Fluvastatin Enhances the Inhibitory Effects of a Selective AT₁ Receptor Blocker, Valsartan, on Atherosclerosis

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Abstract—We investigated the effects of a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (statin) on the inhibitory effects of an angiotensin II type-1 receptor (AT₁) blocker on atherosclerosis and explored cellular mechanisms. We gave apolipoprotein E null mice a high-cholesterol diet for 10 weeks and measured atherosclerotic plaque area and lipid deposition. Neither 1 mg/kg per day of valsartan nor 3 mg/kg per day of fluvastatin had any effect on blood pressure or cholesterol concentration; however, both drugs decreased plaque area and lipid deposition after 10 weeks. We then reduced the doses of both drugs to 0.1 mg/kg per day and 1 mg/kg per day, respectively. At these doses, neither drug had an effect on atherosclerotic lesions. When both drugs were combined at these doses, a significant reduction in atherosclerotic lesions was observed. Similar inhibitory effects of valsartan or fluvastatin on the expressions of nicotinamide-adenine dinucleotide/nicotinamide-adenine dinucleotide phosphate oxidase subunits p22phox and p47phox, production of superoxide anion, the expression of monocyte chemoattractant protein-1, and intercellular adhesion molecule-1 expression were observed. These results suggest that concomitant AT₁ receptor and cholesterol biosynthesis blockade, particularly when given concomitantly, blunts oxidative stress and inflammation independent of blood pressure or cholesterol-related effects. (Hypertension. 2004;44:758-763.)

Key Words: angiotensin ■ atherosclerosis ■ oxidative stress

The angiotensin II (Ang II) type-1 (AT₁) receptor mediates the major cardiovascular actions of Ang II. AT₁ receptor blockers (ARBs) putatively exert a vascular protective effect. We reported previously that the ARB valsartan attenuated neointimal formation, decreased vascular smooth muscle cell (VSMC) proliferation, and diminished vascular inflammation. On the other hand, recent large clinical trials showed that 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) decreased ischemic stroke, myocardial infarction, and mortality in hypercholesterolemic subjects. Possibly, some of the statin-related beneficial effects may be cholesterol independent. Statins may prevent Ang II–induced cellular and organ damage. We demonstrated previously that neointimal formation and VSMC proliferation induced by cuff placement around the femoral artery were significantly inhibited by a valsartan–fluvastatin combination. Either drug alone at these doses did not affect neointimal formation or VSMC proliferation. The drug combination showed a more effective reduction in medial cross-sectional area in genetically hypertensive rats than either drug alone. Furthermore, valsartan showed additive antioxidative effects when given with fluvastatin in terms of LDL oxidation in hypercholesterolemic and hypertensive patients compared with monotherapy. We conceived an experimental study to explore the mechanisms involved with the combination therapy. We studied apolipoprotein E-deficient knockout (ApoEKO) mice, which develop lesions similar to those observed in humans.

Methods

Animals and Treatment

Adult male ApoEKO mice (The Jackson Laboratory, Bar Harbor, ME) and wild-type mice (based on C57BL/6J strain; CLEA Japan, Tokyo, Japan) 6 weeks of age were used. To generate AT₁/ApoEKO mice, ApoEKO and AT₁KO mice (based on C57BL/6J strain; donated by Tanabe Seiyaku Co. Ltd., Osaka, Japan) were bred to yield mice heterozygous at both loci. These double heterozygotes were crossed to yield AT₁−/−, ApoE−/− mice that were intercrossed to yield AT₁−/−, ApoE−/− (AT₁/ApoEKO) mice. The animal studies committee of Ehime University granted approval. The mice received a standard diet (MF; Oriental Yeast Co. Ltd.) or a high-cholesterol diet (1.25% cholesterol, 10% coconut oil in MF) for 10 weeks from 6 weeks of age and water ad libitum. Valsartan, an AT₁ receptor selective ARB, and fluvastatin (provided by Novartis Pharma AG; Basel, Switzerland) were administered daily by gavage from 6 weeks of age. Hydroxylase (Sigma) was given daily by gavage from 6 weeks of age. Plasma cholesterol and blood pressure were measured as described previously.

Atherosclerotic Lesion Size

Mice were euthanized at the age of 16 weeks and the atherosclerotic lesions were analyzed. Atherosclerotic areas in the proximal aorta were determined by means of serial cross-sections of freshly

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frozen sample taken throughout the aortic arch stained with Elastica van Gieson. Lipid areas were determined by serial cross-sections, followed by oil red O staining and counterstaining with hematoxylin. Quantitative analysis was performed with Densitograph imaging software (ATTO Corp). The mean value of 5 sections was taken as the value for each animal. The entire aorta was dissected free from surrounding tissues and opened longitudinally. Atherosclerotic lesion area was quantified by analyzing the open luminal surface of oil red O–stained aorta with Densitograph imaging software. The amount of lesion formation in the entire aorta in each animal was measured as the percentage lesion area per total area of the endothelial surface.

Western Blot and Immunofluorescence
Total proteins were prepared from pooled aortas (6 to 8 arteries for each group). Western blot was performed for monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), p22phox, p47phox, and α-smooth muscle actin. Immunofluorescence was assessed using freshly frozen sections that were incubated with anti-p47phox antibody, washed and incubated with biotin-labeled secondary antibodies, then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system.

Superoxide
Frozen, enzymatically intact, 10-μm-thick sections of cross-sections of proximal aorta were incubated at the same time with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. Ethidium bromide was detected as described previously. Superoxide production was also quantitatively measured using the cytochrome c reduction assay as described previously. Superoxide production was quantified in picomoles per milligram of aorta from the difference between absorbance with or without superoxide dismutase (SOD).

Statistical
Values are expressed as mean±SE in the text and figures. We used 2-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed. A value of P<0.05 was considered to be statistically significant.

Results
Effects of Valsartan or Fluvastatin on Atherosclerosis
Cholesterol levels were markedly increased after high-cholesterol feeding in ApoEKO mice compared with ApoEKO mice with a normal diet (2138±254 mg/dL and 413±45 mg/dL, respectively, 10 weeks after high-cholesterol diet). Valsartan (∼1.0 mg/kg per day) or fluvastatin (∼3.0 mg/kg per day) did not influence the cholesterol concentrations (supplemental Table I, available online at http://www.hypertensionaha.org). Valsartan or fluvastatin at these doses did not affect systolic blood pressure and heart rate. After 10 weeks of diet, marked atherosclerotic lesions and lipid deposition were observed in the proximal aorta in ApoEKO mice (Figures 1 and 2; supplemental Figure I, available online at http://www.hypertensionaha.org). Valsartan at 1.0 mg/kg per day or fluvastatin at 3 mg/kg per day decreased the atherosclerotic area and lipid deposition by ∼60% (Figures 1 and 2; supplemental Figure I).

Next, we examined the possibility of whether fluvastatin–valsartan could exert a synergistic effect. Valsartan and fluvastatin at lower doses (0.1 or 1 mg/kg per day, respectively) significantly decreased atherosclerotic lesions and lipid deposition, whereas valsartan or fluvastatin at these doses alone had no effect (Figures 1 and 2; supplemental Figure I). The combination of higher doses of valsartan and fluvastatin decreased atherosclerotic lesions and lipid deposition by ∼70% (Figures 1 and 2; supplemental Figure I).
Fluvastatin seemed to further decrease atherosclerotic lesion and lipid deposition (Figures 1 and 2; supplemental Figure I). Similar effects of valsartan or fluvastatin on atherosclerotic plaque area were also observed in the descending aorta (Figure 2C). Atherosclerotic lesion formation and lipid deposition in the proximal aorta and atherosclerotic plaque area in the descending aorta in AT1r/ApoEKO mice were 60% to 70% less than in ApoEKO mice. Fluvastatin at 1 mg/kg per day and 3 mg/kg per day further decreased these parameters in AT1r/ApoEKO mice by 30% and 50%, respectively (supplemental Figure II, available online at http://www.hypertensionaha.org), suggesting that antiatherosclerotic effect of fluvastatin is at least partly independent of the inhibition of AT1 receptor activation. On the other hand, an unspecific vasodilator, hydralazine, at a dose of 5 mg/kg per day reduced systolic blood pressure to 82.9 ± 110.0 mm Hg in ApoEKO mice, which is similar to that in AT1r/ApoEKO mice. However, this dose of hydralazine did not affect the atherosclerotic lesion (0.305 ± 0.033 mm²) and lipid deposition in proximal aorta (0.042 ± 0.007 mm²).

**Effect of Valsartan and Fluvastatin on Oxidative Stress and Inflammatory Response**

Superoxide generation in general, and upregulation of nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, in particular, have an essential role in atherosclerotic lesion formation.19,20 To investigate superoxide production in atherosclerotic vessels, in situ superoxide detection was performed with DHE. As shown in Figure 3, the chemiluminescent signal attributable to superoxide production was markedly enhanced in the aorta from ApoEKO mice maintained on a high-cholesterol diet compared with the aorta in ApoEKO mice maintained on a normal diet. Valsartan at 1.0 mg/kg per day or fluvastatin at 3 mg/kg per day decreased superoxide production (Figure 3C and 3D). Valsartan or fluvastatin at lower doses (0.1 or 1 mg/kg per day, respectively) did not decrease superoxide production, whereas coadministration of valsartan and fluvastatin at these doses significantly decreased superoxide production (Figure 3E through 3G). Consistent with the results of in situ superoxide detection with DHE, we observed similar results by quantitative analysis in superoxide production determined with the cytochrome c reduction assay (Figure 4; supplemental Figure IV, available online at http://www.hypertensionaha.org). Combination of higher doses of valsartan and fluvastatin further decreased superoxide production (Figure 4). Superoxide production in AT1r/ApoEKO mice was less compared with that in ApoEKO mice and fluvastatin at 3 mg/kg per day further decreased by 50% this superoxide production (supplemental Figure IV). Fluvastatin at 1 mg/kg per day also tended to decrease superoxide production in AT1r/ApoEKO mice (supplemental Figure IV).

Expression of p22phox, p47phox, MCP-1, and ICAM-1 was evaluated by Western blot (Figure 5; supplemental Figure V, available online at http://www.hypertensionaha.org). Pooled artery samples showed increases in p22phox, p47phox, MCP-1, and ICAM-1 expression in the aorta of ApoEKO mice receiving a high-cholesterol diet. Valsartan at 1.0 mg/kg per day or fluvastatin at 3 mg/kg per day decreased p22phox, p47phox, MCP-1, and ICAM-1 expression, but valsartan or fluvastatin at lower doses (0.1 or 1 mg/kg per day, respectively) did not decrease p22phox, p47phox, MCP-1, and ICAM-1 expression. Coadministration of valsartan and fluvastatin at these doses significantly decreased the expression of these parameters. Next, we examined in situ p47phox expression in atherosclerotic artery and observed that p47phox expression was exaggerated in the aorta of ApoEKO mice maintained on a high-cholesterol diet. Similar inhibitory effects of valsartan or fluvastatin on protein levels of p22phox, p47phox, MCP-1, and ICAM-1 were observed during in situ p47phox expression (supplemental Figure III, available online at http://www.hypertensionaha.org).

**Discussion**

We examined the possibility that statins may enhance the beneficial effects of an ARB on atherosclerosis. We demon-
strated that treatment with even a low dose of valsartan, together with a low dose of fluvastatin that did not influence blood pressure or plasma cholesterol, effectively attenuated atherosclerotic lesion size and lipid deposition with a decrease in oxidative stress and vascular inflammation. These inhibitory effects were not observed with the same doses of valsartan or fluvastatin alone. Moreover, coadministration of higher doses of valsartan and fluvastatin seemed to further decrease atherosclerotic lesion and lipid deposition.

Recent work points to additional cholesterol-independent effects of statins on cellular signal transduction. Increased release and production of ROS may be a key event in pathogenesis of endothelial dysfunction and atherosclerosis. Previous studies suggested that the modulation of subunit of NADH/NADPH oxidase expression is decisively important for the overall activity of NADH/NADPH oxidase. In vascular cells, activation of the AT1 receptor by Ang II is one of the most prominent mechanisms of ROS production in vitro as well as in vivo. Moreover, it has been reported that Ang II–stimulated endothelial NADH/NADPH oxidase activity is regulated through serine phosphorylation of p47phox and its enhanced binding to p22phox in mouse microvascular endothelial cells. We demonstrated that a low dose of valsartan or fluvastatin (1 or 3 mg/kg per day, respectively) decreased p22phox and p47phox expression and superoxide anion production in arteries of high-cholesterol diet–fed ApoEKO mice without changes in blood pressure and plasma cholesterol concentration. Coadministration of lower doses of valsartan and fluvastatin (0.1 and 1 mg/kg per day, respectively) significantly inhibited p22phox and p47phox expression and superoxide anion production, whereas valsartan or fluvastatin alone at these lower doses had no effect. These results suggest that a statin could enhance the inhibitory effect of an ARB on oxidative stress. Such synergistic effects between fluvastatin and valsartan were supported by observations that fluvastatin reduced atherosclerotic changes as well as superoxide production dose dependently in AT1a/ApoEKO mice (supplemental Figures II and IV). The inhibition of atherosclerotic

**Figure 3.** Fluorescent detection of superoxide in proximal aorta from ApoEKO mice treated with HCD for 10 weeks. Representative superoxide detection with DHE in cross-sections of proximal aorta is shown. Similar results were obtained in 5 different animals. A, ApoEKO mouse with standard normal diet. B, ApoEKO mouse with high-cholesterol diet (HCD). C, ApoEKO mouse with HCD plus 1.0 mg/kg per day valsartan. D, ApoEKO mouse with HCD plus 3.0 mg/kg per day fluvastatin. E, ApoEKO mouse with HCD plus 0.1 mg/kg per day valsartan. F, ApoEKO mouse with HCD plus 1.0 mg/kg per day fluvastatin. G, ApoEKO mouse with HCD plus 0.1 mg/kg per day valsartan and 1.0 mg/kg per day fluvastatin. Magnification ×200.

**Figure 4.** Effects of valsartan or fluvastatin on superoxide production in aorta in ApoEKO mice. Aortic samples were taken from ApoEKO mice as in Figures 1 and 2. Superoxide content in aorta of ApoEKO mice is shown. Values are mean±SE (n=6 to 9 per group). Val indicates valsartan; Flu, fluvastatin. *P<0.05 vs normal diet (ND); †P<0.05 vs high-cholesterol diet (HCD) without valsartan or fluvastatin.

**Figure 3.** Fluorescent detection of superoxide in proximal aorta from ApoEKO mice treated with HCD for 10 weeks. Representative superoxide detection with DHE in cross-sections of proximal aorta is shown. Similar results were obtained in 5 different animals. A, ApoEKO mouse with standard normal diet. B, ApoEKO mouse with high-cholesterol diet (HCD). C, ApoEKO mouse with HCD plus 1.0 mg/kg per day valsartan. D, ApoEKO mouse with HCD plus 3.0 mg/kg per day fluvastatin. E, ApoEKO mouse with HCD plus 0.1 mg/kg per day valsartan. F, ApoEKO mouse with HCD plus 1.0 mg/kg per day fluvastatin. G, ApoEKO mouse with HCD plus 0.1 mg/kg per day valsartan and 1.0 mg/kg per day fluvastatin. Magnification ×200.
changes was blood pressure independent because hydralazine reduced blood pressure but did not affect the atherosclerosis.

Little is known about mechanisms involved in ROS generation via NADH/NADPH oxidase in vascular cells. Studies on phagocytes suggest that the small GTP-binding protein Rac1 plays a pivotal role in the activation and assembly of NADH/NADPH oxidase.24,25 We observed that Ang II stimulation induced formation of a complex of Rac1 with Jak2, STAT1, and STAT3, or Rac1 with Tyk2, STAT1, and STAT3, and that this Ang II–induced association of Rac1 with the Jak/STAT families was inhibited by fluvastatin.9 Therefore, the inhibitory effect of fluvastatin on superoxide production with valsartan might involve inhibition of Rac1 assembly with NADH/NADPH oxidase complexes in the artery.

The SOD isoforms glutathione peroxidase and catalase are enzymes residing within the vasculature that finally lead to elimination of free radicals by generation of water and oxygen.26,27 Wassmann et al8 reported that atorvastatin exerts cellular antioxidant effects in cultured rat VSMCs and in the vasculature of spontaneously hypertensive rats mediated by decreased expression of essential NADH/NADPH oxidase subunits and upregulation of catalase expression. Yang et al reported that in a transgenic mouse model, in which catalase was overexpressed, the pressor response to Ang II was diminished with the reduction of H2O2 production in the arterial wall. To examine the possibility that coadministration of a statin with an ARB could regulate the antioxidant enzymes would be intriguing. We have not yet examined the possibility of these effects of fluvastatin or valsartan in ApoEKO mice.

Ang II is a major mediator of oxidative stress, vascular remodeling, and lesion formation. Therefore, anti-inflammatory effects of fluvastatin or valsartan, such as the decrease in MCP-1 and ICAM in ApoEKO mice, could be directly mediated in addition to their antioxidative effects. Recent evidence revealed that treatment with statins decreased VSMC AT1 receptor expression in vitro and in

Figure 5. Detection of inflammatory factors and NADH/NADPH oxidase by Western blot in aorta from ApoEKO mice treated with high-cholesterol diet (HCD) for 10 weeks. A, Dose response of valsartan. B, Effect of combination of lower doses of valsartan and fluvastatin. Reproducible chemiluminescence detection (top) in 4 separate samples and densitometric measurements (bottom) are shown in A and B. Values are mean±SE of densitometric measurements (n=4). p22phox and p47phox: NADH/NADPH oxidase subunit. Val indicates valsartan; Flu, fluvastatin. *P<0.05 vs ApoEKO+HCD without valsartan or fluvastatin.
vivo.7,29 Low-dose fluvastatin did not affect AT1 receptor expression detected with immunohistochemistry and RT-PCR in our study (preliminary data). However, if relatively high doses of fluvastatin would decrease AT1 receptor expression in the atherosclerotic artery, more beneficial effects of statins combined with an ARB during vascular remodeling may occur.

**Perspectives**

Our findings provide supporting evidence to initiate new therapeutic concepts for atherosclerosis that should lead to controlled clinical trials.

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