Pravastatin Enhances Beneficial Effects of Olmesartan on Vascular Injury of Salt-Sensitive Hypertensive Rats, via Pleiotropic Effects


Objective—This work was undertaken to investigate comparative effect of AT1 receptor blocker (ARB), 3-hydroxy-3-methylglutaryl (HMG) coenzymeA (CoA) reductase inhibitor (statin), and their combination on vascular injury of salt-sensitive hypertension.

Methods and Results—Salt-loaded Dahl salt-sensitive hypertensive rats (DS rats) were treated with (1) vehicle, (2) hydralazine (5 mg/kg/d), (3) olmesartan (0.5 mg/kg/d), (4) pravastatin (100 mg/kg/d), and (5) combined olmesartan and pravastatin for 4 weeks. Olmesartan or pravastatin significantly and comparably improved vascular endothelium-dependent relaxation to acetylcholine, coronary arterial remodeling, and eNOS activity of DS rats. Olmesartan prevented vascular eNOS dimer disruption or the downregulation of dihydrofolate reductase (DHFR) more than pravastatin, whereas Akt phosphorylation was enhanced by pravastatin but not olmesartan, indicating differential pleiotropic effects between olmesartan and pravastatin. Add-on pravastatin significantly enhanced the improvement of vascular endothelial dysfunction and remodeling by olmesartan in DS rats. Moreover, pravastatin enhanced the increase in eNOS activity by olmesartan, being associated with additive effects of pravastatin on phosphorylation of Akt and eNOS.

Conclusions—Olmesartan and pravastatin exerted beneficial vascular effects in salt-sensitive hypertension, via differential pleiotropic effects. Pravastatin enhanced vascular protective effects of olmesartan. Thus, the combination of ARB with statin may be the potential therapeutic strategy for vascular diseases of salt-sensitive hypertension. (Arterioscler Thromb Vasc Biol. 2007;27:556-563.)

Key Words: eNOS dimers □ DHFR □ oxidative stress □ vascular injury □ combined ARB and statin

Accumulating evidence indicates that renin–angiotensin system (RAS) plays a crucial role in the pathophysiology of cardiovascular diseases in hypertension, and that RAS blockers, including angiotensin-converting enzyme inhibitors and AT1 receptor blockers (ARB), are the useful therapeutic agents for hypertensive cardiovascular diseases.1 As hypertension is often accompanied by dyslipidemia in the same patients, their treatment frequently involves the combination of RAS blockers with 3-hydroxy-3-methylglutaryl coenzymeA (HMG-CoA) reductase inhibitors (statins), potent inhibitors of cholesterol biosynthesis. Clinical evidence show that statins improve endothelial dysfunction and reduce the incidence of atherosclerotic events.2-5 and these vascular protective effects by statins are at least partially attributed to their pleiotropic vascular effects beyond lowering of plasma cholesterol.4-6 However, the difference in vascular pleiotropic effects between RAS blockers and statins remains to be fully understood. Moreover, the significance and the advantage of their combination therapy in hypertension, particularly salt-sensitive hypertension, are not defined.

Clinically, salt-sensitive hypertensive patients are more prone to cardiovascular diseases than their salt-insensitive counterparts.7-8 Therefore, it is a key clinical issue to elucidate the effect of RAS blockers and statins in salt-sensitive hypertension. Moreover, it is not clear at all whether add-on statin treatment enhances vascular protective effects of RAS blockers in salt-sensitive hypertension. Dahl salt-sensitive hypertensive rats (DS rats) constitute a paradigm of salt-sensitive hypertension in humans and therefore are the useful animal model to investigate the mechanism underlying cardiovascular injury in salt-sensitive hypertension.

The aim of our present work was to compare the impact of olmesartan (an ARB), pravastatin (a statin), and their combination on vascular diseases in DS rats and to examine the relative role of reactive oxygen species (ROS) and eNOS in their pleiotropic effects. We obtained the evidence that olmesartan and pravastatin improved vascular injury of DS rats, via different pleiotropic effects on ROS and eNOS, and pravastatin significantly enhanced vascular effects of olmesartan in DS rats.
Methods

Experimental Animals
All procedures were in accordance with institutional guidelines for animal research. DS rats (Japan SLC Inc, Shizuoka, Japan) were used in the present study. At 7 weeks of age, the diet of DS rats was switched from a 0.3% NaCl (low-salt) to an 8% NaCl (high-salt) diet. Control DS rats were fed a 0.3% NaCl diet, throughout the experiments.

Experiment I: Time Course of Impact of Sodium Loading
The first experiments were performed to examine time-dependent effect of high-salt loading on vascular endothelial function, NADPH oxidase activity, superoxide, eNOS activity and phosphorylation, disruption of eNOS dimers, plasma NO2/NO3, Akt phosphorylation, nitrotyrosine, dihydrofolate reductase (DHFR), and coronary arterial thickening in DS rats. DS rats, fed low-salt diet throughout the experiment, served as control. Blood pressure (BP) was periodically measured by tail-cuff plethysmography (BP-98A; Softron Co). Eight-, 12-, and 16-week-old DS rats, subjected to high-salt diet for 1, 5, and 9 weeks, respectively, were anesthetized with ether, arterial blood was immediately collected by cardiac puncture, and plasma was collected by centrifugation and stored at −80°C until use. Then, the carotid artery and the thoracic aorta were immediately excised to estimate vascular endothelial function and the above mentioned biochemical parameters, and the heart was also removed to examine coronary arterial thickening.

Experiment II: Comparative Effect of an ARB, a Statin, and Their Combination
The second experiments were carried out to compare the effect of olmesartan (Sankyo Co. Ltd), pravastatin (Sankyo Co. Ltd), their combination, or hydralazine on DS rats fed high-salt diet. Twelve-week-old DS rats, which had fed a high-salt diet from 7 weeks of age, were orally given olmesartan (0.5 mg/kg/d), pravastatin (100 mg/kg/d), combined olmesartan (0.5 mg/kg/d) and pravastatin (100 mg/kg/d), or hydralazine (5 mg/kg/d) for 4 weeks. Olmesartan and pravastatin were suspended in 0.5% carboxymethyl cellulose (CMC), and were given to rats by gastric gavage once a day. Hydralazine was given to rats as the drinking water. It is well known that oral administration of 100 mg/kg/d of pravastatin to rats yields plasma pravastatin concentration similar to that seen in patients taking clinical doses of pravastatin (100 mg/d), and does not significantly change plasma cholesterol levels in DS rats. Furthermore, in preliminary experiments, we found that olmesartan, pravastatin, their combination, and hydralazine at the above mentioned dose exerted similar hypotensive effects in DS rats. Therefore, this experimental protocol allowed us to elucidate the lipid-independent and blood pressure-independent effect of each therapy on DS rats. BP and heart rate were measured in conscious rats at 3 to 5 hours after oral dosing, every week. After 4 weeks of treatment, DS rats were anesthetized with ether, then the carotid artery, the thoracic aorta, and the heart were immediately excised to compare the effect of each treatment on vascular endothelial function, NADPH oxidase activity, p22phox, superoxide, eNOS activity, eNOS dimers, phosphorylation of eNOS and Akt, DHFR, extracellular signal regulated-kinase (ERK), nitrotyrosine, phospho-MEK, coronary arterial thickening, perivascular fibrosis, cardiac hypertrophy, and cardiac fibrosis and macrophage infiltration.

A detailed description of the Methods is available in the online data supplement at http://atvb.ahajournals.org.

Results

Time Course of BP, Vascular Endothelial Function, and Coronary Remodeling in DS Rats Fed High-Salt Diet
DS rats were fed high-salt (8% NaCl) diet from 7 weeks of age. We measured BP, endothelial function, and coronary arterial thickening in 8-, 12-, and 16-week-old DS rats, fed high-salt diet for 1, 5, and 9 weeks, respectively (supplemental Figure 1). Compared with control DS rats fed low-salt diet, BP in salt-loaded DS rats progressively increased with time. As shown by vascular relaxing response to acetylcholine (Ach) (supplemental Figure 1-B), vascular endothelium-dependent relaxation in salt-loaded DS rats was already slightly but significantly impaired at 8 weeks of age (P<0.05) and was thereafter progressively impaired with time, which was followed by the significant coronary arterial thickening at 16 weeks of age.

Time Course of Vascular NADPH Oxidase, Superoxide, eNOS Activity, and Plasma NO2/NO3
As shown in Figure 1A and 1B, vascular NADPH oxidase activity and superoxide of DS rats were already significantly increased by 1 week of high-salt loading (P<0.05) (at 8 weeks of age) and remained increased throughout the treatment of high salt. On the other hand, vascular eNOS activity of salt-loaded DS rats was not altered at 8 or 12 weeks of age, but was significantly reduced by 53% at 16 weeks of age (P<0.01), compared with low-salt diet (Figure 1C). As in vascular eNOS activity, plasma NO2/NO3 concentrations were significantly decreased only at 16 weeks of age (Figure 1D).

Time Course of Phospho-Akt, Phospho-eNOS, and the Disruption of eNOS Dimers
As shown in supplemental Figure II, 1, 5, or 9 weeks of high-salt diet did not affect phosphorylation of vascular Akt throughout the treatment. On the other hand, vascular phospho-eNOS levels were significantly reduced in 16-week-old DS rats subjected to 9 weeks of high-salt intake (P<0.05).

Supplemental Figure III shows the detection of vascular eNOS protein dimers and monomers by low-temperature SDS-PAGE followed by Western blot analysis. In low-salt fed DS rats, eNOS existed exclusively as the dimers, whereas...
in high-salt fed 16-week-old DS rats, eNOS was present predominantly in the monomers and eNOS dimers were nearly absent, indicating the significant disruption of eNOS protein dimers by 9 weeks of salt loading. No significant disruption of vascular eNOS dimers was found in 8- and 12-week-old salt-loaded DS rats (data not shown).

**Effect of Olmesartan, Pravastatin, Combined Olmesartan and Pravastatin, and Hydralazine on BP and Plasma Lipids of DS Rats Fed High-Salt Diet**

Each drug was given 12-week-old DS rats which had been already fed high-salt diet for 5 weeks, and drug treatment was carried out for 4 weeks. As shown in supplemental Figure IV, olmesartan, pravastatin, combined olmesartan and pravastatin, and hydralazine slightly reduced BP of DS rats to a comparable degree throughout the treatment, except for greater reduction of BP by hydralazine than the other drug treatments at 13 weeks of age.

After 4 weeks of treatment, plasma cholesterol concentrations in vehicle-, olmesartan-, pravastatin-, combined olmesartan and pravastatin-, and hydralazine-treated salt-loaded DS rats, and low-salt DS rats were 77±8, 76±6, 78±6, 77±8, 74±5, and 71±4 mg/dL, respectively, and plasma triglyceride were 140±20, 124±18, 144±19, 108±9, 119±5, and 136±14 mg/dL, respectively. There was no significant difference in plasma cholesterol or triglyceride levels, among all groups of DS rats.

**Comparative Effect on Serum Creatinine and Plasma Thiobarbituric Acid Reactive Substances**

As shown in supplemental Figure V, serum creatinine and plasma thiobarbituric acid reactive substances (T-BARS; a marker of lipid peroxidation) were significantly higher in 16-week-old salt-loaded DS rats compared with control. Olmesartan, pravastatin, and their combination almost normalized serum creatinine. Olmesartan and pravastatin alone significantly and comparably reduced plasma T-BARS, and their combination reduced it more than either monotherapy.

**Comparative Effect on Vascular Endothelial Function and Coronary Remodeling of DS Rats Fed High-Salt Diet**

As shown in Figure 2, either olmesartan or pravastatin, but not hydralazine, significantly prevented the impairment of vascular endothelium-dependent relaxation to Ach and also prevented the progression of coronary arterial thickening and perivascular fibrosis, to a comparable degree. The combination of olmesartan with pravastatin prevented vascular endothelial dysfunction more than monotherapy with either agent alone and prevented coronary arterial thickening and perivascular fibrosis more than pravastatin alone, despite no difference in BP and plasma lipids among all drug treatment groups.

As shown in supplemental Figure VI, the phosphorylation of vascular MEK and ERK was significantly enhanced in 16-week-old salt-loaded DS rats compared with control. Olmesartan and pravastatin alone similarly prevented the phosphorylation of vascular MEK and ERK compared with control, and their combination decreased the phosphorylation of MEK and ERK more than pravastatin alone.

**Comparative Effect on Vascular NADPH Oxidase, p22phox, Superoxide, and eNOS Activity of DS Rats Fed High-Salt Diet**

As shown in Figure 3, either olmesartan or pravastatin alone, but not hydralazine, significantly ameliorated the increase in vascular NADPH oxidase activity, the increase in p22phox protein expression, the increase in vascular superoxide, or the decrease in vascular eNOS activity of salt-loaded DS rats. The inhibitory effect of olmesartan, regarding NADPH oxidase, p22phox, and superoxide, was greater than that of pravastatin. On the other hand, no significant difference was noted in the beneficial effect on vascular eNOS activity between olmesartan and pravastatin. Add-on pravastatin significantly enhanced the improvement of superoxide (P<0.05) and eNOS activity (P<0.01) by olmesartan alone, whereas pravastatin failed to enhance the suppression of NADPH oxidase and p22phox by olmesartan.
Comparative Effect on Vascular Phospho-Akt, Phospho-eNOS of DS Rats Fed High-Salt Diet

In contrast to no significant effect of olmesartan on vascular Akt phosphorylation, pravastatin alone or olmesartan combined with pravastatin significantly and comparably increased phosphorylation of Akt in DS rats (Figure 4). However, there was no significant difference in vascular total Akt levels in each group. Either olmesartan or pravastatin alone significantly upregulated vascular phospho-eNOS levels and total eNOS, and add-on pravastatin significantly enhanced the upregulation of phospho-eNOS and total eNOS by olmesartan (P<0.05).

Comparative Effect on Vascular eNOS Dimers and DHFR Protein Expression of DS Rats Fed High-Salt Diet

Either olmesartan or pravastatin alone significantly prevented the disruption of vascular eNOS protein dimers in salt-loaded DS rats, but olmesartan prevented it more than pravastatin (P<0.01; Figure 5A). Add-on pravastatin did not significantly enhance the suppressive effect of olmesartan on eNOS dimer disruption. As shown in Figure 5B, vascular DHFR protein levels were significantly downregulated in 16-week-old salt-loaded DS rats compared with control DS rats (P<0.01), although no significant difference in DHFR was

Figure 3. Effect of each treatment on vascular NADPH oxidase (A), p22phox (B), superoxide (C), and eNOS activity (D) of DS rats fed high-salt diet. Top panels in B and C show representative Western blot and fluorescence photomicrographs, respectively, in each group. *P<0.05, **P<0.01 vs V. Each bar represents mean±SEM. A: L, n=9; V, n=9; H, n=4; O, n=9; P, n=9; O+P, n=8. B: L, n=9; V, n=9; H, n=5; O, n=9; P, n=9; O+P, n=8. C: n=5 per group. D: n=4 to 5 per group. Abbreviations are the same as Figure 2. L indicates DS rats fed low-salt diet.

Figure 4. Effect of each treatment on phospho-akt (p-akt), total akt, phospho-eNOS (p-eNOS), and total eNOS of DS rats fed high-salt diet. Top panels show representative Western blot in each group. Each bar represents mean±SEM (n=5 per group). Abbreviations are the same as Figure 2. L indicates DS rats fed low-salt diet.
noted between salt-loaded and control DS rats at the age of 8 and 12 weeks. Olmesartan, but not pravastatin, significantly prevented the downregulation of DHFR in salt-loaded DS rats (P<0.01). Add-on pravastatin did not significantly enhance the upregulation of DHFR by olmesartan.

Comparative Effect on Vascular Nitrotyrosine of DS Rats Fed High-Salt Diet

As shown by Western blot analysis and immunohistochemistry in Figure 6, vascular nitrotyrosine levels were significantly increased in 16-week-old salt-loaded DS rats compared with control DS rats (P<0.01), although no significant difference in nitrotyrosine was noted between salt-loaded and control DS rats at the age of 8 and 12 weeks. Olmesartan and pravastatin significantly reduced vascular nitrotyrosine. The combination of olmesartan with pravastatin did not significantly enhance the suppressive effect of nitrotyrosine by olmesartan, while significantly enhanced the reduction of nitrotyrosine by pravastatin.

Figure 5. Effect of each treatment on eNOS dimer disruption (A) and DHFR protein levels (B) of DS rats fed high-salt diet. Top panels in A and B show representative Western blot in each group. Each bar represents mean±SEM (n=5 per group). Abbreviations are the same as Figure 2. L indicates DS rats fed low-salt diet.

Comparative Effect on Cardiac Hypertrophy, Fibrosis, and Macrophage Infiltration of DS Rats Fed High-Salt Diet

As shown in supplemental Figure VII, olmesartan and pravastatin alone significantly prevented cardiac hypertrophy and fibrosis in salt-loaded DS rats, and their combination prevented them more than either monotherapy. Cardiac macrophage infiltration was significantly and comparably prevented by olmesartan and pravastatin, and their combination prevented it more than pravastatin monotherapy (supplemental Figure VIII). These results suggest that the combination of pravastatin and olmesartan may be useful for the treatment of cardiac remodeling in salt-sensitive hypertension.

Discussion

It is well established that atherosclerosis is closely associated with the impairment of vascular endothelial function, whose hallmark is an impairment of endothelium-dependent vasorelaxation induced by reduced bioavailability of NO. A decline in NO bioavailability is mainly caused by the reduction of NO synthesis by eNOS and the increased inactivation of NO by superoxide. Thus, the dysfunction of eNOS and the activation of NADPH oxidase which generates superoxide play a major role in vascular endothelial dysfunction and vascular remodeling. Previous works on salt-loaded DS rats show that vascular NADPH oxidase and superoxide were significantly increased whereas eNOS activity was inversely significantly decreased in DS rats suffering from severe endothelial dysfunction and vascular hypertrophy. However, in previous works, time course of these parameters in DS rats has not been examined and therefore the relative relationship among NADPH oxidase, superoxide, and eNOS, regarding the role in vascular injury, is not elucidated. In our present work, we found that slight but significant impairment of vascular endothelium-dependent relaxation occurred by 1 week after start of salt loading (supplemental Figure I), being associated with the early increase in NADPH oxidase activity and superoxide (Figure 1). These results, taken together with no change in eNOS activity at this early stage of salt loading, showed that NADPH oxidase–generated superoxide, but not eNOS, was involved in the early onset of vascular endothelial dysfunction in salt-sensitive hypertension. Notably, much later than the onset of increase in NADPH oxidase activity, the remarkable reduction of vascular eNOS activity occurred only in 16-week-old DS rats fed high-salt diet for 9 weeks (Figure 1). At 16 weeks of age, DS rats had much more severe endothelial dysfunction and remarkable coronary arterial remodeling than at the earlier age (supplemental Figure I). Therefore, in contrast to the importance of NADPH oxidase at the early phase of salt loading, vascular eNOS seems to be mainly involved in the exacerbation of vascular endothelial dysfunction and vascular structural changes of salt-sensitive hypertension, indicating differential role between NADPH.
oxidase and eNOS in the progression of vascular injury in salt-sensitive hypertension.

eNOS activity is regulated by not only its phosphorylation but also multiple factors. In this study, to elucidate the underlying mechanism of the decreased eNOS activity in 16-week-old salt-loaded DS rats, we examined the phosphorylation of vascular Akt and eNOS, the disruption of eNOS dimers, peroxynitrite, and DHFR expression in DS rats. Akt appears to be the kinase principle phosphorylating eNOS, leading to endothelium-dependent vasodilation.18 Previous studies show that the formation of eNOS protein homodimers is necessary for enzymatic activity, and peroxynitrite can disrupt eNOS protein dimers through oxidation and displacement of the zinc metal ion, leading to the reduction of eNOS activity.19,20 DHFR is a key enzyme synthesizing tetrahydrobiopterin, eNOS cofactor, and very recent in vitro study on cultured vascular endothelial cells21 indicate that downregulation of DHFR causes the uncoupling of eNOS, leading to the decrease in enzymatic activity.21 However, the in vivo role of Akt, peroxynitrite, disruption of eNOS dimers, and DHFR in hypertension remains unknown. In the present work, Akt phosphorylation was not altered throughout salt loading, indicating the minor role of Akt in the decreased eNOS activity in 16-week-old DS rats. On the other hand, eNOS protein dimers were markedly diminished in 16-week-old DS rats (supplemental Figure III), indicating that the disruption of eNOS dimers significantly occurred in DS rats. Furthermore, this disruption of eNOS dimers was associated with the increase in nitrotyrosine (an indicator of peroxynitrite) and the downregulation of DHFR. Therefore, we obtained the first evidence that the disruption of eNOS dimers was involved in the reduction of eNOS activity in salt-sensitive hypertension, and eNOS dimer disruption might be attributed to the increased peroxynitrite and the downregulation of DHFR.

Although previous studies indicate that monotherapy with ARB (candesartan)16 or statin (atorvastatin)15 improves vascular endothelial dysfunction and hypertrophy, reduces supersoxide, and increases eNOS activity in salt-loaded DS rats, previous studies did not rule out the possible involvement of blood pressure lowering or plasma lipid lowering in these vascular effects. In our present study, we have obtained the evidence that olmesartan and pravastatin improved vascular injury of salt-sensitive hypertension, independently of blood pressure or plasma lipid. Notably, despite similar beneficial effects between olmesartan and pravastatin on vascular endothelial function, coronary remodeling, and eNOS activity in DS rats, olmesartan normalized vascular NADPH oxidase activity, p22phox, superoxide, nitrotyrosine (peroxynitrite), and the disruption of eNOS dimers to a greater extent than pravastatin. Olmesartan significantly enhanced DHFR expression, in contrast to no effect of pravastatin on DHFR. Taken together with the fact that the increase in peroxynitrite19,20 or DHFR downregulation21 leads to the disruption of eNOS dimers, our present data support the notion that the restoration of eNOS activity by olmesartan was mediated by the suppression of eNOS dimer disruption, which was at least partially mediated by the amelioration of peroxynitrite and DHFR downregulation. On the other hand, pravastatin significantly enhanced the phosphorylation of Akt, differing from no effect of olmesartan on Akt. Akt phosphorylation is well known to enhance eNOS activity.20,22-23 Accordingly, the restoration of eNOS activity by pravastatin might be at least in part attributed to the enhancement of Akt phosphorylation. Thus, the mechanism responsible for the restoration of eNOS activity in salt-sensitive hypertension seems to differ between olmesartan and pravastatin.

The therapeutic significance of the combination therapy of RAS blocker with statin remains obscure, and previous clinical and experimental reports are rare and contradictory.24-32 Particularly, the significance of addition of statin to RAS blocker is undefined in salt-sensitive hypertension,32 which encouraged us to investigate whether add-on pravastatin enhances the beneficial effect of olmesartan in DS rats. Of note are the observations that pravastatin significantly enhanced the improvement of endothelium-dependent vascular relaxation and coronary remodeling by olmesartan in DS rats, despite no additive effect of pravastatin on blood pressure and plasma lipid. Therefore, our work provided the first evidence that pravastatin has the potential to enhance vascular protective effect of olmesartan in salt-sensitive hypertension, independent of blood pressure and lipid. Interestingly, pravastatin significantly enhanced the normalization of vascular eNOS activity by olmesartan. In contrast to no effect of olmesartan alone on Akt phosphorylation, pravastatin significantly increased Akt phosphorylation (Figure 4). Therefore, the addition of pravastatin to olmesartan increased the phosphorylation of Akt, as well as pravastatin alone. Moreover, add-on pravastatin significantly enhanced the phosphorylation of eNOS by olmesartan, and this enhancement by pravastatin might be at least in part mediated by the enhancement of the increase in total eNOS protein levels by olmesartan (Figure 4). Thus, the increase in Akt phosphorylation and eNOS phosphorylation seems to be responsible for the increase in eNOS activity by add-on pravastatin.

In the present study, we found no statistically significant additive effect of pravastatin on olmesartan-treated DS rats, with regard to eNOS dimers, DHFR, or ERK. As described above, the formation of eNOS protein homodimers is necessary for eNOS enzymatic activity, and is known to be disrupted mainly by the increase in peroxynitrite19,20 and the reduction of DHFR.21 In this study, we found that vascular peroxynitrite was significantly increased in salt-sensitive hypertension, and that olmesartan and pravastatin alone significantly reduced vascular peroxynitrite. The combination of olmesartan with pravastatin did not significantly enhance the reduction of peroxynitrite by olmesartan. On the other hand, very importantly, the combination of olmesartan with pravastatin more potently inhibited vascular peroxynitrite production more than pravastatin monotherapy, indicating that inhibitory effect of pravastatin on peroxynitrite production was weaker than that of olmesartan. These findings can account for no significant effect of add-on pravastatin on peroxynitrite. Taken together with the fact that peroxynitrite and DHFR are key regulators of eNOS dimer formation, no significant effect of add-on pravastatin on eNOS dimers can be explained by no significant effect of add-on pravastatin on peroxynitrite and DHFR.
Recent in vitro study using cultured vascular endothelial cells indicated that angiotensin II downregulates DHFR.\textsuperscript{21} However, it remains to be determined whether or not the downregulation of DHFR by angiotensin II can apply to in vivo situation. We have obtained the first evidence that the downregulation of DHFR occurred in salt-sensitive hypertension and olmesartan, but not pravastatin, significantly prevented the downregulation of DHFR in salt-sensitive hypertension. Taken together with the findings that renin-angiotensin system is involved in vascular diseases of salt-sensitive hypertension, our present work demonstrated that angiotensin II is implicated in the downregulation of vascular DHFR in salt-sensitive hypertension.

As in the case of ERK, add-on pravastatin did not significantly enhance the inhibition of vascular MEK phosphorylation by olmesartan (supplemental Figure VI). Therefore, no effect of add-on pravastatin on ERK phosphorylation can be explained by no effect of add-on pravastatin on MEK phosphorylation, because MEK is the major upstream activator of ERK.

\textbf{Study limitation}

Pravastatin is a hydrophilic statin, and the direct vascular effects of pravastatin remain to be fully understood. Taken together with previous findings\textsuperscript{10,11} our present results show the direct vascular pleiotropic effects of pravastatin, and this notion is also supported by the findings of MEGA Study\textsuperscript{33} that a low dose of pravastatin suppresses primary cardiovascular events in Japanese hypercholesterolemic patients as much as hydrophobic statins. However, our present in vivo study did not allow us to elucidate the detailed molecular mechanism underlying the effect of pravastatin on vascular injury in salt-sensitive hypertension, because the study on the detailed molecular mechanism was out of scope. Further study is needed to elucidate more detailed molecular mechanism responsible for the effect of olmesartan, pravastatin, and their combination on vascular injury of salt-sensitive hypertension.

In conclusion, we obtained the evidence that olmesartan and pravastatin exerts beneficial effect on vascular endothelial function and remodeling of salt-loaded DS rats, via differential multiple pleiotropic effects, and that add-on pravastatin treatment significantly enhanced the improvement of vascular injury by olmesartan via the phosphorylation of Akt and eNOS. We propose that combination of ARB with statin may be potentially promising therapeutic strategy of salt-sensitive hypertensive patients with hyperlipidemia, beyond blood pressure and lipid control.

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\textbf{Disclosures}

None.

\textbf{References}


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Online Methods

Arterial Ring Preparation and Tension Recording

After removal of the carotid artery from DS rats, the vessel was cut into 5 mm rings with special care to reserve 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, as described ¹. 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. The aortic 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) to obtain cumulative concentration-response curves.

Measurement of vascular superoxide

Carotid artery, removed from DS rats, was immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate carotid arterial superoxide levels in situ, as described in detail ². DHE fluorescence of arterial section was quantified using Lumina Vision version 2.2, analysis software. The mean fluorescence was quantified and expressed relative to values obtained for control rats.

Preparation of arterial protein extracts and western blot analysis

Our detailed method has been described previously ³, ⁴. Briefly, after arterial 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-eNOS (Ser-1177) (x 5000, BD Transduction Laboratories), anti-eNOS (x 5000, BD Transduction Laboratories), anti-phospho-akt (Ser473) (x 5000, Cell Signaling TECHNOLOGY), anti-akt
(x 5000, Cell Signaling TECHNOLOGY), anti-α-tubulin (x 5000, CALBIOCHEM), anti-p22-phox (x2500, SANTA CRUZ BIOTECHNOLOGY, INC), anti-phospho-ERK (x 2500, Cell Signaling TECHNOLOGY), anti-dihydrofolate reductase (DHFR) (x 2500, BD Transduction Laboratories), anti-phospho-MEK1/2 (x 2000, Cell Signaling TECHNOLOGY), anti-nitrotyrosine (x 2000, ZYMED Laboratories). In individual samples, each value was correct for that of α-tubulin.

**Determination of eNOS dimers and monomers**

eNOS dimers and monomers were separated, using low-temperature SDS-PAGE, as described previously⁵, ⁶.

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 polyclonal antibody (x5000, BD Transduction Laboratories) for overnight at 4 °C. Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (x5000, Santa Cruz Biotechnology). The antibody was visualized using an enhanced chemiluminescence method (ECL; Amersham Biosciences). The intensity of the bands was quantified using NIH Image analysis software v1.61.

**NADPH oxidase activity, eNOS activity and plasma NO₂/NO₃**

Arterial tissue was 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 by us ². eNOS activity was determined by measuring the conversion of [3H]-arginine to [3H]-citrulline, using a NOS assay kit (Calbiochem). To determine plasma NO, plasma
NO$_2$/NO$_3$ was measured by a commercially available kit (Dojindo, Co). Protein concentrations were measured by the method of Bradford.

**Plasma thiobarbituric acid reactive substances (T-BARS) and serum creatinine**

To measure plasma T-BARS, plasma 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/ml of plasma. Serum creatinine was measured using a kit (Wako).

**Histological examination**

The hearts 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 coronary arterial thickness, perivascular fibrosis and interstitial fibrosis, as previously described. For identification of monocytes/macrophages, cardiac sections were immunostained with ant-ED-1 antibody, as described.

**Measurement of vascular nitrotyrosine**

Vascular nitrotyrosine was determined by western blot analysis and immunohistochemistry with specific anti-nitrotyrosine antibody, as previously described by Molnar et al. Arterial protein extracts were subjected to 12% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electric transfer to polyvinylidene difluoride membrane, and the membranes were probed with anti-nitrotyrosine (x 2000, ZYMED Laboratories), as described. In individual samples, each value was correct for that of α-tubulin.

Frozen arterial sections were incubated with 1:100 dilution of rabbit anti-nitrotyrosine antibody in 4% goat serum for 30 minutes at room temperature. After washing, Qdot
secondary antibody conjugates (Invitrogen) were applied for 30 minutes, and sections were washed and coverslipped. Fluorescence images were acquired using fixed exposure times using a CCD camera attached to a Zeiss Axiovert inverted microscope. Negative and positive control sections were preincubated with 3-nitrotyrosine (Sigma), or a peroxynitrite solution (Upstate), respectively, to assure specificity of staining.

**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. Differences were considered statistically significant at a value of P<0.05.
Online Figure Legends

**Fig. I** Time course of BP (A), arterial endothelium-dependent relaxation to acetylcholine (EDR) (B), and coronary arterial thickening (C) in DS rats fed high-salt diet from 7 weeks of age

High Na, 8 % NaCl diet; Low Na, 0.3 % NaCl diet. Values are mean±SEM (n=5)

**Fig. II** Time course of phospho-akt (p-akt) and phospho-eNOS (p-eNOS) in DS rats fed 8 % NaCl diet (High Na) or 0.3 % NaCl diet (Low Na)

Top panels show representative western blot in each group. Each bar represents mean±SEM (n=5 per group).

**Fig. III** Determination of vascular eNOS dimers and monomers in 16-week-old DS rats fed high-salt diet or low-salt diet, by low-temperature SDS-PAGE followed by western blot analysis

Top panels show representative western blot in each group. Each bar represents mean±SEM (n=5 per group).

**Fig. IV** Comparative effect of vehicle (V), olmesartan (O), pravastatin (P), combined olmesartan and pravastatin (O+P), and hydralazine (H) on BP of DS rats fed high-salt diet

Values are mean±SEM (n=4-9 per group). L indicates DS rat fed low-salt diet throughout the experiments.

**Fig. V** Effect of vehicle (V), olmesartan (O), pravastatin (P), combined olmesartan and pravastatin (O+P), and hydralazine (H) on serum creatinine (A) and plasma TBARS (B)

Values are mean±SEM (n=5 per group). L indicates DS rat fed low-salt diet throughout the experiments.

**Fig. VI** Effect of vehicle (V), olmesartan (O), pravastatin (P), combined olmesartan and pravastatin (O+P), and hydralazine (H) on phospho-MEK (A) and phospho-p42ERK and phospho-p44ERK (B) of salt-loaded DS rats
Top panels show representative western blot in each group. L indicates DS rat fed low-salt diet throughout the experiments. Each bar represents mean±SEM (n=5 per group).

**Fig. VII** Effect of vehicle (V), olmesartan (O), pravastatin (P), and combined olmesartan and pravastatin (O+P) and hydralazine (H) on cardiac hypertrophy (A) and interstitial fibrosis (B) of salt-loaded DS rats.

Echocardiographic analysis was performed, according to our previous method⁹. AW thickness indicates left ventricular anterior wall thickness in each group. Each bar represents mean±SEM (n=5 per group).

**Fig. VIII** Effect of vehicle (V), olmesartan (O), pravastatin (P), and combined olmesartan and pravastatin (O+P) and hydralazine (H) on cardiac macrophage infiltration of salt-loaded DS rats.

Top panels show representative images of cardiac macrophage infiltration. Each bar represents mean±SEM (n=5 per group).
Online References


* P<0.01 vs V, † P<0.05 vs H
(A) * P<0.01 vs V

Serum creatinine (mg/dl)

High Na

(B) * P<0.01 vs V, † P<0.01 vs H

Plasma TBARS (nmol/ml)

High Na
Low Na  High Na

eNOS
Dimer
Monomer

Ratio of dimer to monomer

P < 0.01
Low Na
High Na

p-akt

p-eNOS

tubulin

Age (weeks)
8 12 16

p-akt / tubulin

0 0.5 1.0 1.5

P<0.05 vs Low Na

# P<0.05 vs Low Na

p-eNOS

Age (weeks)
8 12 16

P<0.01

P<0.01

# P<0.01

Age (weeks)
8 12 16

p-eNOS / tubulin

0 0.5 1.0 1.5

81 2 16

P<0.01
(A) BP

(B) EDR

(C) Coronary arterial thickening

# P<0.05, * P<0.01 vs Low Na

Age (weeks)

Low Na
High Na