Olmesartan Prevents Cardiovascular Injury and Hepatic Steatosis in Obesity and Diabetes, Accompanied by Apoptosis Signal Regulating Kinase-1 Inhibition

Eiichiro Yamamoto, Yi-Fei Dong, Keiichiro Kataoka, Takuro Yamashita, Yoshiko Tokutomi, Shinji Matsuba, Hidenori Ichijo, Hisao Ogawa, Shokei Kim-Mitsuyama

Abstract—Dietary obesity is associated with type 2 diabetes and cardiovascular diseases, although the underlying mechanism is unknown. This study was undertaken to elucidate the role of angiotensin II and apoptosis signal regulating kinase-1 (ASK1) in obesity/diabetes-associated cardiovascular complications and hepatic steatosis. Mice fed a high-fat diet were treated with olmesartan, an angiotensin II type 1 receptor blocker, to elucidate the role of angiotensin II in diabetic mice. Treatment of mice fed a high-fat diet with olmesartan markedly suppressed cardiac inflammation and fibrosis, as well as vascular endothelial dysfunction and remodeling, induced by obesity/diabetes. Moreover, olmesartan suppressed the disruption of the vascular endothelial NO synthase dimer in diabetic mice. Olmesartan also significantly prevented hepatic steatosis and fibrosis in diabetic mice. These beneficial effects of olmesartan on diabetic mice were associated with the attenuation of ASK1 activation in these mice. ASK1-deficient mice and wild-type mice were compared, regarding the effects of a high-fat diet. A comparison between ASK1-deficient and wild-type mice showed that ASK1 deficiency attenuated cardiac inflammation and fibrosis, as well as vascular endothelial dysfunction and remodeling induced by obesity/diabetes. The amelioration of vascular endothelial impairment by ASK1 deficiency was attributed to the prevention of endothelial NO synthase dimer disruption. ASK1 deficiency also significantly lessened hepatic steatosis in diabetic mice. In conclusion, our work provided the evidence that ASK1 is significantly activated in diet-induced diabetic mice and contributes to cardiovascular diseases and hepatic steatosis in diabetic mice. Moreover, the beneficial effects of angiotensin II inhibition on dietary diabetic mice seem to be mediated by the inhibition of ASK1 activation. (Hypertension. 2008;52:573-580.)

Key Words: diabetes ■ obesity ■ angiotensin ■ ASK1 ■ reactive oxygen species ■ vascular endothelial function ■ cardiac injury

Obesity, particularly dietary obesity, is associated with type 2 diabetes and an increased risk of cardiovascular diseases. However, the underlying mechanism is poorly understood. Accumulating experimental and clinical evidence indicate that the renin-angiotensin system is involved not only in hypertension but also in various cardiovascular diseases. Furthermore, emerging experimental and clinical data support the notion that the renin-angiotensin system participates in the pathophysiology of obesity and type 2 diabetes, although the underlying mechanism remains to be elucidated.

Reactive oxygen species (ROS) are supposed to be involved in obesity, insulin resistance, diabetes, and cardiovascular diseases. Apoptosis signal regulating kinase-1 (ASK1), one of the mitogen-activated protein kinase kinases, is markedly activated by ROS and plays a critical role in a variety of cellular responses induced by ROS, including cell apoptosis, growth, differentiation, gene expression, etc. Previously, we have examined the effect of angiotensin II infusion on ASK1-deficient mice and have showed that ASK1, as an ROS-activated intracellular signaling molecule, is involved in angiotensin II–induced cardiac hypertrophy and remodeling, as well as vascular endothelial dysfunction. However, there is no report investigating the role of ASK1 in obesity, diabetes, and their associated cardiovascular diseases.

Hence, in the present study, to elucidate the detailed role of angiotensin II in cardiovascular injury and hepatic steatosis in obese and diabetic mice, we examined the effect of olmesartan, an angiotensin II type 1 receptor blocker (ARB), on obese and diabetic mice fed a high-fat diet. Furthermore, to clarify the potential contribution of ASK1 with the above-mentioned mechanisms is unknown. This study was undertaken to elucidate the role of angiotensin II and apoptosis signal regulating kinase-1 (ASK1) in obesity/diabetes-associated cardiovascular complications and hepatic steatosis. Mice fed a high-fat diet were treated with olmesartan, an angiotensin II type 1 receptor blocker, to elucidate the role of angiotensin II in diabetic mice. Treatment of mice fed a high-fat diet with olmesartan markedly suppressed cardiac inflammation and fibrosis, as well as vascular endothelial dysfunction and remodeling, induced by obesity/diabetes. Moreover, olmesartan suppressed the disruption of the vascular endothelial NO synthase dimer in diabetic mice. Olmesartan also significantly prevented hepatic steatosis and fibrosis in diabetic mice. These beneficial effects of olmesartan on diabetic mice were associated with the attenuation of ASK1 activation in these mice. ASK1-deficient mice and wild-type mice were compared, regarding the effects of a high-fat diet. A comparison between ASK1-deficient and wild-type mice showed that ASK1 deficiency attenuated cardiac inflammation and fibrosis, as well as vascular endothelial dysfunction and remodeling induced by obesity/diabetes. The amelioration of vascular endothelial impairment by ASK1 deficiency was attributed to the prevention of endothelial NO synthase dimer disruption. ASK1 deficiency also significantly lessened hepatic steatosis in diabetic mice. In conclusion, our work provided the evidence that ASK1 is significantly activated in diet-induced diabetic mice and contributes to cardiovascular diseases and hepatic steatosis in diabetic mice. Moreover, the beneficial effects of angiotensin II inhibition on dietary diabetic mice seem to be mediated by the inhibition of ASK1 activation. (Hypertension. 2008;52:573-580.)

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diseases, we compared ASK1-deficient mice with wild-type mice regarding the impact of a high-fat diet. We obtained the evidence that ASK1 is activated in obese and diabetic mice and is responsible for cardiovascular injury and hepatic steatosis by obesity/diabetes and that ASK1 is involved in the protective effect of an ARB against cardiovascular injury and hepatic steatosis.

**Methods**

**Animals**

All of the procedures were in accordance with institutional guidelines for animal research. Male ASK1−/− mice and wild-type mice (C57BL/6J) were used in the present study.

**Effect of Olmesartan on Mice Fed a High-Fat Diet**

Eleven-week-old C57BL/6J mice fed high-fat diet were randomly assigned to 2 groups, and were orally given vehicle or olmesartan (5 mg/kg per day) by gastric gavage for 17 weeks. Olmesartan is a highly specific ARB without peroxisome proliferator-activated receptor-γ-modulating activity. Wild-type mice (C57BL/6J), fed standard chow, served as the control. After 17 weeks of treatment, mice were anesthetized with ether, and blood was collected by cardiac puncture to measure blood glucose, plasma levels of insulin, adiponectin, and total cholesterol. Then, liver, heart, and aorta were rapidly excised from mice to perform biochemical, pharmacological, and pathological examinations.

**Comparative Effect of High-Fat Diet on ASK1−/− Mice and Wild-Type Mice**

Eleven-week-old ASK1−/− mice and wild-type mice (C57BL/6J) were separated into 2 groups and were fed the above-mentioned standard chow or high-fat diet for 17 weeks. After 17 weeks of the treatment, blood and tissue samples were taken in the same manner as the above-mentioned experiments on the effect of olmesartan.

**Effect of Olmesartan on ASK1−/− Mice Fed a High-Fat Diet**

We also examined the effect of olmesartan (5 mg/kg per day) on ASK1−/− mice fed a high-fat diet. After 17 weeks of treatment, blood and tissue samples were collected in the same manner as the above-mentioned experiments. The detailed methods are described in the online supplement available at http://hyper.ahajournals.org.

**Results**

**Effects of Olmesartan on Blood Pressure, Food Intake, Body Weight, Insulin Resistance, and Plasma Adiponectin**

As shown in Figure S1, olmesartan treatment significantly reduced the blood pressure of mice fed a high-fat diet throughout the treatment. Figure S2 indicates food intake, body weight, homeostasis model assessment of insulin resistance (HOMA-IR), and plasma adiponectin after 17 weeks of olmesartan treatment. Olmesartan did not significantly affect food intake of mice fed a high-fat diet throughout the treatment. Seventeen weeks of high-fat diet feeding markedly increased body weight and HOMA-IR in mice compared with a standard diet. Olmesartan statistically significantly prevented the increase in HOMA-IR in mice fed a high-fat diet (P < 0.01). Furthermore, plasma adiponectin levels in olmesartan-treated mice were greater than those in vehicle-treated mice (P < 0.01).

**Effects of Olmesartan on Hepatic Steatosis, Fibrosis, and Transforming Growth Factor-β1 Expression**

As shown in Figure S3, 17 weeks of olmesartan treatment markedly prevented the increase in hepatic weight, hepatic triglyceride content, collagen volume fraction, and transforming growth factor (TGF)-β1 mRNA expression in mice fed a high-fat diet.

**Effects of Olmesartan on Cardiac Injury**

As shown in Figure 1, compared with a standard diet, a high-fat diet significantly increased macrophage infiltration, collagen volume fraction, coronary arterial thickening, and perivascular fibrosis in the heart of mice, and all of these changes by a high-fat diet were significantly prevented by olmesartan treatment.

**Effects of Olmesartan on Vascular Endothelial Dysfunction and Endothelial NO Synthase Dimer Disruption**

A high-fat diet significantly impaired vascular endothelium-dependent relaxation by acetylcholine in mice (P < 0.01), and olmesartan treatment markedly prevented the impairment of vascular endothelial function caused by a high-fat diet (P < 0.05; Figure 2A). Vascular endothelium-independent relaxation by sodium nitroprusside was not impaired by a high-fat diet and was not affected by olmesartan treatment (Figure S4).

As shown in Figure 2B, in mice fed a high-fat diet, vascular endothelial dysfunction was associated with the significant disruption of the endothelial NO synthase (eNOS) dimer (P < 0.05), and olmesartan treatment suppressed high-fat diet–induced eNOS dimer disruption (P < 0.05). On the other hand, phospho-eNOS and total eNOS levels were not different among standard diet–fed mice, and high-fat diet–fed mice treated with vehicle and olmesartan (Figure S5).

**Effects of Olmesartan on Tissue Reduced Nicotinamide-Adenine Dinucleotide Phosphate Oxidase and Oxidative Stress**

Reduced nicotinamide-adenine dinucleotide phosphate oxidase activity in cardiac, vascular, and hepatic tissues was significantly greater in mice fed a high-fat diet than in mice fed a standard diet, and the increase in reduced nicotinamide-adenine dinucleotide phosphate oxidase activity in all of these tissues of high-fat diet–fed mice was significantly attenuated by olmesartan treatment (Figure S6A). As shown in Figure S6B, cardiac, aortic, and hepatic oxidative stresses were significantly enhanced in high-fat diet–fed mice compared with control mice, and olmesartan treatment significantly reduced oxidative stress in all of these tissues of diabetic mice.

**Effects of Olmesartan on Tissue ASK1 Phosphorylation**

As shown in Figure 3, ASK1 phosphorylation levels in the heart, aorta, and liver were greater in mice fed a high-fat diet than those fed a standard diet, and olmesartan treatment significantly prevented the enhancement of ASK1 phosphorylation in all of these tissues of high-fat diet–fed mice.
Blood Pressure, Food Intake, Body Weight, HOMA-IR, and Plasma Adiponectin in ASK1-Deficient Mice

As shown in Figure S7, there was no significant difference in blood pressure between wild-type and ASK1-deficient mice, regardless of the standard diet or high-fat diet. As shown in Figure S8A, food intake in high-fat diet–fed wild-type and ASK1-deficient mice was 2.1 \pm 0.1 and 2.2 \pm 0.1 g/d per mouse, respectively, indicating no significant difference in food intake between both groups. As shown in Figure S8B, there was no significant difference between wild-type and ASK1-deficient mice on a standard diet in body weight. However, body weight gain by the high-fat diet was markedly less in ASK1-deficient mice than in wild-type mice \((P<0.01)\). Visceral fat weight in ASK1-deficient mice fed a high-fat diet was also less than wild-type mice fed a high-fat diet (4400 \pm 200 versus 5744 \pm 171 mg; \(P<0.01)\), although no difference was noted between visceral fat weight of the 2 strains of mice fed a standard diet. The increase in HOMA-IR by high-fat diet was less in ASK1-deficient mice than in wild-type mice \((P<0.01)\; \text{Figure S8C})\). As shown in Figure S8D, in contrast to the significant reduction of plasma

Figure 1. Macrophage infiltration (A), interstitial fibrosis (B), coronary arterial thickening (C), and perivascular fibrosis (D) in the heart of standard diet–fed mice and high-fat diet–fed mice treated with vehicle or olmesartan. HF indicates high-fat diet; SD, standard diet; Veh, mice fed high-fat diet and treated with vehicle; Olm, mice fed high-fat diet and treated with olmesartan. Top panels in A show representative photomicrographs of cardiac sections immunostained with anti-CD68 antibody. Top panels in B and C show representative cardiac sections stained with Sirius red. Magnification, \(\times 400\). Bar=100 \(\mu\)m in B and 50 \(\mu\)m in C. Values are means\pm SEMs \((n=6 \text{ to } 8)\).
adiponectin in wild-type mice fed a high-fat diet, plasma adiponectin in ASK1-deficient mice was not significantly decreased by a high-fat diet.

Cardiac Inflammation, Fibrosis, and Coronary Arterial Remodeling in ASK1-Deficient Mice
Cardiac macrophage infiltration, interstitial fibrosis, coronary arterial thickening, and perivascular fibrosis were all much less in ASK1-deficient mice fed a high-fat diet than wild-type mice fed a high-fat diet (Figure 4).

Vascular eNOS Dimer Disruption and Superoxide in ASK1-Deficient Mice
In contrast to the marked impairment of vascular relaxation by acetylcholine in wild-type mice fed a high-fat diet \( (P<0.01) \), vascular relaxation by acetylcholine was not significantly impaired in ASK1-deficient mice fed a high-fat diet (Figure 5A). There was no significant difference in vascular endothelium-independent relaxation by sodium nitroprusside between wild-type and ASK1-deficient mice, regardless of the standard diet or high-fat diet (Figure S9). High-fat diet markedly caused the increase in disruption of the eNOS dimer in wild-type mice \( (P<0.01) \) but failed to cause it in ASK1-deficient mice (Figure 5B). The increase in vascular superoxide levels by high-fat diet was less in ASK1-deficient mice than in wild-type mice \( (P<0.05) \); Figure 5C). Vascular phospho-eNOS and total eNOS levels did not differ between wild-type and ASK1-deficient mice, regardless of standard diet or high-fat diet (Figure S10).

Hepatic Steatosis, Fibrosis, and TGF-\( \beta1 \) Expression in Wild-Type and ASK1-Deficient Mice
The high-fat diet augmented liver weight, hepatic triglyceride, and hepatic fibrosis to much less extent in ASK1-deficient mice than in wild-type mice (Figure 6A through 6C). Moreover, the increase in hepatic TGF-\( \beta1 \) mRNA expression induced by a high-fat diet was absent in ASK1-deficient mice (Figure 6D).

Effects of Olmesartan on ASK1-Deficient Mice Fed a High-Fat Diet
As shown in Figure S11, olmesartan treatment did not significantly affect cardiac macrophage infiltration and interstitial fibrosis, coronary arterial thickening, perivascular fibrosis, vascular endothelium-dependent relaxation, liver weight, and hepatic fibrosis in ASK1-deficient mice fed a high-fat diet.

Discussion
Cardiovascular remodeling and vascular endothelial dysfunction are well known to occur in diabetes and are mainly implicated in the pathogenesis of cardiovascular diseases.\(^{5,33–35}\) In our present work, of note, despite no significant effect of olmesartan on food intake, olmesartan markedly prevented cardiac inflammation, cardiac interstitial fibrosis, coronary arterial remodeling, and vascular endothelial dysfunction in obese and diabetic mice (Figures 1 and 2). On the other hand, similar blood pressure lowering by hydralazine treatment failed to prevent cardiac remodeling, vascular endothelial dysfunction, and hepatic fibrosis in high-fat diet–fed mice (see Table S1). Thus, our work provided the evidence for the critical role of angiotensin II in diabetes-associated cardiovascular remodeling and endothelial dysfunction.

eNOS plays a major role in the regulation of vascular endothelial function.\(^{35,36}\) The formation of eNOS protein homodimers is essential for the enzymatic activity of eNOS to generate NO. Under normal conditions, eNOS exists mainly as the dimeric form, and it can generate NO to scavenge superoxide, leading to vascular protective effects.\(^{33,36,37}\) However, it has been reported that high-fat diet–induced diabetes markedly causes disruption of the eNOS dimer.\(^{31}\) Thus, eNOS dimer disruption in diabetes seems to be involved in vascular endothelial impairment, although the underlying mechanism is unclear. Notably, olmesartan treatment significantly suppressed the disruption of the vascular eNOS dimer in diabetic mice, demonstrating the crucial role of angiotensin II in diabetes-associated eNOS dimer disruption. Because eNOS enzymatic activity is also regulated by the phosphorylation of eNOS, we also measured the phosphorylation of eNOS in diabetic mice and found that vascular eNOS phosphorylation and total eNOS levels were not affected by diet-induced diabetes or olmesartan treatment. All of these findings support the notion that the prevention of
vascular endothelial dysfunction by olmesartan in diet-induced diabetes might be at least partially mediated by the suppression of eNOS dimer disruption.

Interestingly, in this work, we found that the enhancement of oxidative stress in cardiac, vascular, and hepatic tissues of obese and diabetic mice was significantly attenuated by olmesartan (Figure S6), suggesting that ROS might play some role in the beneficial effects of olmesartan on diabetic mice. ASK1 is one of the major protein kinases markedly activated by ROS and plays a pivotal role in a variety of cellular responses induced by ROS. Furthermore, using ASK1−/− mice, we have found that ASK1, as a ROS-activated intracellular signaling molecule, is implicated in angiotensin II–induced cardiovascular remodeling and vascular endothelial dysfunction. However, to the best of our knowledge, there is no report on the potential role of ASK1 in diabetes-associated cardiovascular diseases. Of note are the observations that ASK1 was significantly activated in cardiac and vascular tissues of diet-induced diabetic mice and that the marked amelioration by olmesartan of high-fat diet–induced cardiac injury and vascular endothelial dysfunction was associated with the significant suppression of cardiovascular ASK1 activation. These findings suggest the potential contribution of ASK1 to the protective effects of olmesartan against diabetes-associated cardiovascular injury. All of these findings encouraged us to assess the direct role of ASK1 in diet-induced diabetic mice using ASK1-deficient mice. In this work, we demonstrated that ASK1-deficient mice fed a high-fat diet exhibited much less cardiac inflammation, interstitial fibrosis, and coronary arterial remodeling than wild-type mice fed a high-fat diet, in spite of comparable food intake and blood pressure between both strains of mice (Figures 4, S7, and S8A). Moreover, we also found that ASK1 deficiency abolished the impairment of vascular endothelial function by diet-induced diabetes, being associated with the inhibition of the disruption of vascular eNOS function.
Figure 5. Endothelium-dependent relaxation with acetylcholine (A), eNOS dimer/monomer ratio (B), and superoxide levels (C) in aorta of wild-type mice and ASK-deficient mice. WT indicates wild-type mice; ASK, ASK1−/− mice; HF, high-fat diet; SD, standard diet. Top panels in B and C indicate representative Western blot of eNOS and representative photomicrograph of aortic sections stained with dihydroethidium, respectively. For the estimation of the vascular eNOS dimmer/monomer ratio, aortic tissues were pooled from 3 mice to obtain 1 sample. Magnification, ×400. Bar=100 μm. Values are means±SEMs (n=6 to 8 in A and C and n=3 in B).

In conclusion, ARB protects against cardiovascular injury and hepatic steatosis in obese and diabetic mice fed a high-fat diet. ASK1 deficiency mimicked these beneficial effects of ARB on diet-induced diabetic mice. Furthermore, angiotensin II is specifically responsible for ASK1 activation in cardiac, vascular, and hepatic tissues of diet-induced diabetic mice. From all of these findings, we propose that the protective effects of ARB against cardiovascular injury and hepatic steatosis in obesity and diabetes might be at least partially mediated by the inhibition of ASK1 activation and that ASK1 is a novel therapeutic target for cardiovascular complications and hepatic steatosis in diabetes. Hence, our present work provides a novel insight into the molecular mechanism responsible for diet-induced obesity, diabetes, and cardiovascular diseases.
of great clinical relevance to elucidate the mechanism responsible for obesity/diabetes-associated cardiovascular diseases and hepatic steatosis. In our present study, we have obtained the evidence that angiotensin II and ASK1 are implicated in cardiovascular remodeling and vascular endothelial dysfunction in obese and diabetic mice and have suggested that ASK1 is also implicated in the mechanism underlying the beneficial effects of ARB on these complications. Thus, our present work provided the novel insight into not only the role of angiotensin II in diabetes and its associated complications but also the molecular mechanism of these diseases.

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Disclosures
None.

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Olmesartan prevents cardiovascular injury and hepatic steatosis in obesity and diabetes, accompanied by ASK1 inhibition

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Online Methods

Animals

All procedures were in accordance with institutional guidelines for animal research. Male ASK1-/- mice and wild type mice (C57BL/6J) were used in the present study. Generation of ASK1-/- mice has been described previously. ASK1-/- mice were backcrossed into the C57BL/6J background at least 10 generations to reduce genetic variation.

Effect of olmesartan on mice fed high-fat diet

Eleven-week-old C57BL/6J mice were fed high-fat diet (535 kcal/100 g, 60 % energy as fat [primarily lard], 28 % energy as carbohydrate [primarily sucrose]) (KBT Oriental Co., Ltd, Saga, Japan), were randomly assigned to 2 groups, and were orally given (1) vehicle and (2) olmesartan (5 mg/kg/day) by gastric gavage for 17 weeks. Wild type mice (C57BL/6J), fed standard chow (373 kcal/100 g, 16.9 % energy as fat, 63.9 % energy as carbohydrate) (KBT Oriental Co., Ltd, Saga, Japan), served as the control. Food intake and body weight were periodically measured. Rectal temperature was regularly measured using an electron thermistor equipped with rectal probe (RET-3, Physitem Instruments, New Jersey, USA). Blood pressure (BP) was also periodically measured by tail-cuff plethysmography (BP-98A; Softron Co, Tokyo, Japan), as described. After 17 weeks of the treatment, mice were anesthetized with ether, and blood was collected by cardiac puncture to measure blood glucose, plasma levels of insulin, adiponectin, and total cholesterol. Then, liver, heart, and aorta were rapidly excised from mice, to perform biochemical, pharmacological, and pathological examinations, as described below in detail.

Comparative effect of high-fat diet on ASK1-/- mice and wild type mice

Eleven-week-old ASK1-/- mice and wild type mice (C57BL/6J) were separated into 2 groups, and were fed the above mentioned standard chow or the above mentioned high-fat...
diet for 17 weeks. After 17 weeks of the treatment, mice were anesthetized with ether, and blood and tissue samples were taken in the same manner as the above mentioned experiments on the effect of olmesartan.

**Effect of olmesartan on ASK1-/- mice fed high-fat diet**

We also examined the effect of olmesartan on ASK1-/- mice fed high-fat diet. ASK1-/- mice fed high-fat diet were given vehicle or olmesartan (5 mg/kg/day) by gastric gavage for 17 weeks. After the treatment, blood and tissue samples were collected in the same manner as the above mentioned experiments.

**Homeostasis Model Assessment of Insulin Resistance (HOMA-IR)**

HOMA-IR, a simple assessment of insulin sensitivity, was calculated by using the following formula: fasting plasma glucose (mg/dl) x insulin (ng/ml) /405. Plasma insulin levels were quantified by using the commercial ELISA kit (MORINAGA, Tokyo, Japan).

**Vessel ring preparation and organ chamber experiments**

Isometric tension studies were performed, as previously described. In brief, thoracic aortas from 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 1 g was maintained throughout the experiment. Vessel rings were precontracted with L-phenylephrine (10\(^{-7}\) mol/L). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) (10\(^{-9}\) mol/L to 10\(^{-4}\) mol/L) or sodium nitroprusside (10\(^{-9}\) mol/L to 10\(^{-4}\) mol/L) to obtain cumulative concentration-response curves.

**Tissue NADPH oxidase activity**
Hepatic, cardiac, or aortic tissues 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 10 µM lucigenin as electron acceptor, as described\(^4\). Protein concentrations were measured by the method of Bradford.

**Measurement of tissue superoxide**

Heart and aorta, removed from mice, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate cardiac and aortic superoxide levels in situ, as described in detail\(^4\). DHE fluorescence of cardiac and aortic sections was quantified using Lumina Vision version 2.2, analysis software. The mean fluorescence was quantified and expressed relative to values obtained for control mice.

**Measurement of thiobarbituric acid reactive substances (T-BARS)**

T-BARS was measured, as previously described\(^4\). In brief, hepatic tissues were homogenized (5 % wt/vol) in a solution containing 0.15 mol/L KCl and 0.02 mol/L Tris-HCl (pH 7.4). The homogenate was mixed with 15 % trichloroacetic acid and 0.375 % thiobarbituric acid. Butylated hydroxytoluene (0.01 %) was added to the assay mixture to prevent autoxidation of the sample, followed by heating at 100 °C for 15 min. After cooling, the mixture was centrifuged, and the absorbance of the organic phase was measured at 535 nm. The concentration was determined by the malondialdehyde standard curve and expressed as nmol/mg tissue.

**Preparation of aortic protein extracts and Western blot analysis of ASK1 and eNOS**

The detailed method was previously described\(^2\). 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: anti-phospho ASK1 \(^5\), anti-eNOS (x 5000, BD Transduction Laboratories), anti-total eNOS (x 4000, BD Transduction Laboratories), and anti-\(\alpha\)-tubulin (x 5000, CALBIOCHEM). In individual samples, each value was correct for that of \(\alpha\)-tubulin.

**Determination of vascular eNOS dimer and monomer**

Aortic eNOS dimer and monomer were separated, using low-temperature SDS-PAGE followed by Western blot analysis, as described previously \(^3\). For immunoblot analysis of the dimeric and monomeric form of eNOS protein, arterial 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 semidry 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 (x 5000, 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.

**Histological examination**

The heart and the liver from mice were fixed in 4 % paraformaldehyde overnight. Then, they were embedded in paraffin, sectioned into 5-\(\mu\)m slices, stained with hematoxylin and eosin. Hepatic section and cardiac section were also stained with sirius red F3BA (0.5 % in saturated aqueous picric acid, Aldrich Chemical Company) for the measurement of collagen volume fraction, as described \(^2\). Furthermore, in cardiac sections, coronary arterial thickness and perivascular fibrosis were quantitated, as described \(^2\).

For the quantification of macrophage, cardiac sections were also immunostained with anti-CD68 antibody (x 500, SEROTEC), as described \(^4\).
Measurement of plasma adiponectin, serum cholesterol, and hepatic triglyceride

Plasma adiponectin was determined by using a kit (Otsuka, Tokushima, Japan). Plasma total cholesterol levels were measured by using a kit (Wako Pure Chemical, Osaka Japan). Hepatic triglyceride was measured with a kit (Wako Pure Chemical, Osaka Japan).

Quantitative real time RT-PCR

Total RNA was extracted from hepatic tissue, according to the manufacturer’s instruction. One µg of RNA sample was reverse transcribed to first-strand cDNA, using QuantiTect® Reverse Transcription Kit (QIAGEN), according to the manufacturer’s recommended protocol. Thermal Cycler Dice® Real Time System (Takara) was used for 2 step RT-PCR. cDNA was amplified using SYBR® Premix Ex TaqTM with specific oligonucleotide primers for target sequences of transforming growth factor-β1 (TGF-β1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described. Amplification conditions included 10 seconds at 95 ºC and run for 40 cycles at 95 ºC for 5 seconds and 60 ºC for 30 seconds, and then dissociation 15 seconds at 95 ºC and 30 seconds at 60 ºC on the Thermal Cycler Dice® Real Time System. Specificity of the SYBR® Premix Ex TaqTM assays was confirmed by melting point analysis. Each threshold cycles (Ct) value was normalized to GAPDH Ct value and a control sample. Relative quantization by second derivative maximum method was expressed as fold-induction compared to control conditions. Gene expression of the housekeeping gene GAPDH (Applied Biosystems) was used for normalization.

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 comparison among more than 2 groups,
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 References


<table>
<thead>
<tr>
<th>End-point</th>
<th>SD (n= 6)</th>
<th>Veh (n= 6)</th>
<th>Hyd (n=6)</th>
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<tbody>
<tr>
<td>Blood pressure (mmHg)</td>
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<tr>
<td>1 week</td>
<td>113±2</td>
<td>114±2</td>
<td>94±3*</td>
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<td>5 week</td>
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<td>96±2*</td>
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<td>Body weight (g)</td>
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<td>LV/tibia (mg/mm)</td>
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<td>Endothelium-dependent relaxation (maximal relaxation) (%)</td>
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<td>Liver weight (mg)</td>
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<td>1397±26</td>
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<tr>
<td>Hepatic fibrosis (%)</td>
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To examine the role of blood pressure in cardiovascular and hepatic injury of high-fat diet fed mice, we examined the preventive effect of hydralazine on cardiovascular and hepatic injury of mice fed high-fat diet.

Abbreviations used; SD, mice fed standard diet; Veh, mice fed high-fat diet and simultaneously treated with vehicle; Hyd, mice fed high-fat diet and simultaneously orally given hydralazine (10 mg/kg/day). Each value represents mean±SEM. * P<0.01 vs Veh.
**Figure S1.** Blood pressure in standard diet fed mice and high-fat diet fed mice treated with vehicle or olmesartan

Abbreviations: HF, high-fat diet; SD, standard diet; Veh, mice fed high-fat diet and treated with vehicle; Olm, mice fed high-fat diet and treated with olmesartan. Values are means±SEM(n=7-10)
Figure S2. Food intake (A), body weight (B), HOMA-IR (C), and plasma adiponectin (D) in standard diet fed mice and high-fat diet fed mice treated with vehicle or olmesartan. Abbreviations used are the same as in Figure S1. NS, not significant. Values are means±SEM(n=6-8).
Figure S3. Hepatic weight (A), triglyceride (B), fibrosis (C), and TGF β 1 mRNA expression (D) in standard diet fed mice and high-fat diet fed mice treated with vehicle or olmesartan.

Upper panels in (C) indicate representative photomicrograph of hepatic sections stained with sirius red in each group of mice. Magnification = x 400. Bar = 100 µm. Abbreviations used are the same as in Figure S1. Values are means±SEM(n=6-8).
Figure S4. Endothelium-independent relaxation by sodium nitroprusside in aorta of standard diet fed mice and high-fat diet fed mice treated with vehicle or olmesartan.

Abbreviations used are the same as in Figure S1. SNP, sodium nitroprusside. Values are means±SEM(n=5-8).
**Figure S5.** Aortic phospho-eNOS and total eNOS in standard diet fed mice and high-fat diet fed mice treated with vehicle or olmesartan

Upper left and right panels indicate representative Western blot of phospho-eNOS and total eNOS, respectively. Abbreviations used are the same as in Figure S1. Values are means±SEM(n=3).
Figure S6. NADPH oxidase activity (A) and oxidative stress (B) in the heart, aorta, and liver of standard diet fed mice, and high-fat diet fed mice treated with vehicle or olmesartan.

Abbreviations used are the same as in Figure S1. Upper panels in (B) indicate representative photomicrographs of cardiac and aortic sections stained with DHE. Magnification = x 400. Bar = 100 µm. Values are means±SEM(n=6-8).
Figure S7. Blood pressure in wild type mice and ASK1 deficient mice

Abbreviations used: WT, wild type mice; ASK, ASK1-/- mice; HF, high-fat diet; SD, standard diet. Values are means±SEM (n=7-10).
Figure S8. Food intake (A), body weight (B), HOMA-IR (C), and plasma adiponectin (D) in wild type mice and ASK1 deficient mice. 

Abbreviations used are the same as in Figure S7. NS, not significant. Values are means±SEM (n=7-8)
Figure S9. Vascular endothelium-independent relaxation by sodium nitroprusside in wild type mice and ASK1 deficient mice

Abbreviations used are the same as in Figure S7. SNP, sodium nitroprusside. Values are means±SEM (n=6-9).
**Figure S10.** Aortic phospho-eNOS and total eNOS in wild type mice and ASK1 deficient mice. Upper left and right panels indicate representative Western blot of phospho-eNOS and total eNOS, respectively. Abbreviations used are the same as in Figure S7. Values are means±SEM(n=3).
Figure S11. Effects of olmesartan on cardiac macrophage infiltration (A), interstitial fibrosis (B), coronary arterial thickening (C), perivascular fibrosis (D), vascular endothelium-dependent relaxation by acetylcholine (E), liver weight (F), and hepatic fibrosis (G) of ASK1 deficient mice fed high-fat diet.

Abbreviations used: Veh, vehicle-treated ASK1 deficient mice fed high-fat diet; Olm, olmesartan-treated ASK1 deficient mice fed high-fat diet; n.s., not significant. Values are means±SEM (n=5-9).