Apoptosis Signal-Regulating Kinase-1 Is Involved in Vascular Endothelial and Cardiac Remodeling Caused by Nitric Oxide Deficiency


Abstract—Long-term treatment with  N\textsuperscript{\textcircled{O}}-nitro-L-arginine methylester (L-NAME), an NO synthase inhibitor, induces hypertension and cardiovascular injury. However, its precise mechanism is unknown. Using apoptosis signal-regulating kinase-1 (ASK1)-deficient mice, we investigated the role of ASK1 in cardiovascular injury caused by L-NAME treatment. L-NAME was orally administered to ASK1-deficient and C57BL/6J (wild) mice for 8 weeks. L-NAME treatment increased blood pressure of wild and ASK1-deficient mice to a similar extent, indicating no role of ASK1 in NO-deficient hypertension. L-NAME treatment significantly impaired acetylcholine-induced carotid arterial relaxation in wild mice (P<0.01), being associated with the decreased endothelial NO synthase (eNOS) activity (P<0.01) and the increased disruption of eNOS dimer (P<0.01), whereas these changes by L-NAME were substantially attenuated in ASK1-deficient mice. Thus, ASK1 is involved in the impairment of vascular endothelial function by reducing eNOS activity and disrupting eNOS dimer. L-NAME treatment increased vascular reduced nicotinamide-adenine dinucleotide phosphate oxidase activity and superoxide in wild mice to a greater extent than in ASK1-deficient mice. L-NAME treatment in wild mice caused cardiac hypertrophy, myocyte apoptosis, macrophage infiltration, coronary arterial remodeling, interstitial fibrosis, and the expression of monocyte chemoattractant protein-1 and transforming growth factor-\(\beta\)1, whereas these cardiac changes by L-NAME were absent in ASK1-deficient mice. Cardiac reduced nicotinamide-adenine dinucleotide phosphate oxidase activation and superoxide elevation by L-NAME were much less in ASK1-deficient mice than in wild mice. Our work provided the first evidence that ASK1 is implicated in vascular endothelial dysfunction and cardiovascular remodeling induced by NO deficiency by regulating eNOS and reduced nicotinamide-adenine dinucleotide phosphate oxidase. (Hypertension. 2007;50:519-524.)

Key Words: cardiovascular diseases ■ endothelium-derived relaxing factor ■ NO synthase ■ oxidative stress ■ signal transduction

Nitric oxide, particularly produced by endothelial NO synthase (eNOS), plays a central role not only in physiology of vascular endothelial function and blood circulation but also plays a protective role against hypertension and cardiovascular injury.\(^1\)\(^2\) \textit{N}\textsuperscript{\textcircled{O}}-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue, is the agent most frequently used as an NO synthase inhibitor in vitro and in vivo. It has been well established that NO deficiency by long-term oral treatment with L-NAME causes hypertension and cardiovascular remodeling in experimental animals.\(^3\)\(^4\)\(^5\) Furthermore, this model induced by chronic L-NAME treatment is regarded as a useful and popular model to investigate the mechanism of cardiovascular diseases caused by NO deficiency. Interestingly, cardiovascular injury induced by chronic L-NAME treatment cannot be prevented by normalization of blood pressure with coadministration of hydralazine, a vasodilator.\(^6\) Thus, cardiovascular damage by L-NAME is not mainly attributed to blood pressure elevation but to systemic and local neurohumoral factors, such as the renin-angiotensin system.\(^6\)\(^7\)\(^8\) However, the detailed mechanism underlying cardiovascular injury caused by NO deficiency with L-NAME treatment remains to be determined. Particularly, available information is scarce concerning the intracellular signaling molecule responsible for L-NAME-induced cardiovascular damage.

Apoptosis signal-regulating kinase-1 (ASK1), a mitogen-activated protein kinase kinase kinase, has been initially identified as a proapoptotic kinase.\(^9\) However, recently, ASK1 has been reported to also be implicated in a variety of cellular functions, including cell proliferation, survival, dif-
The effect of long-term L-NAME treatment on ASK1-synthase inhibition. To demonstrate our hypothesis, we examined the role of ASK1 in cardiovascular injury during long-term NO deficiency. However, there is no report concerning the role of ASK1 in cardiovascular injury under an NO-deficient state.

In the present work, we hypothesized that ASK1 may contribute to cardiovascular injury during long-term NO synthase inhibition. To demonstrate our hypothesis, we examined the effect of long-term L-NAME treatment on ASK1-deficient mice and obtained the first evidence that ASK1 plays a crucial role in vascular endothelial dysfunction and cardiovascular remodeling under the NO-deficient state.

Methods

Animals

ASK1−/− mice were backcrossed into C57BL/6J background by ≥10 generations to reduce genetic variation. Wild-type mice on the same genetic background (C57BL/6J) were purchased from KBT Oriental (Hamamatsu, Japan). All of the procedures were in accordance with institutional guidelines for animal research.

Chronic L-NAME Treatment

L-NAME (Dojindo) dissolved in the drinking water (1 mg/mL) was orally given to 8-week-old ASK1−/− mice and age-matched wild-type mice for 8 weeks. Throughout the experiment, blood pressure of the conscious mice was measured every week with the tail-cuff method (BP98A; Softron Co). During the treatment, water intake was 4.19±0.12, 4.05±0.09, 4.24±0.09, and 4.23±0.06 mL/d in wild-type mice without and with L-NAME treatment and ASK1−/− mice without and with L-NAME treatment (n=10 to 11 per group), respectively, indicating no statistically significant difference among 4 groups of mice. Thus, wild-type and ASK1−/− mice consumed the same amount of L-NAME. After 8 weeks of L-NAME treatment, mice were anesthetized with ether, and the blood was obtained by cardiac puncture to measure plasma nitrogen oxide. Then, the heart, carotid arteries, and thoracic aorta were rapidly excised from each mouse to evaluate vascular relaxing response, reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase activity, superoxide, and eNOS activity and to perform Western blot analysis, histological examination, and real-time PCR, as described in detail in the online supplemental data available at http://hyper.ahajournals.org.

Results

Effect of L-NAME on Blood Pressure

ASK1-deficient mice were indistinguishable in appearance from wild-type mice, and no developmental abnormalities were observed in histological analysis. As shown in Figure S1, under basal condition, there was no significant difference in blood pressure between wild-type and ASK1-deficient mice. Eight weeks of L-NAME treatment significantly elevated the blood pressure of wild-type and ASK1-deficient mice, to a comparable degree, throughout the treatment (Figure S1).

Effect of L-NAME on Vascular Endothelial Function

In mice without L-NAME treatment, there was no significant difference in carotid arterial endothelium-dependent relaxation by acetylcholine (Figure 1) or endothelium-independent relaxation by S-nitroso-N-acetyl-DL-penicillamine (NO donor; Figure S2) between wild-type and ASK1-deficient mice. Chronic L-NAME treatment significantly impaired acetylcholine-induced endothelium-dependent relaxation in wild-type mice, whereas it failed to impair it in ASK1-deficient mice (Figure 1). L-NAME treatment did not significantly impair S-nitroso-N-acetyl-DL-penicillamine-induced carotid arterial relaxation in wild-type or ASK1-deficient mice (Figure S2).

Effect of L-NAME on Vascular eNOS Activity, Plasma Nitrogen Oxide, eNOS Uncoupling, Phospho-eNOS, and Total eNOS

As shown in Figure 2A and 2B, chronic L-NAME treatment significantly decreased vascular eNOS activity (P<0.01) and plasma nitrogen oxide (P<0.01) in wild-type mice but did not reduce them in ASK1-deficient mice. As shown in Figure 2C, low-temperature SDS-PAGE coupled with Western blot analysis indicated that the ratio of eNOS dimer to monomer was significantly reduced by L-NAME treatment in wild-type (P<0.01) and ASK1-deficient mice (P<0.05). However, the decrease in eNOS dimer:monomer ratio by L-NAME was significantly attenuated in ASK1-deficient mice compared with wild-type mice (P<0.05).

As shown by Western blot analysis in Figure S3, without L-NAME treatment, no significant difference was noted between wild-type and ASK1-deficient mice in vascular phospho-eNOS and total eNOS levels. L-NAME treatment did not alter vascular phospho-eNOS and total eNOS levels in wild-type and ASK1-deficient mice.

Effect of L-NAME on Vascular NADPH Oxidase Activity and Superoxide Levels

As shown in Figure 3, L-NAME treatment in wild-type mice significantly increased vascular NADPH oxidase activity (P<0.01), accompanied by the increase in vascular superox-
ide levels ($P<0.01$). L-NAME–treated ASK1-deficient mice exhibited less vascular NADPH oxidase activity ($P<0.05$) and less superoxide levels ($P<0.05$) than L-NAME–treated wild-type mice. The presence of apocynin in the reaction mixture virtually abolished the above mentioned NADPH oxidase activity in all 4 of the groups (data not shown), confirming the specificity of the assay for NADPH oxidase activity.

**Effect of L-NAME on Vascular p38 and c-Jun N-Terminal Kinase Phosphorylation**

Because ASK1 is well known to preferentially activate p38 and c-Jun N-terminal kinase, we determined the phosphorylation of p38 and c-Jun N-terminal kinase in mice with or without L-NAME treatment. Western blot analysis in Figure S4 shows that L-NAME treatment significantly increased vascular phospho-p38 and phospho-c-Jun N-terminal kinase in wild-type mice but not in ASK1-deficient mice.

**Effect of L-NAME on Cardiac Hypertrophy and Remodeling and Coronary Remodeling**

As shown in Figure 4 and Figure S5, chronic L-NAME treatment in wild-type mice markedly increased left ventricular weight corrected for body weight ($P<0.01$), the number of TUNEL-positive cardiomyocytes ($P<0.01$), macrophage infiltration ($P<0.01$), interstitial fibrosis ($P<0.01$), coronary arterial thickening ($P<0.01$), and perivascular fibrosis ($P<0.01$), whereas chronic L-NAME treatment did not change these parameters in ASK1-deficient mice.

**Effect of L-NAME on Cardiac Monocyte Chemoattractant Protein-1 and Transforming Growth Factor-$\beta$1 mRNA Expression**

Real-time RT-PCR analysis indicated that L-NAME administration significantly increased cardiac monocyte chemoattractant protein-1 (MCP-1; $P<0.01$) and transforming growth factor-$\beta$1 (TGF-$\beta$1; $P<0.01$) mRNA expression in wild-type mice but did not increase them in ASK1-deficient mice (Figure S6).

**Effect of L-NAME on Cardiac NADPH Oxidase Activity and Superoxide Levels**

Figure S7 indicates that L-NAME treatment significantly increased cardiac NADPH oxidase activity ($P<0.05$) and superoxide ($P<0.01$) in wild-type mice, whereas it did not...
significantly increase them in ASK1-deficient mice. The presence of apocynin or diphenyleneiodonium in the reaction mixture virtually abolished the above-mentioned NADPH oxidase activity in all 4 of the groups (data not shown), confirming the specificity of the assay for NADPH oxidase activity.

**Discussion**

The major findings of our present work were that ASK1 plays a pivotal role in the impairment of vascular endothelial function and cardiovascular hypertrophy and remodeling caused by chronic L-NAME treatment and that the protective effects of ASK1 deficiency on L-NAME–induced cardiovascular injuries were mediated by the normalization of eNOS activity, the suppression of eNOS dimer disruption, and the inhibition of NADPH oxidase activation. Thus, our present work provided the novel molecular mechanism underlying cardiovascular damage by NO deficiency.

ASK1 is known to be activated by various stress stimuli relevant to the pathophysiology of cardiovascular diseases and to participate in various cellular responses, such as apoptosis, growth, inflammation, gene expression, etc.

Furthermore, using ASK1-deficient mice, we have reported previously the important role of ASK1 in cardiovascular injury and angiogenesis. Therefore, ASK1 seems to be one of the key molecules responsible for the pathophysiology of cardiovascular diseases. However, to the best of our knowledge, the role of ASK1 under an NO-deficient state has not been reported. Moreover, our previous work did not address the detailed mechanism responsible for the contribution of ASK1 to cardiovascular diseases, and the precise role of ASK1 in cardiovascular diseases is poorly understood.

Therefore, in this work, we assessed the detailed role of ASK1 in cardiovascular injury caused by chronic L-NAME treatment.

In this work, L-NAME treatment increased blood pressure of ASK1-deficient mice to a comparable degree to wild-type mice throughout the treatment. Thus, our work indicates that ASK1 plays no role in the development of hypertension caused by L-NAME treatment. Our present findings on L-NAME treatment are in good agreement with our previous findings that ASK1 deficiency does not affect hypertension induced by angiotensin II infusion, confirming no role of ASK1 in blood pressure regulation. Furthermore, in our present work, no difference in blood pressure between ASK1 and wild-type mice allowed us to examine the role of ASK1 in L-NAME–induced cardiovascular injury, independent of blood pressure.

To the best of our knowledge, there is no report concerning the role of ASK1 in vascular endothelial function and eNOS regulation. In the present work, chronic L-NAME treatment caused the marked impairment of vascular endothelial function in wild-type mice, as shown by the decreased vascular relaxation with acetylcholine but not with S-nitroso-N-acetyl-DL-penicillamine (Figure 1 and Figure S2), which was associated with the significant reduction of vascular eNOS activity (Figure 2A). Of note are the observations that ASK1-deficient mice treated with L-NAME exhibited no impairment of vascular endothelial function and no reduction of eNOS activity. Hence, our present work provided the first evidence that ASK1 deficiency protects against the impairment of vascular endothelial function by preventing the reduction of eNOS activity.

eNOS activity is known to be regulated by multiple mechanisms, including phosphorylation of eNOS, eNOS
protein expression, and eNOS uncoupling. In this work, to elucidate the underlying mechanism of preservation of eNOS activity in L-NAME–treated ASK1-deficient mice, we compared vascular phospho-eNOS and total eNOS levels and the ratio of eNOS dimer:monomer between both strains of mice. Regardless of L-NAME treatment, there was no difference in vascular phospho-eNOS and total eNOS protein levels between either strain of mice, thereby indicating no alteration of phosphorylation and protein expression of eNOS by L-NAME. eNOS under a physiological state exists as a dimeric form and generates NO from arginine, but excessive oxidative stress observed under various pathological conditions is known to disrupt the eNOS dimer to generate the eNOS monomer. eNOS monomer has no enzymatic activity to generate NO and instead produces superoxide (eNOS uncoupling). In the present study, low-temperature SDS-PAGE analysis showed that L-NAME treatment reduced the ratio of eNOS dimer to monomer in wild-type mice and ASK1-deficient mice, indicating the disruption of eNOS dimer by chronic L-NAME treatment. However, of note, L-NAME–treated ASK1-deficient mice exhibited less disruption of the eNOS dimer than L-NAME–treated wild-type mice (Figure 2C). These findings indicate that the absence of reduction of eNOS activity in L-NAME–treated ASK1-deficient mice might be at least in part attributed to the attenuation of eNOS dimer disruption (eNOS uncoupling).

Previously, we have reported that angiotensin II–induced cardiac hypertrophy, apoptosis, fibrosis, and coronary remodeling are significantly attenuated in ASK1-deficient mice. Vascular remodeling induced by cuff injury is also lessened in ASK1-deficient mice. Furthermore, hind limb ischemia-induced angiogenesis is attenuated in ASK1-deficient mice, being associated with the attenuation of expression of MCP-1 and macrophage infiltration. In the present work, we found that ASK1 deficiency inhibited cardiac hypertrophy, myocyte apoptosis, interstitial fibrosis, and coronary arterial remodeling under L-NAME treatment. These present findings were consistent with our previous findings on angiotensin II infusion. Furthermore, in this work, we also examined cardiac macrophage infiltration and expression of MCP-1 which plays a major role in macrophage infiltration. We obtained the evidence that ASK1 deficiency suppressed macrophage infiltration and the enhanced expression of MCP-1 caused by L-NAME, findings similar to our previous report on the hind limb ischemia model. We also examined cardiac TGF-β1 expression, because TGF-β1 is a major growth factor responsible for tissue fibrosis. We found that the enhancement of TGF-β1 expression caused by L-NAME is weak in ASK1-deficient mice, suggesting the contribution of TGF-β1 to less cardiac fibrosis in L-NAME–treated ASK1-deficient mice. Hence, the prevention of cardiac injury by ASK1 deficiency might be at least partially attributed to the inhibition of MCP-1 and TGF-β1 expressions. Thus, our present findings extend the important role of ASK1 in pathophysiology of cardiac diseases.

NO and reactive oxygen species (ROS) play a counterregulatory role in vascular endothelial function and cardiovascular hypertrophy and remodeling. NO synthase inhibition by L-NAME treatment causes NO deficiency, leading to the condition of excessive ROS in cardiovascular tissue. Accumulating evidence indicates that NADPH oxidase is a major enzyme producing superoxide (ROS) and NADPH oxidase-mediated ROS causes vascular endothelial dysfunction and remodeling and cardiac hypertrophy and remodeling. Therefore, in the present work, we compared the effect of L-NAME treatment on NADPH oxidase activity and superoxide levels between wild-type and ASK1-deficient mice. We obtained the evidence that L-NAME–treated ASK1-deficient mice showed no increase in cardiac and vascular NADPH oxidase activity in contrast to the significant increase in NADPH oxidase in L-NAME–treated wild-type mice. Furthermore, cardiac and vascular superoxide levels in ASK1-deficient mice treated with L-NAME were significantly smaller than those in wild-type mice with L-NAME. These results show that ASK1 deficiency attenuates ROS production caused by L-NAME by decreasing NADPH oxidase activity. Taken together with the fact that NADPH oxidase–mediated ROS plays a key role in vascular endothelial dysfunction and cardiovascular remodeling, our present results support the notion that the absence of an increase in NAPDH oxidase activity might be at least in part involved in the attenuation of cardiovascular remodeling in ASK1-deficient mice treated with L-NAME.

In conclusion, we have first examined the role of ASK1 in cardiovascular injury under the NO-deficient state and provided the first evidence that ASK1 plays a pivotal role in the impairment of vascular endothelial function, vascular remodeling, and cardiac hypertrophy and remodeling. Furthermore, these protective effects of ASK1 deficiency seem to be attributed to the suppression of eNOS dimer disruption and the inhibition of NAPDH oxidase activation. Thus, our work provided a novel insight into the molecular mechanism underlying cardiovascular injury caused by NO deficiency.

**Perspectives**

The decrease in NO synthesis in cardiovascular tissue commonly occurs in metabolic syndrome, including hypertension, diabetes, obesity, and hyperlipidemia, and plays a key role in the pathophysiology of cardiovascular diseases, such as vascular endothelial dysfunction, and cardiovascular hypertrophy and remodeling. Therefore, it is of great clinical relevance to elucidate the precise mechanism underlying cardiovascular diseases caused by NO deficiency. ASK1 is well known to be activated by stress stimuli, such as angiotensin II, ROS, etc, and participates in a variety of cellular responses. In our present work, by using ASK1-deficient mice, we obtained the first evidence that ASK1 deficiency prevents the impairment of vascular endothelial function, vascular remodeling, cardiac hypertrophy, apoptosis, inflammation, and fibrosis under an NO-deficient state with chronic L-NAME treatment. Furthermore, we showed that these protective effects of ASK1 deficiency in L-NAME–treated mice were attributed to the suppression of eNOS dimer disruption and NADPH oxidase activation. Therefore, our present work provides a novel insight into the molecular mechanism of cardiovascular diseases and highlights ASK1 as a potential therapeutic target molecule for cardiovascular injury caused by NO deficiency.
Disclosures

None.

References

Apoptosis Signal-Regulating Kinase-1 Is Involved in Vascular Endothelial and Cardiac Remodeling Caused by Nitric Oxide Deficiency

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*Hypertension*. 2007;50:519-524; originally published online July 23, 2007; doi: 10.1161/HYPERTENSIONAHA.107.092049

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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Online Expanded Materials and Methods

Vessel Ring Preparation and Organ Chamber Experiments

Isometric tension studies were performed, as previously described by us. In brief, carotid arteries from each group of mice were cut into 5 mm rings with special care to preserve the endothelium, and mounted in organ baths filled with modified Tyrode buffer (pH 7.4; NaCl 121 mmol/L, KCl 5.9 mmol/L, CaCl₂ 2.5 mmol/L, MgCl₂ 1.2 mmol/L, NaH₂PO₄ 1.2 mmol/L, NaHCO₃ 15.5 mmol/L, and D(+)-glucose 11.5 mmol/L) aerated with 95% O₂ and 5% CO₂ at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. A resting tension of 0.5 g was maintained throughout the experiment. Vessel rings were precontracted with prostaglandin F2α (10⁻⁵ M). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) (10⁻⁹ mol/L to 10⁻⁵ mol/L) or S-nitroso-N-acetylpenicillamine (SNAP) (10⁻⁸ M~10⁻⁴ M) to obtain cumulative concentration-response curves.

Vascular and cardiac NADPH oxidase activity

Aortic tissue pooled from 3 mice and left ventricular tissue from individual mice were homogenized with an Ultraturrax T8, centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of 10 µM NADPH and 5 µM lucigenin as electron acceptor, as described. To confirm that the measured activity was attributed to NADPH oxidase, the assay was also performed in the presence of specific NADPH oxidase inhibitors, diphenyleneiodonium (DPI) (10 µM) or apocynin (1 mM). Protein concentrations were measured by the method of Bradford.

Measurement of vascular and cardiac superoxide

Carotid arteries and cardiac tissues, removed from mice, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate tissue superoxide levels in situ, as described. DHE fluorescence of arterial and cardiac sections were quantified using Lumina Vision version 2.2, analysis software. The mean fluorescence was quantified and expressed relative to values obtained for control mice.

Preparation of arterial protein extracts and Western blot analysis of eNOS, p38, and JNK

Our detailed method has been described previously. Briefly, after aortic protein extracts were subjected to sodium-dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: phospho-eNOS (Ser-1177) (x 5000, BD Transduction Laboratories), eNOS (x 5000, BD Transduction Laboratories), phospho-p38 (x 2000, Cell Signaling), phospho-c-Jun N-terminal kinase (JNK) (x 2000, Cell Signaling), p38 (x 2000, Cell Signaling), JNK (x 2000, Cell Signaling), α-tubulin (x 5000, CALBIOCHEM). In individual samples, each value was corrected for that of α-tubulin, since α-tubulin is regarded as a useful internal control for Western blot analysis.

**Determination of eNOS dimer and monomer**

eNOS dimer and monomer were separated, using low-temperature sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis.

For immunoblot analysis of the dimeric and monomeric form of eNOS protein, aortic samples were not heated and the temperature of the gel was maintained at 4 °C during electrophoresis (low-temperature SDS-PAGE). The proteins were transferred by semidy electroblotting to polyvinylidene difluoride membranes for 90 min. The blots were then blocked and incubated with anti-eNOS monoclonal antibody for overnight at 4 °C. Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (x5000, Santa Cruz Biotechnology). The antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham Biosciences). The intensity of the bands was quantified using NIH Image analysis software v1.61.

**NOS activity (arginine-to-citrulline conversion) and plasma NOx**

Ca²⁺-dependent and -independent NOS activity was determined by measuring the conversion of [¹⁴C] arginine to [¹⁴C] citrulline during the enzymatic formation of NO, using a NOS Activity Assay Kit™ (Cayman Chemical Company, Ann Arbor, MI).

To determine plasma NO, plasma NOx was measured by using a total NO Assay Kit-FX™ (Dojindo, Kumamoto, Japan).

**Histological Examination**

The hearts from each mouse were fixed in 4 % paraformaldehyde overnight. Then, they were embedded in paraffin, sectioned into 5-µm slices, stained with Sirius Red F3BA (0.5 % in saturated aqueous picric acid, Aldrich Chemical Company) for assessment of cardiac interstitial fibrosis and coronary remodeling. To evaluate coronary remodeling, coronary arterial thickening was assessed by calculating the wall-to-lumen ratio (the medial thickness to the internal diameter). The area of perivascular fibrosis per vessel was assessed by using Lumina Vision version 2.2.
ASK1, NO deficiency, and cardiovascular injury

analysis software.

Apoptosis in cardiac section was detected with the TdT-mediated dUTP nick-end labeling (TUNEL) by utilizing Apoptosis in situ Detection Kit Wako (Wako, Osaka, Japan).

For detection of macrophage infiltration, cardiac sections were blocked with 0.3% H$_2$O$_2$ for 30 minutes, washed in water, treated with 5% bovine serum albumin in PBS, washed with PBS, and incubated with the primary antibodies (CD68 rat anti-mouse macrophages, Serotec; x500) at 37°C for 5 hours. After being washed with PBS, the primary antibody was detected by using a biotinylated anti-rat IgG at room temperature for 30 minutes. Sections were washed with PBS, reacted with horseradish peroxidase–conjugated streptavidin, and developed with 3,3′-diaminobenzidine. Negative controls were prepared by substitution of the primary antibody with an irrelevant antibody.

**Real-Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction**

Total RNA was isolated from left ventricular samples (30 mg each) using Isogen (Nippon Gene, Tokyo, Japan). Complementary DNAs were synthesized by reverse transcription of 1µg total RNA according to the QuantiTect Reverse Transcription Handbook (QIAGEN Inc., Hilden, Germany). Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to evaluate the expression levels of transforming growth factor (TGF)-beta1 and of monocyte chemoattractant protein-1 (MCP-1) by using Thermal Cycler Dice Real Time System (Takara Biochemicals, Kyoto, Japan). The following sets of primers were used for PCR: TGF beta1; forward, 5′-GTG TGG AGC AAC ATG TGG AAC TCT A -3′; reverse, 5′-TTG GTT CAG CCA CTG CCG TA -3′. MCP-1; forward, 5′-GCA TCC ACG TGT TGG CTC A -3′; reverse, 5′-CTC CAG CCT ACT CAT TGG GAT CA -3′. SyBr Green assays were performed with the qPCR Master Mix for SYBR Green I (Takara). Specificity of the SyBr Green assays was confirmed by melting point analysis. mRNA for the housekeeping gene glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Applied Biosystems, California, U.S.A.) was also quantitated for normalization, since GAPDH is regarded as a useful internal control for mRNA quantification. mRNA quantification was carried out by standard curve method. The amplification was in a linear relationship with the initial template concentration, and all testing samples were located within range. The efficiency was 93.9 %, 104.9 %, and 98.4 % for MCP-1, TGF-beta 1, and GAPDH, respectively. The correlation coefficient was 0.998, 0.990, and 0.998 for MCP-1, TGF-beta 1, and GAPDH, respectively. Thus, these findings confirmed the validity of our method.
ASK1, NO deficiency, and cardiovascular injury

Statistical analysis

All data are presented as mean±SEM. The data on time course experiments were analyzed by two-way ANOVA, followed by Fisher’s PLSD test, using StatView for Windows (SAS Institute, Inc. Cary, U.S.A.). In all other data, statistical significance was determined with one-way ANOVA, followed by Fisher’s PLSD test. In all tests, differences were considered statistically significant at a value of P<0.05.
Online Reference


**Online Figure Legends**

**Figure S1** Time course of blood pressure in wild type and ASK1 deficient mice

Wild (-), wild type mice not treated with L-NAME; Wild (+), wild type mice treated with L-NAME; ASK1-/- (-), ASK1 deficient mice not treated with L-NAME; ASK1-/- (+), ASK1 deficient mice treated with L-NAME. Values are mean±SEM (n=10-11 per group)

**Figure S2** Carotid arterial endothelium-independent relaxation by S-nitroso-N-acetylpenicillamine in wild type and ASK1 deficient mice

Abbreviations are the same as in Figure S1. SNAP, S-nitroso-N-acetylpenicillamine. Values are mean±SEM (n=6 per group)

**Figure S3** Vascular phospho-eNOS (p-eNOS) and total eNOS (t-eNOS) in wild type and ASK1 deficient mice

Thoracic aorta was pooled from 3 mice to obtain one protein sample. Abbreviations are the same as in Figure S1. NS, not significant. Values are mean±SEM (n=4 per group)

**Figure S4** Vascular phospho p38 (A) and phospho JNK (B) in wild type and ASK1 deficient mice

Abbreviations are the same as in Figure S1. NS, not significant. Values are mean±SEM (n=4 per group)

**Figure S5** Representative images of photomicrographs of TUNEL-positive nuclei on myocardial sections (A), myocardial sections immunostained with CD68 (B), and Sirius Red F3BA stain of myocardial sections (C and D)

Scale bars = 100 µm. Abbreviations are the same as in Figure S1.

**Figure S6** Cardiac mRNA of MCP-1 (A) and TGF-β1 (B) in wild type and ASK1 deficient mice, measured by real time PCR

Abbreviations are the same as in Figure S1. MCP-1, monocyte chemoattractant protein 1; TGF-β1, transforming growth factor β 1. NS, not significant. Values are mean±SEM (n=4-6 per group in (A), n=3-4 in (B))

**Figure S7** Cardiac NADPH oxidase activity (A) and superoxide levels (B) in wild type and ASK1 deficient mice

Abbreviations are the same as in Figure S1. NS, not significant. Scale bar = 100µm in (B). Values are mean±SEM (n=6 per group in (A), n=7-10 in (B))
Figure S1

Blood pressure (mmHg)

Wild (-) vs Wild (+)
P<0.01

ASK1-/- (-) vs ASK1-/- (+)
P<0.01

Treatment (weeks)

L-NAME

Wild (-)
Wild (+)
ASK1-/- (-)
ASK1-/- (+)
Figure S2

Vascular relaxation (%)

SNAP (-log mol/L)

Wild (-)
Wild (+)
ASK1 -/- (-)
ASK1 -/- (+)

L-NAME
Figure S3

Wild ASK1-/-
L-NAME (-) (+) (-) (+)

α-tublin

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Graphs showing the expression levels of p-eNOS and t-eNOS relative to α-tublin under different conditions of L-NAME and ASK1 status.

NS
Figure S4

(A)

B

p-p38

p-p38/t-p38

P<0.01

NS

L-NAME

Wild ASK1-/-

(-) (+) (-) (+)

(B)

p-JNK

p-JNK/t-JNK

P<0.01

NS

L-NAME

Wild ASK1-/-

(-) (+) (-) (+)
Figure S5

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<td>L-NAME</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td></td>
<td>(+)</td>
<td>(+)</td>
</tr>
</tbody>
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(A)  (B)  (C)  (D)
Figure S6

(A) MCP-1/GAPDH

(B) TGF-β1/GAPDH

<table>
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<tr>
<th>L-NAME</th>
<th>Wild</th>
<th>ASK1 -/−</th>
</tr>
</thead>
<tbody>
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<td>1</td>
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<tr>
<td>(+)</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

P<0.01
P<0.05
NS

<table>
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<tr>
<th>L-NAME</th>
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<td>(+)</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

P<0.01
P<0.05
NS
Figure S7

(A) 

(x10² CPM/mg protein)

NADPH oxidase

P<0.05

NS

Wild (--) (++)
ASK1 --/- (--) (++)

(B) 

Superoxide (Relative fluorescence)

P<0.01

NS

L-NAME (--) (++)
Wild
ASK1 --/- (--) (++)