockout mice compared with Raf CKO:ASK+/+ mice (Fig-
In order to better understand the function of Raf-1 in mature cardiac muscle, we used the Cre-loxP system to specifically inactivate the c-raf-1 gene in cardiac muscle. Our data indicate that the c-raf-1 gene is not essential for mouse heart development or the regulation of developmental cardiomyocyte hypertrophic growth. Our data, however, do not exclude the role of the MEK/ERK pathway in cardiac development or hypertrophy, since MEK1/2 and ERK can be activated in Raf CKO hearts.

Raf CKO mice exhibited cardiac dysfunction and heart dilatation at 5 weeks of age. Raf CKO hearts demonstrated a heightened level of apoptosis at 3, 4, and 5 weeks but not at 2 weeks and after 6 weeks of age, even though cardiac contractility progressively decreased during the 10 weeks. These findings suggest that apoptosis is one of the early causes of cardiac dysfunction rather than a terminal event associated with the end stage. Mice are undergoing marked postnatal growth and hypertrophic stimulation during the first month after birth. We assume that such stress might transiently activate stress-activated signaling molecules such as ASK1 in Raf CKO mice and induce cardiomyocyte apoptosis. To ensure that the resultant anatomical and functional alterations were not in part due to positional effects of the Cre gene, we crossed c-raf-1lox/lox mice with mice expressing the Cre recombinase under the control of the myosin light chain 2v (MLC2v) promoter (22). We observed similar phenotypes in the c-raf-1lox/lox:MLC2v-Cre+/– mice.

Accumulating evidence suggests that apoptosis may be an important mode of cell death during heart failure in both humans and animal models (1). However, the extent to which apoptosis plays a critical role in the pathogenesis of heart failure has remained an open question. Our study demonstrates that induction of apoptosis leads to the development and progression of cardiac dysfunction. A number of biochemical studies have indicated a role for Raf-1 in apoptosis (2, 3). Recent knockout studies of Raf-1 demonstrated that c-raf-1–/– mice die in embryogenesis and show vascular defects in the yolk sac and placenta as well as increased apoptosis in embryonic tissues (4, 5). These findings suggest that Raf-1 plays a key role in preventing apoptosis, which is in agreement with the findings of our study of the heart. However, it is possible that ablation of Raf-1 affected gene expression of calcium-handling proteins, other ion-handling proteins, and contractile proteins, which regulate cardiac contractility. We observed no changes in protein expression of calcium-handling proteins such as ryanodine receptor, SERCA, phospholamban, and calsequestrin (data not shown).

As a limitation of knockout study, we could not exclude the possibility that ablation of one gene leads to alteration of the expression profile of unrelated or related genes. In this study, Raf CKO hearts did not develop compensatory hypertrophy despite cardiac dysfunction and dilatation, which suggests that Raf-1 is involved in compensatory cardiomyocyte growth through a MEK/ERK-independent pathway. This could be another important mechanism of cardiac dysfunction in Raf CKO mice. Further study will be necessary to elucidate a role of Raf-1 in compensatory hypertrophy in response to increased hemodynamic stress.

Raf-1 activation of the MEK/ERK pathway has been associated with inhibition of apoptosis (2, 3). However, studies using conventional c-raf-1–/– mice suggest that effector other than the MEK/ERK cascade must mediate the antiapoptotic function of Raf-1 (4, 5). Our results indicate that Raf-1 promotes cell survival in the heart, as in other tissues, through a MEK/ERK-independent mechanism. In this study, we detected a transient increase in ASK1 activity in Raf CKO hearts. Furthermore, ablation of ASK1 resulted in the rescue of the functional, structural, and histological phenotypes observed in Raf CKO mice. These suggested that ASK1 might play an important role in the apoptosis-induced abnormalities observed in Raf-1–knockout

Discussion

In order to better understand the function of Raf-1 in mature cardiac muscle, we used the Cre-loxP system to specifically inactivate the c-raf-1 gene in cardiac muscle. Our data indicate that the c-raf-1

Figure 3

Apoptosis in Raf CKO hearts. (A) Number of TUNEL-positive cells in Raf CKO hearts (black bars) at 2, 3, 4, 5, 6, 7, 8, and 10 weeks of age compared with CTL hearts (white bars) (n = 3). *P < 0.05 versus CTLs at the same age. (B) Confocal analysis of Raf CKO ventricular myocardium. Triple staining (propidium iodide, TUNEL, and anti–α-sarcomeric actin antibody) was performed. Staining for propidium iodide and anti–α-sarcomeric actin antibody is shown in red, and that for TUNEL in green. In the overlay image, a nucleus stained by both TUNEL and propidium iodide is shown in yellow. Scale bar: 20 μm. (C) Immunoblot analysis of the apoptosis-related proteins in Raf CKO hearts. The expression levels of Bcl-2, Bax, and actin as a control in the indicated tissues were examined with their respective antibodies. (D) The Bax/Bcl-2 ratio is shown (n = 3).
hearts. Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cells (23), while oxidative stress and TNF-induced apoptosis are suppressed in ASK1–/– cells (6, 9). We have previously shown that expression of a constitutively active mutant of ASK1 in cardiomyocytes results in apoptosis, while isolated ASK–/– cardiomyocytes are resistant to H$_2$O$_2$ (21). The ablation of ASK1 in mice prevents heart dilatation and cardiac dysfunction following pressure overload or myocardial infarction, possibly by inhibiting the ASK/JNK signaling pathway (21). The prevention of LV remodeling was accompanied by a reduction in the appearance of apoptotic cardiomyocytes in ASK–/– hearts. Thus, the activation of ASK1 in Raf CKO mice appears to be a cause of cardiac dysfunction and heart dilatation. However, it is still possible that, in mouse hearts in which both the ASK1 and the Raf-1 genes have been ablated, the beneficial effect of ASK1 ablation on LV remodeling independently cancels the detrimental effect of Raf-1 ablation. Alternative pathways aside from ASK1, which could contribute to alteration of cell survival, might mediate the phenotypes observed in Raf CKO mice. As a candidate, we examined Akt activation in Raf CKO mice. We observed no difference in Akt activation between Raf CKO mice and CTLs.

We detected a transient activation of JNK and p38 in Raf CKO mice. We observed no activation of JNK and p38 in the Raf-1/ASK1 double-knockout mice. Knockout studies indicate the role of the JNK pathway in proapoptotic signaling (24). The ASK1/JNK signaling pathway has been reported to be a crucial element of cardiac (21) and neuronal apoptosis (20). Thus, we can hypothesize that the activation of the ASK1/JNK signaling pathway might mediate apoptosis in Raf CKO hearts. In addition to JNK activation, it is also possible that p38 activation might be involved in the apoptotic pathway.

The molecular target for activated ASK1 to induce apoptosis remains to be elucidated. It has been reported that overexpression of a dominant negative mutant of ASK induced cytochrome c release from mitochondria and activation of caspase-9 and caspase-3, but not of caspase-8, in nonmuscle cells (25). This indicates that mitochondria-initiated activation of caspases might be a main mechanism operating in the execution of ASK1-induced apoptosis. The ratio of Bcl-2 protein to Bax protein determines survival or death after an apoptotic stimulus (19). In our study, we observed a decrease in the ratio of Bcl-2 to Bax in Raf CKO hearts, which promotes cell death. However, the mechanism underlying this increase in Bax protein level in response to ASK1 activation in Raf CKO mice remains to be clarified.

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mote cell survival through its protein-protein interactions, which are not related to Raf-1 kinase activity (7). It is possible to hypothesize that Raf-1 promotes cardiomyocyte survival by antagonizing ASK1. Deletion of the N-terminal domain of ASK1 caused the constitutive activation of kinase activity and induction of apoptosis. Raf-1 has been shown to bind to the N-terminal domain of ASK1 (7). Thus, Raf-1 might promote an inactive conformation of ASK1 through the interaction of Raf-1 with the N-terminal domain of ASK1. In addition, the Fas death receptor–associated protein Daxx has been found to activate ASK1 through interaction with the N-terminal portion of ASK1 and lead to apoptosis (26). It is, therefore, also possible that Raf-1 interferes with the binding of ASK1 with Daxx; another possibility is that the antagonizing function of Raf-1 against ASK1 is mediated through Akt activation. It has been reported that Raf-1 is associated with Akt (27), which phosphorylates and negatively regulates ASK1 (28). These findings indicate that Raf-1 may recruit Akt to phosphorylate ASK1, resulting in reduced ASK1 kinase activity. However, we could not detect any change of Akt activation in Raf CKO mice. It has been reported that H-Ras interacts with ASK1 to cause the inhibition of both ASK1 activity and ASK1-induced apoptosis (29). We observed no significant difference in the protein expression level of H-Ras between Raf CKO and CTL hearts (data not shown); this excludes the possibility that altered expression levels of H-Ras in Raf CKO hearts could contribute to enhancement of ASK1 activity. To summarize, Raf CKO mice exhibited cardiac dysfunction and dilatation with increased apoptosis. Activation of the ASK1 signaling pathway seems to play an important role in apoptosis in Raf-1–knockout hearts.

Methods

Generation of the Raf CKO mice. The construction of c-raf-1floxflox and ASK1-depleted mice has been previously described in detail (6, 9). The transgenic mice expressing the Cre recombinase under the control of α-mysin heavy chain promoter (α-MHCCre mice) in C57BL/6J background were generated as previously reported (17). To confirm the cardiac-specific expression of the Cre recombinase in the α-MHCCre mice, they were mated with a transgenic mouse line carrying a reporter gene construct, which directs expression of the *Escherichia coli lacZ* gene following Cre-mediated excision of the loxP-flanked chloramphenicol acetyltransferase gene (30). Crossing the α-MHCCre mice with the reporter mice resulted in Cre-mediated recombination in the heart, but not in other tissues, as determined by X-gal staining (data not shown). The c-raf-1floxflox mice were mated with α-MHCCre mice to obtain c-raf-1floxflox/α-MHCCre(+) mice, which harbor cardiac muscle–specific Raf-1 knockout. The ASK−/− mice were in the C57BL/6J background and were crossed with c-raf-1floxflox/α-MHCCre(+) mice to obtain double-knockout mice [c-raf-1floxflox/α-MHCCre(+)·ASK−/−].

This study was carried out under the supervision of the Animal Research Committee of Osaka University and in accordance with the Guidelines for Animal Experiments of Osaka University and the Japanese Animal Protection and Management Law (No. 25).

Echocardiography and hemodynamic analysis. Sequential murine transthoracic echocardiography (SONOS 5500, equipped with a 15-MHz linear transducer; Philips Medical Systems) was performed on anesthetized mice intraperitoneally injected at 5, 10, and 15 weeks with 2.5% avertin (8 μl/g), as previously described (31). Hemodynamic measurements were performed on mice intraperitoneally anesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine, as done previously (32).

Histological analysis. The heart samples were removed in diastole and immediately fixed with buffered 3.7% formalin, embedded in paraffin, and sectioned at 3 μm thickness. H&E or Masson trichrome staining was performed on serial sections.
Evaluation of apoptosis. To determine the quantity of cells undergoing nuclear fragmentation, TUNEL assay was performed on paraffin-embedded heart sections using an in situ apoptosis detection kit (Takara Bio Inc.). The number of TUNEL-positive nuclei was counted by examination of the entire section with a ×40 objective. Triple staining with propidium iodide (Vector Laboratories Inc.), TUNEL, and anti-α-tubulin III axon antibody (Sigma-Aldrich) was performed.

Western blots. Mouse cardiac homogenates were prepared as previously described (33). Total protein homogenates (50 μg/lane) were subjected to Western blot analysis using antibodies against mouse Bax (N-20), p38 (N-20), ERK1 (K-23), JNK1 (FL), and H-Ras (C-20) from Santa Cruz Biotechnology Inc.; antibodies against MEK1/2, phospho–MEK1/2, phospho-ERK, phospho-JNK, phospho-p38, Akt, and phospho-Akt from Cell Signaling Technology Inc.; and antibody against Raf-1 from Transduction Laboratories. Western blots were developed with the ECL kit, ECL Plus kit, or ECL Advance kit (Amersham Biosciences Corp.). Quantification of signals was performed by densitometry of scanned autoradiographs with the aid of Scion Image software (version 4.02, Scion Corp.). In some studies, mice were treated before excision with endothelin-1 (50 μg/kg/min, intra-arterial perfusion) for 3 minutes to activate MAPK.

In vitro kinase assay. The activity of ASK1 was measured by immune complex kinase assay as described previously (8). Immunoprecipitation of endogenous ASK1 was performed on 500 μg of myocardial extracts as reported previously (23). Immune complex kinase activity was measured as previously described (34).

Statistical analysis. Results are shown as mean ± SEM. Paired data were evaluated by Student’s t test. A 1-way ANOVA with the Bonferroni’s post hoc test or repeated-measures ANOVA was used for multiple comparisons. A value of P less than 0.05 was considered statistically significant.

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Osamu Yamaguchi and Tetsuya Watanabe contributed equally to this work.

Address correspondence to: Kinya Otsu, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-3635; Fax: 81-6-6879-3645; E-mail: kotsu@medone.med.osaka-u.ac.jp.