Aliskiren Enhances the Protective Effects of Valsartan Against Cardiovascular and Renal Injury in Endothelial Nitric Oxide Synthase–Deficient Mice

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Abstract—The protective effect of aliskiren, a direct renin inhibitor, against hypertensive cardiovascular and renal injury remains to be defined. This study was undertaken to examine the protective effects of the combination of aliskiren and valsartan, an angiotensin receptor blocker, against cardiovascular and renal injury. Endothelial NO synthase–deficient mice, subjected to cuff injury of femoral artery, were divided into 5 groups and were treated with the following: (1) vehicle; (2) aliskiren (25 mg/kg per day); (3) valsartan (8 mg/kg per day); (4) combined aliskiren (12.5 mg/kg per day) and valsartan (4 mg/kg per day); and (5) hydralazine (10 mg/kg per day) for 4 weeks. Aliskiren and valsartan alone markedly and similarly suppressed cardiac hypertrophy, inflammation and fibrosis, and coronary remodeling; prevented cuff injury–induced arterial intimal thickening; and reduced urinary albumin excretion, glomerular inflammation, and glomerulosclerosis in endothelial NO synthase–deficient mice. These beneficial effects of aliskiren and valsartan were associated with the significant attenuation of oxidative stress in these tissues. Hence, aliskiren and valsartan markedly exert the protective effects against cardiovascular and renal injury through the reduction of oxidative stress. Furthermore, compared with monotherapy with aliskiren or valsartan, the combination of a half dose of these drugs more greatly improved the above-mentioned cardiovascular and renal injuries of endothelial NO synthase–deficient mice, which were associated with greater attenuation of tissue oxidative stress by the combination therapy. Thus, the combination of aliskiren and valsartan exerts the synergistic organ-protective effects through synergistic attenuation of oxidative stress. The combination of aliskiren and valsartan seems to be a promising therapeutic strategy for hypertensive organ injury caused by endothelial NO synthase dysfunction. (Hypertension. 2009;54:633-638.)

Key Words: eNOS ■ oxidative stress ■ combination therapy ■ synergistic effect ■ inflammation

Accumulating experimental and clinical evidence support the idea that the renin-angiotensin system (RAS) plays a key role in the pathophysiology and development of hypertension, cardiac diseases, atherosclerosis, and renal diseases in a broad range of patients. Mechanistically, conventional RAS blockers, angiotensin-converting enzyme inhibitor and angiotensin II type 1 (AT1) receptor blocker (ARB), attenuate feedback inhibition of renal renin release by suppressing the production and the action, respectively, of angiotensin II, which results in the increase in plasma renin activity. Moreover, plasma renin activity is regarded as a risk factor for myocardial infarction in hypertensive patients. Therefore, it is still unclear whether treatment with angiotensin-converting enzyme inhibitor or ARB allows optimized RAS suppression in patients with hypertension or cardiovascular and renal diseases.

Aliskiren is the first in a new class of orally effective direct renin inhibitors approved for the treatment of hypertension. In contrast to conventional RAS blockers, aliskiren blocks the renin system by directly inhibiting plasma renin activity and preventing the formation of both angiotensin I and angiotensin II, as shown by basic and clinical findings. However, it is unknown whether aliskiren is superior to conventional RAS blockers in terms of the prevention of cardiovascular and renal diseases.

Therefore, in the present study, to examine the protective effect of direct renin inhibition against hypertensive organ injury, we compared the effects of aliskiren, valsartan, and their combination on cardiovascular and renal injury of endothelial NO synthase (eNOS)-deficient mice with hypertension. We obtained evidence that the organ-protective effects of the combination of aliskiren and valsartan were greater than those of either monotherapy alone.

Methods

Animals and Drugs
Male eNOS−/− mice and wild-type mice (C57BL/6J) were used for this study. All of the procedures were in accordance with
Effects of Aliskiren, Valsartan, and Their Combination on eNOS−/− Mice

This study was undertaken to compare the effect of each drug treatment on organ injury of eNOS−/− mice, under similar hypotensive effects. Therefore, in preliminary experiments, we examined the effects of various doses of aliskiren, valsartan, their combination, and hydralazine on blood pressure of eNOS−/− mice to determine the equihypotensive dose of each drug. Mice at the age of 12±2 weeks were used for this experiment. According to our previous method, as described in the online Data Supplement (please see http://hyper.ahajournals.org), we developed a model of vascular remodeling by placing a nonconstrictive polyethylene cuff (2-mm length, PE50, Becton Dickinson) loosely around the left femoral artery of eNOS−/− mice. eNOS−/− mice, subjected to the above-mentioned cuff placement, were randomly assigned to 5 groups, including the following: (1) saline-infused group (control group); (2) hydralazine (10 mg/kg per day)-treated group; (3) valsartan (AT1 receptor blocker, 8 mg/kg per day)-treated group; (4) aliskiren (a direct renin inhibitor, 25 mg/kg per day)-treated group; and (5) combined valsartan (4 mg/kg per day) and aliskiren (12.5 mg/kg per day)-treated group. We chose the dose of 25 mg/kg per day for aliskiren monotherapy, because 25 mg/kg per day of aliskiren is the sufficient dose to sufficiently inhibit renin activity in mice in vivo.10 Drug treatment was performed for 4 weeks. Valsartan, aliskiren, and saline (control) were given to mice via osmotic minipump (ALZA Co). Hydralazine was given to mice as the drinking water. Throughout the experiment, systolic blood pressure of the conscious mice was measured every week with the tail-cuff method (BP98A; Softron Co). After 4 weeks of the drug treatment, a 24-hour urine sample was collected from each mouse with metabolic cages. Then, mice were anesthetized with ether, and the heart, kidney, and cuff-injured femoral arteries were rapidly excised from each mouse to perform histological examination and to evaluate various biochemical and molecular parameters, as described in the online Data Supplement.

Results

Cardiovascular and Renal Phenotypes of eNOS−/− Mice

Blood pressure of eNOS−/− mice was significantly higher than that of wild-type mice (132±3 versus 101±2 mm Hg; P<0.01). Figure S1 (please see the online Data Supplement) shows the detail of phenotypes of eNOS−/− mice. Fifteen-week-old eNOS−/− mice displayed the enhancement of cardiac interstitial fibrosis, coronary arterial thickening, and cardiac macrophage infiltration compared with wild-type mice (Figure S1A). Intimal thickening of the femoral artery caused by cuff injury for 4 weeks was greater in eNOS−/− mice than in wild-type mice (Figure S1B). eNOS−/− mice had the prominent glomerulosclerosis and the increased glomerular macrophage infiltration compared with wild-type

![Image of cardiovascular and renal phenotypes](https://example.com/image1.png)

Figure 1. Left ventricular weight (A) and cardiac interstitial fibrosis (B) of each group of eNOS−/− mice. Veh indicates vehicle-treated group; Hyd, hydralazine-treated group; Val, valsartan (8 mg/kg per day)-treated group; Ali, aliskiren (25 mg/kg per day)-treated group; Com, combined valsartan (4 mg/kg per day) and aliskiren (12.5 mg/kg per day)-treated group. A, Left ventricular (LV) weight in individual animals was corrected for the respective tibia length. B (top), Representative photomicrographs of cardiac sections stained with Sirius red. Values are mean±SEM (each group included 10 to 13 mice).

![Image of coronary arterial thickening](https://example.com/image2.png)

Figure 2. Coronary arterial thickening (A) and perivascular fibrosis (B) of each group of eNOS−/− mice. Top, Representative photomicrographs of cardiac sections stained with Sirius red. Abbreviations used are the same as in Figure 1. Values are mean±SEM (each group included 10 to 13 mice).
mice (Figure S1C). Moreover, superoxide levels in cardiac, injured arterial, and glomerular tissues were greater in eNOS−/−
mice than in wild-type mice (Figure S1). Thus, the phenotype of eNOS−/− mice was characterized not only by hypertension but also by cardiovascular and renal injuries and the enhancement of oxidative stress in these tissues.

Expression of AT1 Receptor mRNA and Protein in Cardiac and Renal Tissues of eNOS−/− Mice
As shown in Figure S2, AT1a receptor mRNA levels in both cardiac and renal tissues were greater in eNOS−/− mice than in wild-type mice. Cardiac and renal AT1 receptor protein levels in eNOS−/− mice were also larger than those in wild-type mice.

Effect on Blood Pressure and Plasma Angiotensin II
Aliskiren (25 mg/kg per day) alone, valsartan (8 mg/kg per day) alone, the combination of a half-dose of aliskiren (12.5 mg/kg per day) and valsartan (4 mg/kg per day), and hydralazine significantly reduced blood pressure of eNOS−/− mice throughout the treatment (Figure S3). There were no significant differences in the hypotensive effects among all of these drug treatments throughout the experiment.

As shown in Figure S4, plasma angiotensin II levels in eNOS−/− mice were markedly reduced by aliskiren monotherapy (P<0.01), whereas angiotensin II levels were significantly increased by valsartan monotherapy (P<0.01). Moreover, plasma angiotensin II concentrations in the combination group of valsartan plus aliskiren were lower than those in the valsartan monotherapy group (P<0.01) and the vehicle group (P<0.05). These results confirmed that aliskiren at the doses used in this study sufficiently inhibited plasma renin.

Effect on Cardiac Hypertrophy and Remodeling
As shown in Figure 1, aliskiren or valsartan alone significantly and comparably reduced cardiac weight and cardiac interstitial fibrosis of eNOS−/− mice. On the other hand, hydralazine failed to reduce them. The combination of a half-dose of aliskiren and valsartan reduced cardiac weight and cardiac interstitial fibrosis more than monotherapy with either agent. As shown in Figure 2, aliskiren and valsartan monotherapy, but not hydralazine, significantly and similarly reduced coronary arterial thickening and perivascular fibrosis of eNOS−/− mice. Furthermore, the combination of a half-dose of aliskiren and valsartan reduced these parameters more than either monotherapy. Figure 3 shows cardiac macrophage infiltration and cardiac superoxide levels of each group of eNOS−/− mice. In contrast to no effect of hydralazine on these parameters, valsartan and aliskiren monotherapy similarly and significantly reduced cardiac macrophage infiltration and superoxide levels of eNOS−/− mice. Moreover, their combination reduced cardiac macrophage infiltration and superoxide levels of eNOS−/− mice more than either monotherapy.

Effect on Cuff Injury–Induced Arterial Intimal Hyperplasia
Figure 4 shows the ratio of intima:media area of cuff-injured femoral artery from each group of mice after 4 weeks of the treatment. Hydralazine did not reduce cuff-induced arterial intimal thickening of eNOS−/− mice, whereas aliskiren and valsartan monotherapies significantly reduced arterial intimal thickening of eNOS−/− mice. Furthermore, the reduction of the ratio of intima:media area was greater in eNOS−/− mice...
effects of these agents. Thus, our present work highlights the combination of aliskiren and valsartan as a promising therapeutic strategy for cardiovascular and renal diseases.

Effect on Cardiac and Renal NADPH Oxidase Activities of eNOS<sup>-/-</sup> Mice

As shown in Figure 8, cardiac and renal NADPH oxidase activities of eNOS<sup>-/-</sup> mice were significantly higher than those of wild-type mice. Valsartan and aliskiren monotherapies significantly and similarly reduced NADPH oxidase activity of both cardiac and renal tissues of eNOS<sup>-/-</sup> mice, whereas hydralazine did not significantly reduce it. Furthermore, the combination of these agents attenuated cardiac and renal NADPH oxidase activities more than either monotherapy alone.

Discussion

The major finding of our current study was that the combination of aliskiren and valsartan exerted greater organ-protective effects than monotherapy with a higher dose of either agent and was associated with greater attenuation of oxidative stress by the combination. Thus, our present work highlights the combination of aliskiren and valsartan as a promising therapeutic strategy for cardiovascular and renal diseases.

Accumulating clinical and experimental evidence indicates that eNOS, through NO production, plays a protective role not only in hypertension but also in cardiovascular and renal diseases. Importantly, essential hypertension is characterized by the defect of eNOS and the diminished NO production and is significantly associated with polymorphism in the eNOS gene. Thus, it is a clinically very key issue whether RAS blockers protect against cardiovascular injury caused by eNOS dysfunction. In the current work, aliskiren and valsartan monotherapies both markedly prevented cardiac hypertrophy, inflammation and fibrosis, renal glomerulosclerosis and inflammation, and vascular intimal hyperplasia caused by cuff injury in eNOS-deficient mice, whereas the equihypotensive dose of hydralazine failed to prevent them. All of these findings show that these organ-protective effects of aliskiren and valsartan in eNOS-deficient mice were in part mediated by their direct inhibition of RAS independent of blood pressure, although the blood pressure-lowering effect might still be partially required for the protective effects of these agents.

Recent clinical data show the additive beneficial effects of the combination of aliskiren with conventional RAS blockers on patients with hypertension, type 2 diabetic nephropa-
remodeling, cuff injury-induced femoral arterial intimal thickening, renal glomerulosclerosis, and inflammation of eNOS-deficient mice to a greater extent than either monotherapy alone. These findings provide the first evidence that the combination of aliskiren and valsartan exerts greater organ-protective effects than monotherapy with higher dose of either agent alone.

Oxidative stress is involved in the onset and development of cardiovascular and renal diseases. NO plays antiatherogenic role by counteracting oxidative stress. In the present study, as expected, we found the enhancement of superoxide, the initial product of reactive oxygen species, in the heart, the kidney, and cuff-injured femoral artery of eNOS-deficient mice (Figure S1), supporting the contribution of oxidative stress to organ injuries in eNOS-deficient mice. Notably, the combination of aliskiren and valsartan exerted more attenuation of cardiac, arterial, and renal superoxide in

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Figure 7. Urinary albumin excretion (A), glomerulosclerosis index (B), glomerular macrophage infiltration (C), and glomerular superoxide levels (D) of each group of eNOS−/− mice. A, Urinary albumin excretion in individual mice was corrected for the respective urinary creatinine excretion. B (top), Representative photomicrographs of renal sections stained with periodic acid-Schiff. C (top), Representative photomicrographs of renal sections stained with dihydroethidium. Abbreviations used are the same as in Figure 1. Values are mean±SEM (each group included 10 to 13 mice).

Figure 8. NADPH oxidase activity of cardiac (A) and renal (B) tissues of wild-type and eNOS−/− mice. Abbreviations used are the same as in Figure 1. WT indicates wild-type mice. Values are mean±SEM (each group included 6 mice).
eNOS-deficient mice than either monotherapy alone (Figures 3, 6, and 7), which was attributed to greater suppression of NADPH oxidase activity by the combination therapy (Figure 8). These findings show that greater organ-protective effects of the combination therapy in eNOS-deficient mice than either monotherapy are at least in part attributed to more attenuation of NADPH oxidase–mediated oxidative stress by the combination therapy.

**Study Limitation**

Importantly, hypertensive patients and animals are characterized by partial attenuation of eNOS, differing from complete absence of eNOS in eNOS-deficient mice. Therefore, much caution should be given regarding the interpretation and the implication of our current results obtained from eNOS-deficient mice (a particular hypertensive model). The mechanisms underlying vascular protection of aliskiren and ARBs in hypertensive animals or patients are partially mediated by the improvement of eNOS function, such as the enhancement of eNOS activity and the inhibition of eNOS uncoupling.11,20,26–28 Thus, the use of eNOS-deficient mice in this work did not allow us to examine the potential of the eNOS-mediated benefit of the combination of these agents. Additional study using other hypertensive models is needed to confirm the benefits of their combination therapy.

In conclusion, we obtained evidence that the combination of aliskiren and valsartan exerted greater protective effects against cardiovascular and renal diseases caused by eNOS deficiency than monotherapy with a double dose of either agent through greater attenuation of tissue oxidative stress.

**Perspectives**

Risk factors, such as hypertension, diabetes mellitus, or dyslipidemia, are significantly associated with eNOS dysfunction. Our current experimental findings highlight the combination therapy of aliskiren with valsartan as the promising therapeutic strategy for cardiovascular and renal diseases.

**Sources of Funding**

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology and by a grant from Novartis Pharmaceutical Corporation.

**Disclosures**

None.

**References**

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Hypertension. 2009;54:633-638; originally published online July 13, 2009;
doi: 10.1161/HYPERTENSIONAHA.109.133884

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/54/3/633

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2009/07/13/HYPERTENSIONAHA.109.133884.DC1

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Online supplement

Aliskiren enhances the protective effects of valsartan against cardiovascular and renal injury in eNOS deficient mice

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**Animals and drugs**

Male eNOS-/- mice\(^1\)\(^-\)\(^2\) and wild-type mice (C57BL/6J) were used for this study. All procedures were in accordance with institutional guidelines for animal research. Valsartan and aliskiren were kindly gifted from Novartis (Basel, Switzerland). Hydralazine was purchased from Sigma-Aldrich Co. (Missouri, USA).

**Effect of aliskiren, valsartan and their combination on eNOS-/- mice**

This study was undertaken to compare the effect of each drug treatment on organ injury of eNOS-/- mice, under similar hypotensive effects. Therefore, in preliminary experiments, we examined the effect of various doses of aliskiren, valsartan, their combination, and hydralazine on blood pressure of eNOS-/- mice, to determine the equihypotensive dose of each drug. Mice at the age of 12±2 weeks were used for this experiment. According to our previous method,\(^3\) as described later, we developed a model of vascular remodeling by placing a non constructive-polyethylene cuff (2 mm length, PE50; Becton Dickinson, Mountain View, CA, USA) loosely around left femoral artery of eNOS-/- mice. eNOS-/- mice, subjected to the above mentioned cuff placement, were randomly assigned to 5 groups, including (1) saline-infused group (control group), (2) hydralazine (10 mg/kg/day)-treated group, (3) valsartan (AT1 receptor blocker, 8 mg/kg/day)-treated group, (4) aliskiren (a direct renin inhibitor, 25 mg/kg/day)-treated group, and (5) combined valsartan (4 mg/kg/day) and aliskiren (12.5 mg/kg/day)-treated group. Previous report \(^4\) indicates that the administration of 25 mg/kg/day of aliskiren in mice sufficiently inhibits plasma renin in vivo. As the main purpose of our work was to examine the efficacy of renin inhibition with aliskiren on cardiovascular and renal diseases, it was appropriate to use the dose of aliskiren enough to inhibit plasma renin in vivo. Therefore, in this work, we used the above mentioned hypotensive doses of aliskiren and valsartan. Drug treatment was performed for 4 weeks. Valsartan, aliskiren, and saline (control) were given to mice via osmotic minipump (ALZA Co., California, USA). Hydralazine was given to mice as the drinking water. Throughout the experiment, systolic blood pressure of the conscious mice was measured every week with the tail-cuff method (BP98A; Softron Co). After 4 weeks of the drug treatment, 24 hour-urine sample was collected from each mouse with metabolic cages. Then, mice were anesthetized with ether, and the heart, kidney, and cuff-injured femoral arteries were rapidly excised from each mouse to perform histological examination and to evaluate various biochemical and molecular parameters, as described in Online Supplement.

**Periadventitial vascular injury procedure**

The surgical procedure for cuff-induced periadventitial injury was performed, according to the method previously described with some modification \(^3\). In brief, mice were anesthetized with pentobarbital by intraperitoneal injection. The right and left femoral arteries of the mice were dissected from the surrounding tissue. A non constructive-polyethylene cuff (2 mm length, PE50; Becton Dickinson, Mountain View, CA, USA) was placed loosely around the left femoral artery.

**Morphometry and cell proliferation of cuff-injured arteries**

Paraffin-embedded sections were used for morphometry and proliferating cell analysis of cuff-injured femoral arteries, as described \(^3\). The harvested injured arteries were cut into cross sections and were stained with Elastica Van Gieson. For each artery section, the areas of the intima, media, and lumen were measured utilizing image-analysis software (Lumina Vision). Three cross-section cuts, at least 250 \(\mu\)m intervals, were obtained from each artery, and then the measurements of the sections from each animal were averaged for morphometrical analysis.

For proliferating cell nuclear antigen (PCNA) staining, we used PCNA staining kit (ZYMED Laboratories, San Francisco, CA, USA), and counter-stained with methylgreen.
Histological examination on cardiac and renal tissues

The heart and kidney from each mouse were fixed in 4 % paraformaldehyde overnight and embedded in paraffin. Cardiac sections (5 µm thick) were 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), as previously described 5. The area of perivascular fibrosis per vessel was assessed by using image-analysis software (Lumina Vision version 2.2, Mitani Co., Fukui, Japan; Image J, National Institutes of Health, Bethesda, Maryland, USA).

Renal sections (5 µm thick) were stained with hematoxylineosin and periodic acid-Schiff (PAS) and were analyzed for degree of glomerulosclerosis, defined as disappearance of cellular elements from the tuft, capillary loop collapse, folding of the glomerular basement membrane with accumulation of amorphous material, as described 6. The grades were 0, 0 %; I, 1 to 25 %; II, 26 % to 50 %; III, 51 to 75 %; and IV, 76 % to 100 % of glomeruli involved. The glomerulosclerosis score was calculated as \((1 \times \% \text{ grade I}) + (2 \times \% \text{ grade II}) + (3 \times \% \text{ grade III}) + (4 \times \% \text{ grade IV})\). One hundred glomeruli were examined from each animal.

Measurement of cardiac and glomerular macrophage infiltration.

Cardiac and renal tissues, removed from mice, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). For detection of macrophage infiltration, the frozen sections were stained with the primary antibodies (rat anti-mouse CD68, Serotec; x500) at 4°C overnight 7. After incubation with the primary antibodies, HRP conjugated anti-rat IgG secondary antibody (BioSource, Camarillo, CA, USA) was utilized. The reactions were visualized with 3,3′-diaminobenzidine (DakoCytomation, Carpinteria, CA, USA), and counter-stained with hematoxylin. Negative controls were prepared by substitution of the primary antibody with an irrelevant antibody.

Measurement of tissue superoxide

Dihydroethidium (DHE) was used to evaluate tissue superoxide levels in situ, as described previously 8. DHE fluorescence of cardiac, renal and femoral arterial sections was quantified using image-analysis software (Lumina Vision). The mean fluorescence was quantified and expressed relative to values obtained from vehicle saline-treated eNOS−/− mice.

Measurement of NADPH oxidative activity

The heart and the kidney were 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 8. 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 and renal protein extracts and Western blot analysis

Our detailed method has been described previously. As primary antibodies, anti-AT1 receptor antibody (x2000, Santa Cruz) and anti-α-tubulin (x5000, Oncogene) were used. 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.

Real-time quantitative reverse transcriptase–polymerase chain reaction

Total RNA was isolated from left ventricle and kidney samples (30 mg each) using
Isogen (Nippon Gene, Tokyo, Japan). Complementary DNAs were synthesized by reverse transcription of 1 µg 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 angiotensin type 1a (AT1a) receptor, by using Thermal Cycler Dice Real Time System (Takara Biochemicals, Kyoto, Japan). The following sets of primers were used for PCR; AT1a receptor; forward, 5′-GGACACTGCCATGCCCATAAC-3′; reverse, 5′-TGAGTGCGACTTGGCCTTTG-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. Ct value and relatively calculated by setting a calibrator sample in each run using standard curve method.

**Measurement of urinary albumin excretion**

The mice were housed in metabolic cages for 24-hour to collect urine. Urinary albumin excretion was measured by using a mouse Albumin ELISA Quantitation kit (albuwell, Exocell, Philadelphia, USA), according to the manufacturer’s instruction.

**Measurement of plasma angiotensin II**

Angiotensin II concentrations were measured in plasma from 5 experimental groups of eNOS-/- mice subjected to cuff injury and treated with each drug for 4 weeks and from wild type mice subjected to cuff injury in the same manner as eNOS-/- mice. The measurement of angiotensin II in plasma was carried out by high performance liquid chromatography coupled with a specific radioimmunoassay, according to our established method previously described in detail. 9, 10

**Statistical Analysis**

All data are presented as mean±SEM. Comparison between 2 groups was analyzed by unpaired Student’s t-test using Prism (GraphPad Software Inc., San Diego, CA, USA). 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.
References


Online Figure S1. Cardiovascular and renal phenotype of eNOS-/- mice (A), (B), and (C) indicate representative photomicrographs of cardiac, femoral arterial, and renal sections, respectively, of wild type mice (WT) and eNOS-/- mice (eNOS-/-). (A) eNOS-/- mice display the enhancement of cardiac interstitial fibrosis, coronary arterial remodeling, cardiac macrophage infiltration, and the increased cardiac superoxide, compared with wild type mice. (B) Femoral arterial intimal thickening and superoxide production induced by 4 weeks of cuff injury are greater in eNOS-/- mice than wild type mice. (C) eNOS-/- mice shows the significant glomerulosclerosis, the increased glomerular macrophage infiltration, and the increased glomerular superoxide levels, compared with wild type mice. Experiments were performed on 5 wild type mice and 5 eNOS -/- mice, and similar results were obtained in all mice.
Online Figure S2. AT1a receptor mRNA and AT1 receptor protein in the heart (A) and the kidney (B) of wild type mice and eNOS-/- mice. Abbreviation used: WT, wild type mice; eNOS-/-, eNOS-/- mice. AT1 receptor protein in individual samples was corrected for tubulin. Values are means ± SEM (each group included 10-13 mice).
Online Figure S3. Time course of blood pressure of each group of eNOS-/- mice

Abbreviations used: Veh, vehicle-treated group; Hyd, hydralazine-treated group; Val, valsartan (8 mg/kg/day)-treated group; Ali, aliskiren (25 mg/kg/day)-treated group; Com, combined valsartan (4 mg/kg/day) and aliskiren (12.5 mg/kg/day)-treated group. Values are means ± SEM (each group included 10-13 mice).
Online Figure S4. Plasma angiotensin II levels in wild type mice and each group of eNOS-/- mice

Abbreviations used: WT, wild type mice; Veh, vehicle-treated group; Hyd, hydralazine-treated group; Val, valsartan (8 mg/kg/day)-treated group; Ali, aliskiren (25 mg/kg/day)-treated group; Com, combined valsartan (4 mg/kg/day) and aliskiren (12.5 mg/kg/day)-treated group. Values are means±SEM (n=5 per group).