Critical Role of Apoptosis Signal-Regulating Kinase 1 in Aldosterone/Salt-Induced Cardiac Inflammation and Fibrosis

Taishi Nakamura, Keiichiro Kataoka, Masaya Fukuda, Hisato Nako, Yoshiko Tokutomi, Yi-Fei Dong, Hidenori Ichijo, Hisao Ogawa, Shokei Kim-Mitsuyama

Abstract—The molecular mechanism underlying aldosterone/salt-induced cardiovascular injury remains to be defined. This work was undertaken to determine the role of apoptosis signal-regulating kinase 1 (ASK1) in the mechanism underlying aldosterone-induced cardiac injury in vivo. We compared the in vivo effects of 4 weeks of aldosterone/salt treatment on wild-type and ASK1-deficient mice. Aldosterone infusion plus high salt intake in wild-type mice significantly increased blood pressure and urinary albumin excretion and decreased plasma potassium concentrations, and these effects of aldosterone/salt were not affected by ASK1 deficiency. Thus, ASK1 seems to play a minor role in aldosterone-induced hypertension and renal injury. ASK1 deficiency also failed to affect aldosterone-induced cardiac hypertrophy. However, ASK1 deficiency markedly ameliorated aldosterone-induced cardiac injury, eg, the enhancement of cardiac macrophage infiltration, monocyte chemotactic protein 1 expression, interstitial fibrosis, perivascular fibrosis, and transforming growth factor-β1 and collagen type I expressions. Thus, ASK1 participates in aldosterone-induced cardiac inflammation and fibrosis. Furthermore, the enhancement of NADPH oxidase–mediated cardiac oxidative stress caused by aldosterone infusion was markedly lessened by ASK1 deficiency, which was associated with the significant amelioration by ASK1 deficiency of aldosterone-induced cardiac Nox2 upregulation. Furthermore, aldosterone/salt treatment significantly enhanced cardiac expression of the angiotensin-converting enzyme and angiotensin II type 1 receptor in wild-type mice, whereas the enhancement of these proteins by aldosterone/salt was abolished by ASK1 deficiency. Our results demonstrate that ASK1 is implicated in aldosterone/salt-induced cardiac inflammation and fibrosis through the enhancement of NADPH oxidase-mediated oxidative stress and the upregulation of the cardiac renin-angiotensin system. (Hypertension. 2009;54:544-551.)

Key Words: oxidative stress ▪ inflammation ▪ cross-talk ▪ angiotensin ▪ cardiac injury

Experimental and clinical studies show that aldosterone is one of the key players responsible for the pathophysiology of not only hypertension but also various cardiovascular diseases. Importantly, aldosterone in the setting of high salt intake, is particularly involved in cardiac inflammation and fibrosis, at least in part through the mechanisms not related to blood pressure and renal sodium retention. It has been shown that excess aldosterone plus high salt intake induces cardiac macrophage infiltration and upregulates cardiac expression of proinflammatory, fibrosis-related, or oxidative stress-related genes. Furthermore, aldosterone is also known to upregulate cardiac renin-angiotensin system components. Thus, the deleterious cardiac effects of aldosterone are thought to be attributed to these pleiotropic effects. However, the underlying molecular mechanisms for aldosterone-induced cardiac inflammation and fibrosis are still uncertain.

Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase and is well known to be activated by oxidative stress, leading to the activation of the downstream cascades, eg, p38 and c-Jun amino-terminal kinase. The activation of ASK1 causes a variety of cellular responses, eg, differentiation, proliferation, apoptosis, inflammation, or gene expression. We have reported previously that ASK1 is involved in angiotensin II–induced cardiac hypertrophy and fibrosis, as well as vascular endothelial dysfunction. We have also reported that ASK1 participates in vascular intimal hyperplasia caused by adventitial injury and ischemia-induced angiogenesis. Thus, ASK1 is regarded as one of the key molecules participating in the pathophysiology of various cardiovascular diseases. Furthermore, ASK1 deficiency causes the resistance to lipopolysaccharide-induced septic shock by attenuating the induction of proinflammatory cytokines, thereby indicating the critical role of ASK1 in inflammatory responses. However, to our knowledge, there is no available information on the role of ASK1 in the in vivo deleterious effects of aldosterone.
In the current study, we first examined the potential role of ASK1 in the in vivo effects of aldosterone. We obtained the first evidence that ASK1 is responsible for aldosterone/salt-induced cardiac inflammation and fibrosis, being mediated by the enhancement of NADPH oxidase–mediated oxidative stress and the upregulation of the cardiac renin-angiotensin system.

**Materials and Methods**

**Animals and Study Design**

Male ASK1 deficient (ASK1−/−) mice14,15 and wild-type mice (C57BL/6) were used in the present study. ASK1−/− mice were backcrossed into the C57BL/6 background for ≥10 generations to reduce genetic variation. Wild-type mice on the same genetic background were purchased from KBT Oriental (Hamamatsu, Japan). All of the procedures were performed in accordance with institutional guidelines for animal research by the Kumamoto University Animal Care and Use Committee.

Thirteen-week-old ASK1 deficient mice and age-matched wild-type mice were maintained on 1% NaCl drinking water, because a high salt intake is requisite to the development of cardiac inflammation and fibrosis by aldosterone infusion.5,21,22 Both strains of mice were divided into 3 groups, including the following: (1) saline infusion (control); (2) aldosterone infusion; and (3) aldosterone infusion plus 0.3% KCl supplementation. Aldosterone (Sigma; 0.2 mg/kg per day) dissolved in 5% ethanol or saline alone as the control was infused continuously into both strains via osmotic minipumps (model 1002, Alzet Durect Corp) for 4 weeks. Throughout the experiment, blood pressure and heart rate of the conscious mice were periodically measured with the tail-cuff method (BP98A, Softron). To measure 24-hour urinary albumin excretion, mice received 4 weeks of aldosterone infusion and were also individually housed in metabolic cages under standardized conditions of a 12:12-hour light-dark cycle. After 4 weeks of aldosterone infusion, mice were anesthetized with ether, arterial blood was immediately collected by cardiac puncture, and plasma was collected by centrifugation and stored at −80°C for further studies. After perfusion with PBS, the heart was immediately excised from each mouse for the measurement of various parameters, as described in the online Data Supplement, available at http://hyper.ahajournals.org.

**Results**

**Effect of Aldosterone on Blood Pressure**

As shown in Figure 1, there was no significant difference in blood pressure between wild-type and ASK1−/− mice under basal condition. Aldosterone infusion increased blood pressure of wild-type and ASK1−/− mice to a similar extent throughout the infusion. Potassium supplementation did not affect blood pressure elevation by aldosterone infusion in both wild-type and ASK1-deficient mice. Significant difference was noted between both strains of mice. Moreover, aldosterone infusion markedly augmented urinary albumin excretion in both strains of mice to a similar degree. KCl supplementation successfully prevented aldosterone-induced hypokalemia in wild-type and ASK1-deficient mice to a similar extent. KCl supplementation significantly attenuated an aldosterone-induced increase in urinary albumin excretion in wild-type and ASK1-deficient mice to a similar extent.

**Effect of Aldosterone on Cardiac ASK1 and Mitogen-Activated Protein Kinases**

As shown in Figure 2A and 2B, aldosterone infusion in wild-type mice significantly increased the phosphorylation of cardiac ASK1 (P<0.01), which was accompanied by a significant increase in the phosphorylation of cardiac p38 (P<0.01). As expected, cardiac ASK1 was not detected in ASK1−/− mice. Cardiac p38 phosphorylation was much smaller in ASK1−/− mice than in wild-type mice. Unlike wild-type mice, aldosterone infusion did not apparently increase phosphorylation of cardiac p38 in ASK1−/− mice. Aldosterone infusion significantly increased cardiac extracellular signal–regulated kinase phosphorylation in wild-type and ASK1−/− mice to a comparable degree (Figure 2C).

**Effect of Aldosterone on Cardiac Hypertrophy and Inflammation**

As shown in Figure 3A, aldosterone infusion increased left ventricular weight in both wild-type (P<0.01) and ASK1−/− (P<0.01) mice, to a similar degree. KCl supplementation did not significantly affect aldosterone-induced cardiac hypertrophy in wild-type and ASK1-deficient mice. Echocardiographic assessment in Table S3 indicates that, regardless of aldosterone infusion or no infusion, there was no significant difference between wild-type and ASK1−/− mice regarding left ventricular wall thickness, diameter and mass index, and fractional shortening. On the other hand, the upregulation of cardiac B-type natriuretic peptide mRNA by aldosterone...
infusion was smaller in ASK1\(^{-/-}\) mice than in wild-type mice \((P<0.05;\) Figure 3B).

Aldosterone infusion significantly increased cardiac macrophage infiltration in wild-type mice \((P<0.01;\) Figure 3C), being associated with the enhanced expression of cardiac monocyte chemotactic protein (MCP) 1 mRNA \((P<0.01;\) Figure 3D). Cardiac macrophage infiltration and MCP-1 expression in aldosterone-infused wild-type mice were only slightly attenuated by KCl supplementation. Aldosterone-induced cardiac macrophage infiltration in ASK1\(^{-/-}\) mice was less than that in wild-type mice \((P<0.01),\) which was associated with less increase in cardiac MCP-1 mRNA expression in ASK1\(^{-/-}\) mice \((P<0.01).\)

Effect of Aldosterone on Cardiac Fibrosis

Figure 4 indicates cardiac interstitial and perivascular fibrosis, as well as fibrosis-related gene expression in wild-type and ASK1\(^{-/-}\) mice. Aldosterone infusion in wild-type mice significantly enhanced cardiac interstitial fibrosis \((P<0.01)\) and perivascular fibrosis \((P<0.01),\) whereas these effects of aldosterone infusion were smaller in ASK1\(^{-/-}\) mice than in wild-type mice \((P<0.01).\) Furthermore, the upregulation of cardiac transforming growth factor (TGF)-\(\beta1\) and collagen type 1 mRNA by aldosterone infusion was also less in ASK1\(^{-/-}\) mice than in wild-type mice \((P<0.01;\) Figure 4C and 4D, respectively).

Effect of Aldosterone on Cardiac Superoxide, NADPH Oxidase Activity, and NADPH Oxidase Subunits

As shown in Figure 5, aldosterone infusion in wild-type mice significantly increased cardiac superoxide levels \((P<0.01),\) being associated with the significant increase in cardiac NADPH oxidase activity \((P<0.01).\) These increases were not altered by KCl supplementation. However, these effects of aldosterone were less in ASK1\(^{-/-}\) mice than in wild-type mice.

As shown in Figure 6A, aldosterone infusion significantly enhanced cardiac Nox2 protein expression in both strains of mice, but this effect of aldosterone was less in ASK1\(^{-/-}\) mice than in wild-type mice \((P<0.01).\) Cardiac Nox4 protein expression was increased by aldosterone infusion in both wild-type and ASK1\(^{-/-}\) mice to a similar extent (Figure 6B).

Cardiac p22 phox expression was smaller in ASK1\(^{-/-}\) mice than in wild-type mice under basal condition, and aldosterone infusion did not significantly affect cardiac p22 phox protein expression in wild-type or ASK1\(^{-/-}\) mice (Figure 6C).

Effect of Aldosterone on Cardiac Angiotensin-Converting Enzyme, Angiotensin II Type 1 Receptor, and Mineralocorticoid Proteins

Aldosterone infusion in wild-type mice significantly increased cardiac protein and mRNA levels for the angiotensin-converting enzyme (ACE; \(P<0.01\)) and angiotensin II type 1 (AT1) receptor \((P<0.01;\) Figure 7). However, aldosterone infusion failed to alter cardiac protein and mRNA levels for the ACE and AT1 receptor in ASK1\(^{-/-}\) mice. Aldosterone infusion did not significantly alter cardiac mineralocorticoid protein levels (Figure S1). There was no significant difference between wild-type and ASK1\(^{-/-}\) mice in cardiac mineralocorticoid protein levels, regardless of aldosterone/salt treatment (Figure S1).

Discussion

The major findings of our current work were that ASK1 deficiency, independent of blood pressure and renal sodium retention, markedly lessened cardiac macrophage infiltration, interstitial fibrosis, and perivascular fibrosis in aldosterone-infused mice.
infused mice and that ASK1 deficiency significantly prevented the enhancement of cardiac NADPH oxidase–mediated oxidative stress and the upregulation of cardiac ACE and AT1 receptor in aldosterone-infused mice. Our present findings provided the first evidence for the critical role of ASK1 in aldosterone-induced cardiac inflammation and fibrosis.

In this study, aldosterone/salt treatment induced hypokalemia. Therefore, it is possible that cardiac inflammation and fibrosis might be consequent to cardiac myocyte necrosis caused by hypokalemia. However, the prevention of hypokalemia with potassium supplementation had little effect on cardiac inflammation and fibrosis induced by aldosterone/salt treatment. Thus, it is unlikely that cardiac inflammation and fibrosis in this study might be secondary to myocyte necrosis. We have demonstrated previously that ASK1 is implicated in cardiac hypertrophy and fibrosis caused by chronic angiotensin II infusion, thereby indicating the critical contribution of ASK1 to angiotensin II–induced cardiac injury.16 However, there is no report investigating the role of ASK1 in the mechanism of in vivo effects of aldosterone. This encouraged us to first examine the potential role of ASK1 in aldosterone-induced cardiac injury in vivo.

In our current work, we found that the absence of ASK1 did not significantly affect aldosterone-induced blood pressure elevation (Figure 1) or aldosterone-induced hypokalemia (Table S2), indicating the minor role of ASK1 in aldosterone-induced hypertension and hypokalemia. Furthermore, the increase in urinary albumin excretion by aldosterone infusion was not altered by ASK1 deficiency, thereby providing the evidence for the minor role of ASK1 in aldosterone-induced renal injury. Moreover, ASK1 deficiency failed to suppress aldosterone-induced cardiac hypertrophy (Figure 3A and Table S3). In contrast, notably, ASK1 deficiency markedly attenuated the augmentation of cardiac macrophage infiltration, collagen expression, interstitial fibrosis, and perivascular fibrosis in aldosterone-infused mice (Figures 3 and 4). These effects of ASK1 deficiency in aldosterone-infused mice were associated with the suppression of the upregulation of cardiac mRNA expression of

![Figure 3. Left ventricular weight (A), cardiac B-type natriuretic peptide mRNA (B), macrophage infiltration (C), and cardiac MCP-1 mRNA (D) in wild-type and ASK1−/− mice subjected to 4 weeks of aldosterone infusion or no infusion. Abbreviations used are the same as in Figure 1. +KCl indicates aldosterone infusion plus KCl supplementation; NS, not significant. Top panels (perivascular space and interstitium) in C indicate representative photomicrographs of cardiac sections immunostained with CD68. Aldosterone/salt treatment in wild-type mice increased macrophage infiltration, particularly in the perivascular space. Magnification, ×400. Scale bar=100 μm. mRNA levels of cardiac B-type natriuretic peptide (C) and MCP-1 (D) in individual mice were normalized to GAPDH mRNA levels. Values are mean±SEM (n=6 to 7).](http://hyper.ahajournals.org/doi/figure/10.1161/HYPERTENSIONAHA.110.166872)
MCP-1, a proinflammatory chemokine inducing macrophage infiltration, and TGF-β1, a growth factor inducing tissue fibrosis. These results demonstrate that ASK1, independent of blood pressure or hypokalemia, is involved in aldosterone-induced cardiac inflammation and fibrosis, at least in part through the enhancement of cardiac MCP-1 and TGF-β1 expression. Thus, our current findings provided the novel molecular mechanism underlying cardiac injury caused by aldosterone/salt in vivo.

Accumulating evidence show that reactive oxygen species play a key role in the initiation and progression of cardiovascular diseases. Aldosterone is well known to activate the cardiac renin-angiotensin system by aldosterone. Moreover, AT1 receptor blockade significantly prevents aldosterone-induced cardiac collagen expression and cardiac fibrosis in vivo.13,30,31 The mechanism by which aldosterone augments cardiac and vascular AT1 receptor expression,13,30,31 and ACE expression,13,32 indicating activation of the cardiac renin-angiotensin system by aldosterone. Moreover, AT1 receptor blockade significantly prevents aldosterone-induced cardiac fibrosis.

Thus, aldosterone-induced cardiac fibrosis seems to be partially attributed to the cross-talk between aldosterone and the cardiac renin-angiotensin system. However, the mechanism by which aldosterone upregulates the cardiac AT1 receptor and ACE is still unknown. Our present results provide the first evidence that ASK1 deficiency significantly lessened aldosterone-induced cardiac fibrosis in vivo.13

Not only oxidative stress but also the cardiac renin-angiotensin system is thought to be partially involved in the pathogenesis of aldosterone-induced cardiac injury.10,12 Aldosterone augments cardiac and vascular AT1 receptor expression, and ACE expression, indicating activation of the cardiac renin-angiotensin system by aldosterone. Moreover, AT1 receptor blockade significantly prevents aldosterone-induced cardiac collagen expression and cardiac fibrosis in vivo. Thus, aldosterone-induced cardiac fibrosis seems to be partially attributed to the cross-talk between aldosterone and the cardiac renin-angiotensin system. However, the mechanism by which aldosterone upregulates the cardiac AT1 receptor and ACE is still unknown. Our present results provide the first evidence that ASK1 deficiency significantly lessened aldosterone-induced augmentation of cardiac ACE and AT1 receptor expression. Collectively, the amelioration by ASK1 deficiency of aldosterone-induced cardiac inflammation and fibrosis seems to be
partially mediated by the attenuation of aldosterone-induced cardiac ACE and AT1 receptor upregulation.

**Study Limitation**

Our present in vivo study did not allow us to define the cell types responsible for the increased expression of cardiac ACE, AT1 receptor, MCP-1, and NADPH oxidase. However, previous reports indicate that aldosterone enhances ACE expression in cardiomyocytes, vascular endothelial cells, and macrophages; increases AT1 receptor expression in cardiomyocytes and vascular smooth muscle cells; and activates NADPH oxidase (Nox2) in cardiomyocytes, macrophages, and vascular smooth muscle cells. Moreover, aldosterone infusion in vivo increases the expression of P22 phox.

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**Figure 5.** Cardiac superoxide (A) and NADPH oxidase activity (B) in wild-type and ASK1−/− mice subjected to 4 weeks of aldosterone infusion or no infusion. Abbreviations used are the same as in Figure 1. +KCl indicates aldosterone infusion plus KCl supplementation. Top panels in A indicate representative photomicrographs of dihydroethidium-stained cardiac sections. Dihydroethidium fluorescence was quantified to evaluate superoxide levels in situ and expressed relative to the values obtained for control wild-type mice subjected to saline infusion. Magnification, ×400. Scale bar=100 μm. Values are mean±SEM (n=6 to 7).

**Figure 6.** Cardiac NADPH oxidase subunit Nox2 (A), Nox4 (B), and p22-phox (C) protein levels in wild-type and ASK1−/− mice subjected to 4 weeks of aldosterone infusion or no infusion. Abbreviations used are the same as in Figure 1. NS indicates not significant. Top panels in each figure indicate representative Western blot. NADPH oxidase subunit levels in individual samples were corrected for tubulin protein levels. Values are mean±SEM (n=4).
MCP-1 and NADPH oxidase subunit Nox2 in inflammatory cells and endothelial cells in the perivascular space of coronary arteries.28 Thus, the underlying mechanism of aldosterone/salt-induced cardiac inflammation and fibrosis involves a very complex interaction among cardiomyocytes, fibroblasts, inflammatory cells, and vascular cells in a paracrine fashion. Additional study is warranted to elucidate the precise relative role of these cells in aldosterone-induced cardiac injury.

In conclusion, we first examined the potential role of ASK1 in the effects of aldosterone/salt treatment on blood pressure, renal injury, and cardiac injury. Our current findings provided the first evidence that ASK1, independent of hypertension and hypokalemia, significantly participates in aldosterone-induced cardiac inflammation and fibrosis, being mediated by the enhancement of cardiac NADPH oxidase-mediated oxidative stress and the activation of the cardiac renin-angiotensin system.

Perspectives
Clinical evidence indicates that aldosterone blockers exert beneficial effects on heart failure and postmyocardial infarction, showing that aldosterone is one of the key players responsible for human cardiovascular diseases, as well as hypertension. Therefore, for the development of a novel therapeutic strategy of cardiovascular diseases, it is clinically very critical to elucidate the detailed mechanism underlying aldosterone-induced cardiovascular injury. Our present work not only provides a novel insight into the molecular mechanism of cardiovascular injury caused by aldosterone but also highlights ASK1 as a promising therapeutic target for cardiovascular diseases.

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Disclosures
None.

References


ONLINE SUPPLEMENTS

Critical role of apoptosis signal-regulating kinase 1 in aldosterone/salt-induced cardiac inflammation and fibrosis

Short title: Aldosterone, cardiac injury, and ASK1

Taishi Nakamura¹, Keiichiro Kataoka¹, Masaya Fukuda¹, Hisato Nako¹, Yoshiko Tokutomi¹, Yi-Fei Dong¹, Hidenori Ichijo², Hisao Ogawa³, Shokei Kim-Mitsuyama¹

¹ Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan
² Laboratory of Cell Signaling, Tokyo University Graduate School of Pharmaceutical Sciences, Tokyo, Japan
³ Department of Cardiovascular Medicine, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

All correspondence to: Shokei Kim-Mitsuyama, MD., PhD.

Department of Pharmacology and Molecular Therapeutics
Kumamoto University Graduate School of Medical Sciences
1-1-1 Honjo, Kumamoto 860-8556, Japan
Tel. 81-96-373-5082
Fax. 81-96-373-5082
E-mail: kimmitsu@gpo.kumamoto-u.ac.jp
Online methods

**Measurement of urinary albumin**

Urinary albumin concentrations were quantified by using commercially available kit (AssayMax Mouse Albumin ELISA Kit; Assaypro LLC). Urinary albumin excretion was corrected for urinary creatinine excretion.

**Analysis of plasma biochemistry**

Plasma was analyzed for Na⁺, K⁺ (ion-selective electrode system), urea (ultra violet spectrophotometry), and creatine (enzyme assay) by 7180 Clinical Analyzer; Hitachi Highechnologies Corp., Tokyo, Japan.

**Echocardiographic assessment**

Transthoracic echocardiographic studies were performed with an echocardiographic system equipped with 12-MHz echocardiographic probe (PHILIPS SONOS-4500), as previously described in detail. Mice were anesthetized with a mixture of ketamine HCl (50 mg/kg i.p.) and xylazine HCl (5 mg/kg i.p.). M-mode tracings were recorded to assess LV morphology after the chest was shaved. Echocardiographic LV mass was determined by the cube formula, as well as LV volumes. LV fractional shortening was calculated as follows: (EDd-ESd)/EDd x100.

**Measurement of cardiac superoxide production**

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

**Cardiac NADPH oxidase activity**

The heart was homogenized with a POLYTRON homogenizer PT1200E (KINEMATICA AG), centrifuged, and cardiac NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of modified Krebs/HEPES buffer (pH 7.4) equilibrated at 37°C, 10 μM NADPH, and 10 μM lucigenin as electron acceptor, as described previously. The chemiluminescence was then recorded every 15 seconds for 5 minutes, with the use of a luminescence reader (BLR-201, Aloka). Values of chemiluminescence were expressed as cpm/mg protein. Protein concentrations were measured by the method of Bradford.

**Preparation of cardiac protein extracts and Western blot analysis**

Our detailed method has been described previously. Antibodies used were as follows: anti-phospho ASK1 (x1000), Anti-phospho p38 (x2000, Cell signaling Technology Inc), anti-p38 (x2000, Cell signaling Technology Inc), anti-phospho ERK (x2000, Cell signaling Technology Inc), anti-ERK (x2000, Cell signaling Technology Inc), anti-α-tubulin (x5000, Oncogene), anti-p22phox (x2000, Santa Cruz Biotechnology Inc), anti-Nox2 (x2000, BD Transduction Laboratories), anti-Nox4 (x1000, Abcam), anti-ACE (x2000, Abcam), anti-AT1 receptor (x2000, Santa Cruz Biotechnology Inc), anti-mineralocorticoid (MR) (x1000, Santa Cruz Biotechnology Inc). The antibody was visualized using an enhanced chemiluminescence method (ECL Plus; Amersham Biosciences). The intensity of the bands was quantified using NIH Image analysis software v1.61. In individual samples, each value was corrected for that of α-tubulin.
**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 and perivascular fibrosis as previously described. The area of cardiac interstitial fibrosis and perivascular fibrosis per vessel was assessed by using Lumina Vision version 2.2 analysis software. For detection of macrophage infiltration, frozen cardiac sections were stained with the primary antibodies (CD68 rat anti-mouse macrophages, Serotec; x 500) at 4°C overnight. After incubation with the primary antibody, sections were reacted with horseradish peroxidase-conjugated anti-rat IgG secondary antibody (BioSource, Camarillo, CA, USA), and visualized with 3,3’-diaminobenzidine (Dako Cytomation, Carpinteria, CA, USA). Negative controls were prepared by substitution of the primary antibody with an irrelevant antibody.

**Quantitative real time RT-PCR**

Frozen cardiac tissue was homogenized after adding TRIzol Regent (Invitrogen). Total RNA was extracted from the tissue according to the manufacturer’s suggested protocol followed by phenol-guanidine isothiocyanate-chloroform extraction and ethanol precipitation. Briefly, 1 μg total RNA was reverse transcribed to complementary DNAs using QuantiTect® Reverse Transcription Kit (QIAGEN Inc., Hilden, Germany).

Real time PCR was performed to evaluated the expression levels of ACE, AT1 receptor, BNP, procollagen type I, MCP-1, mineralocorticoid receptor, and TGF-β1 by using Thermal Cycler Dice® Real Time System (TaKaRa Bio Inc., Shiga, Japan) as described previously. cDNA was amplified using SYBR® Premix Ex Taq™ (Perfect Real Time) PCR kit (TaKaRa Bio Inc.) with specific primers for target sequences (described in Online Table S1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To confirm amplification specificity of the PCR products from each primer pair were subjected to a melting curve analysis. The threshold cycle (Ct) value, which was determined using crossing point method, was normalized to the respective housekeeping GAPDH (Applied Biosystems, California, U.S.A) Ct value and relatively calculated by setting a calibrator sample in each run.

**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.
References


**Table S1.** The sequence of primers used in real-time PCR assays

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Abbreviations used: ACE, angiotensin-converting enzyme; AT1a, Angiotensin II receptor type 1a; BNP, natriuretic peptide precursor type B; Colla1, procollagen type I, alpha 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1; TGF-β1, transforming growth factor-β 1; F, forward primer; R, reverse primer; Acc No, GenBank accession number
Online Table S2. Body weight, urinary albumin excretion, and plasma sodium and potassium concentrations after 4 weeks of aldosterone infusion

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Urinary albumin/Cr indicates urinary albumin excretion corrected for urinary creatinine excretion. Abbreviations used: A (-), saline-infused group; A (+), aldosterone-infused group; A (+) + KCl, aldosterone-infused and KCl-supplemented group. Na, sodium; K, potassium; BUN, blood urea nitrogen. Values are means ± SEM (n=6-7). * P<0.05, § P<0.01 vs Wild-type mice, A (+). ΩP<0.05, §P<0.01 vs ASK1-/- mice, A (+).
Online Table S3. Transthoracic echocardiographic data after 4 weeks of aldosterone infusion

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<td>PW thickness (mm)</td>
<td>0.62±0.03</td>
<td>0.83±0.01 j</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>2.77±0.10</td>
<td>3.05±0.05*</td>
</tr>
<tr>
<td>LVESd (mm)</td>
<td>1.28±0.07</td>
<td>1.39±0.04</td>
</tr>
<tr>
<td>LV mass index</td>
<td>1.73±0.25</td>
<td>2.98±0.08 j</td>
</tr>
<tr>
<td>(mg/g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FS (%)</td>
<td>54±3</td>
<td>55±1</td>
</tr>
</tbody>
</table>

A (-) and A (+) indicate mice subjected to saline and aldosterone infusion, respectively.

Abbreviations used: IVS, Intraventricular septum; PW, posterior wall; LVEDd, left ventricular end-diastolic diameter; LVESd, left ventricular end-systolic diameter; FS, fractional shortening. Values are means±SEM (n=5-6). * P<0.05, j P<0.01 vs Wild, A (-). Ω P<0.05, § P<0.01 vs ASK1-/-, A (-)
Online Figure S1. Cardiac mineralocorticoid receptor (MR) protein levels in wild-type and ASK1-/- mice subjected to 4 weeks of aldosterone infusion or not.

Abbreviations used: Wild, wild-type mice; ASK1-/-, ASK1-/- mice; A (-), saline infusion; A (+), aldosterone infusion. NS, not significant. Upper panels indicates representative western blot analysis of MR. Values are means±SEM (n=6-7).