Statin Protects Endothelial Nitric Oxide Synthase Activity in Hypoxia-Induced Pulmonary Hypertension

Takahisa Murata, Kazuya Kinoshita, Masatoshi Hori, Masayoshi Kuwahara, Hirokazu Tsubone, Hideaki Karaki, Hiroshi Ozaki

Objective—We investigated the effects of fluvastatin on hypoxia-induced (1 to 3 weeks, 10% O\textsubscript{2}) pulmonary hypertension with focus on endothelial nitric oxide synthase (eNOS) activity.

Methods and Results—Oral fluvastatin treatment (1 mg/kg daily) prevented the causing and progression of pulmonary hypertension as determined by the right ventricular pressure, right ventricular hypertrophy, and muscularization of pulmonary artery. We also revealed that fluvastatin treatments prevented the hypoxia-induced decrease in cGMP production in the rat lung and restored the endothelium-dependent relaxation in the pulmonary artery. We revealed that this beneficial effect was not dependent on the increase in eNOS mRNA or protein expression, but was dependent on the inhibition of the eNOS-tight coupling with caveolin-1, the eNOS dissociation from heat shock protein 90, and the decrease in eNOS Ser\textsuperscript{1177}—phosphorylation induced by hypoxia. Furthermore, in a whole-mount immunostaining the hypoxia-induced eNOS protein condensation with caveolin-1 of pulmonary endothelial cells was restored by the fluvastatin-treatment.

Conclusion—These results suggest that the fluvastatin exerts beneficial effects on chronic hypoxia-induced pulmonary hypertension by protecting against the eNOS activity at the post-transcriptional level. (Arterioscler Thromb Vasc Biol. 2005;25:2335-2342.)

Key Words: endothelial nitric oxide synthase ■ fluvastatin ■ hypoxia

Hypoxia commonly occurs in patients with cardiopulmonary disease and in normal individuals at high altitudes. Chronic hypoxia causes sustained pulmonary hypertension, which is characterized by elevated pulmonary arterial pressure, in association with right ventricular hypertrophy, polycythemia, and pulmonary vascular muscularization. Various forms of pulmonary hypertension pose a significant medical problem and the current options for effective prevention and therapy are limited.\textsuperscript{1,2}

There is evidence that endothelial dysfunction is intimately involved in the onset and progression of pulmonary hypertension through abnormalities in the production, release, or degradation of endothelium-derived factors, primarily nitric oxide (NO) and endothelin-1.\textsuperscript{3,4} In fact, endothelial NO synthase (eNOS) is a candidate gene for vascular gene-expression at the transcriptional level of eNOS, which has been demonstrated in many studies.\textsuperscript{1,2,7} The decrease in eNOS protein expression and activity in response to hypoxia has been shown to be associated with various pathological conditions, such as restenosis, atherogenesis, and angina pectoris.\textsuperscript{5,6}

eNOS and caveolin-1.

HMG-CoA reductase inhibitors, have been developed as lipid-lowering drugs.\textsuperscript{10} However, recent experiments and clinical trials have demonstrated that statins also exert vasculoprotective effects independent of their cholesterol-lowering effects.\textsuperscript{10–12} Although the mechanisms underlying the pleiotropic effects of statins are not completely understood, several studies have suggested that statins may improve endothelial function through NO-dependent pathways.\textsuperscript{12–14} Other recent studies have reported that statins may have potential use in the treatment of pulmonary hypertension.\textsuperscript{15,16} However, the detailed mechanisms of this beneficial effect have not yet been clarified.

The aim of this study was to determine whether an HMG-CoA reductase inhibitor, fluvastatin, could prevent hypoxia-induced pulmonary hypertension by protecting against eNOS dysactivity at the post-transcriptional level. Our results showed that fluvastatin exerts beneficial effects
on chronic hypoxia-induced pulmonary hypertension through its protective effect on endothelial NO production.

Materials and Methods

Materials
The chemicals used were anti-eNOS, anti-HSP90, anti-Akt, and anti-phosphorylated Akt (at Ser1177) antibodies (BD Transduction Laboratories), anti-phosphorylated eNOS (at Ser1177) antibody (New England Biolabs), and anti-caveolin-1 antibody (Santa Cruz Biotechnology).

Animal and Treatments
Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Yokohama, Japan) at 7 weeks of age. Rats were placed in transparent plastic chambers and adapted to normobaric hypoxia by gradually decreasing the oxygen concentration to 10% to 11% over a period of 1 week. After adaptation, rats were exposed to an additional 1 week or 3 weeks of hypoxia. Normoxic rats were housed in identical cages in the same room, and breathed room air for 1 week or 3 weeks. Fluvastatin (1 mg/kg) dissolved in 0.5% carboxymethyl cellulose solution was given orally to the rats once per day by intraperitoneal injection. To record the systemic arterial pressure (Psyst), the left femoral artery was isolated and cannulated with a polyethylene catheter. The mean Psyst was recorded in spontaneously breathing rats as the heart was being monitored with an electrocardiograph (ECG).

Tissue Procession
The heart and lungs were removed en bloc, and lungs were used for the experiments including cGMP measurement, RT-polymerase chain reaction, and Western blot. In the other experiments (whole-mount immunostaining and muscle tension), the right and left main intrapulmonary arteries, <1.5 mm diameter, were dissected from the lungs and used.

Measurement of cGMP Content
We measured the cGMP content indicating the NO production in rat lung. After dissection, the lungs were immediately frozen in liquid nitrogen (LN2) and homogenized in 6% trichloroacetic acid solution as previously described. Briefly, the supernatants were incubated with anti-eNOS polyclonal antibody following immunoprecipitation with protein A (Amersham Pharmacia), whereas the pellets were used to determine protein content.

Immunoprecipitation and Western Blot
Immunoprecipitation and Western blotting were performed as described previously. Briefly, the lungs were homogenized, and centrifuged, and the supernatant was used for Western blot analysis to measure the eNOS and phospho-Ser1177eNOS protein expression. Equal amounts (80 μg) of protein from the total homogenates of the lungs were loaded on the SDS-PAGE. In the immunoprecipitation experiments, the supernatant was incubated with anti-eNOS polyclonal antibody following immunoprecipitation with protein A (Amersham Pharmacia). The immunoprecipitated protein was loaded and subjected to Western blot analysis to detect eNOS-bound caveolin-1 or HSP90. In each experiment, to ensure that the equal amounts of proteins were loaded, we confirmed that the same densitometric intensities of actin were observed in each preparation. The densitometric intensity was quantified by NIH Imaging 1.55.

Whole-Mount Immunostaining
Whole-mount immunostaining was performed as reported previously. Briefly, the arterial rings were cut, opened, and fixed with 4%
paraformaldehyde. The fixed arteries were incubated in 0.3% Triton X-100 and 10% normal goat serum dissolved in phosphate-buffered saline and then probed with primary antibody. The images were captured using a Carl Zeiss confocal laser scanning microscope LSM510 imaging system (×630). Five-μm-thick images from the endothelial cell surface were digitized under constant exposure time, gain, and offset (4 fields each from 5 animals). After capturing the images, we measured protein colocalization by counting the pixels of red and green merged signals and the cell area by a LSM510 program.

Muscle Tension

The vascular muscle tension was recorded as in a previous article. Briefly, the muscle tension was recorded isometrically with a force-displacement transducer under a resting tension of 10 mN. At the end of the experiment, 100 μmol/L papaverine was added to determine the basal tone. Data are shown as the percent relaxation of the steady-state precontraction.

Statistical Analysis

The results of the experiments are expressed as means ± SEM. Statistical evaluation of the data were performed by unpaired Student t test for comparisons between 2 groups, and by 1-way analysis of variance (ANOVA) followed by Dunnett’s test for comparisons between >2 groups. A value of P < 0.05 was regarded as significant.

Results

Cardiac Weight and Muscularization of Distal Pulmonary Artery

The degree of arterial remodeling (arterial medial thickness) of the distal pulmonary arteries ranging from 100 to 200 μm in outer diameter was significantly elevated by hypoxic exposure, and was worsened with age (Figure 1A-1; typical photo, A-2; summary; n = 5, 18 vessels from each rat).

Fluvastatin treatment at 1 mg/kg daily resulted in significantly lower medial thickness indexes of the distal pulmonary arteries compared with those in the hypoxic control; Fluvastatin had no such significant protective effects against the pulmonary hypertension at the lower dose (0.3 mg/kg daily treatment, data not shown, n = 4) or at the lower frequency of administration (0.3 to 3 mg/kg at 2-day intervals, data not shown, n = 4), which may be explained by its short half-life. However, treatment with 3 mg/kg daily fluvastatin did not result in an additional improvement over treatment with 1 mg/kg daily fluvastatin; the 3-week hypoxic elevation in wall thickness diameter was decreased from 21.2 ± 0.5 to 17.8 ± 0.3% (n = 5 each).

As expected, the right ventricular weight indexes (RV/LV+S) were increased in the 3-week hypoxic control rats compared with the 3-week normoxic controls, indicating right ventricular hypertrophy (Figure 1B; n = 8 each). The elevation in RV/LV+S was reduced by 1 mg/kg daily fluvastatin administration (n = 8).

The effects of fluvastatin administered 1 week after the hypoxic (10% to 11% O2)-exposure were also examined. As demonstrated in Figure 1, post-fluvastatin (1 mg/kg daily) treatment attenuated the development of arterial medial thickness, and right ventricular hypertrophy.

Right Ventricular Pressure, Systemic Blood Pressure, and ECG

As shown in Figure 2A-1, P RV was higher in rats exposed to 3-week hypoxia than in age-matched normoxic rats (n = 4 each). The chronic administration of fluvastatin (1 mg/kg...
daily) to hypoxic rats attenuated the hypoxic elevation of $P_{RV}$ (n=4). In normoxic rats, 1 mg/kg daily fluvastatin did not affect the $P_{RV}$. One mg/kg daily fluvastatin treatment did not change $P_{RV}$ both in normoxic and hypoxic rats (n=4 each, Figure 2A-2).

An ECG shows the increase in R-wave amplitude by chronic hypoxia, which suggests right ventricular hypertrophy and hypertension (3-week normoxic control; 0.67 ± 0.03 mV, 3-week hypoxic control; 1.03 ± 0.06 mV, n=5 each), and 1 mg/kg daily fluvastatin treatment inhibited the hypoxia-induced increase in the R-wave amplitude (3-week hypoxic fluvastatin; 0.85 ± 0.03 mV, n=5).

cGMP Levels
We measured the cGMP content to estimate the NO production in rat lung (Figure 2B-1; 1-week and 2B-2; 3-week), and confirmed the significant decrease in NO production in 1- and 3-week hypoxic control rats (n=5 each). In hypoxic rats, 1 mg/kg daily fluvastatin-treatment restored the cGMP content to the similar levels to that of normoxic control rats (n=5 each).

**Figure 3.** The effects of fluvastatin on the eNOS protein expression (A-1; 1-week, B-1; 3-weeks) and Ser$^{1177}$ phosphorylation of eNOS (A-2; 1-week, B-2; 3-weeks). 1.0 represents the densitometric intensity of the normoxic control lung. **,§Significantly different from the normoxic control and hypoxic control with $P<0.01$, respectively.

**eNOS Protein Expression and eNOS Phosphorylation**
We examined the eNOS protein expression and eNOS Ser$^{1177}$-phosphorylation. Initially, we checked both of chronic hypoxia and fluvastatin treatments do not affect the actin protein expression (Figure 3A-1, n=6 each). The amount of eNOS protein expression did not change among the 1-week normoxic control, hypoxic control, normoxic fluvastatin-treated, and hypoxic fluvastatin-treated lungs (Figure 3A-1, n=5 each). In the 1-week normoxic control lung, Ser$^{1177}$-phosphorylated eNOS was detected at a resting condition. One-week hypoxia abolished the eNOS phosphorylation, and 1 mg/kg daily fluvastatin-treatment recovered the decrease of the phosphorylation by hypoxia (Figure 3A-2, n=5 each).

In the 3-week hypoxic control rat lung, the eNOS protein expression was increased to 1.86 ± 0.18 of the age-matched normoxic control rat lung (Figure 3B-1, n=4 each). In the 3-week hypoxic fluvastatin-treated rat lung, the amount of eNOS proteins expression was significantly greater than in the age-matched normoxic control (1.40 ± 0.20 of the normoxic control, n=5), but the amount was inferior to that of the hypoxic control. However, in the 3-week hypoxic control rat lung, the amount of phosphorylated eNOS was slightly less than the normoxic control (0.83 ± 0.15 of normoxic control; Figure 3B-2, n=5), and in the hypoxic fluvastatin-treated rat lung, the amount of phosphorylated eNOS protein was increased to 1.22 ± 0.19 of the normoxic control (n=5).

**Interaction of eNOS With Caveolin-1 and HSP90 Proteins**
We next investigated the interaction of eNOS with caveolin-1 or HSP90 using immunoprecipitation procedure (Figure 4). Both in the 1-week and 3-week hypoxic lung, tight coupling between eNOS and caveolin-1 (1-week; 1.81 ± 0.14, 3-week; 1.70 ± 0.13 of the normoxic control, Figure 4A-1, B-1, n=5 each) and dissociation of HSP90 from eNOS (1-week; 0.20 ± 0.07, 3-week; 0.70 ± 0.17 of the normoxic control, Figure 4A-2, B-2, n=5 each) were observed. One-week and 3-week 1 mg/kg daily fluvastatin treatment significantly improved the hypoxia-induced impairment in eNOS-cavelin-1 interaction (1-week; from 1.81 ± 0.14 to 1.11 ± 0.15, 3-week; from 1.70 ± 0.13 to 1.07 ± 0.14, n=5 each) and eNOS–HSP90 interaction (1-week; from 0.20 ± 0.07 to 1.03 ± 0.17, 3-week; from 0.70 ± 0.17 to 1.120 ± 0.20, n=5 each).

**Akt Phosphorylation**
We also examined the effects of 1-week and 3-week hypoxia in the presence or absence of fluvastatin (1 mg/kg daily) on the amount of Akt Ser$^{73}$ phosphorylation in the rat lung (Figure 4A-3, B-3, n=5 each), but no differences were observed in any of the lungs.

**Localization of eNOS Protein in Endothelial Cell**
The localization of eNOS on the caveolae in the plasma membrane in endothelial cells is considered to be important for its activation. Figure 5A and 5B show the immunohistochemical images demonstrating the intracellular localization of eNOS and eNOS inhibitory protein, caveolin-1, in the endothelium. eNOS was located on the plasma membrane in the 1-week (Figure 5A) or 3-week (Figure 5B) normoxic control, hypoxic control, normoxic fluvastatin-treated, and
hypoxic fluvastatin-treated pulmonary arteries. The endothelial cells were closely blocked together like cobblestones in the 1- and 3-week normoxic arteries. In the 1-week hypoxic arteries, however, many endothelial cells were shriveled, and the eNOS protein was condensed on the periphery of the cell membrane (normoxic control; 8.2±1.0%, hypoxic control; 12.9±1.0% eNOS colocalized with caveolin-1, n=12 each; P<0.01). Fluvastatin-treatment (1 mg/kg daily) restored these morphological changes (normoxic fluvastatin-treated; 8.1±1.1%, hypoxic fluvastatin-treated; 8.8±1.3% eNOS colocalized with caveolin-1; 20 endothelial cells from each mouse, n=5 each; P<0.01).

Figure 4. The effects of fluvastatin on the eNOS-caveolin-1 (A-1; 1-week, B-1; 3-weeks) and eNOS-HSP90 interactions (A-2; 1-week, B-2; 3-weeks) and Ser473 phosphorylation of Akt (A-3; 1-week, B-3; 3-weeks). 1.0 represents the densitometric intensity of the normoxic control lung. **, §Significantly different from the normoxic control and hypoxic control with P<0.01, respectively.

Figure 5. The effect of fluvastatin on the condensation of the eNOS protein on the periphery of the cell membrane in the 1-week (A) or 3-week (B) hypoxic pulmonary endothelium in situ. Antibodies recognizing eNOS (green) and caveolin-1 (red) were used. Bar=20 μm.
We also counted the endothelial cell number per area. In all of the 1-week endothelium, there was no significant difference (normoxic control; $3.41 \pm 10^{-3} \times 10^{3}$, hypoxic control; $3.36 \pm 10^{-3} \times 10^{3}$, normoxic fluvastatin-treated; $3.45 \pm 10^{-3} \times 10^{3}$, hypoxic fluvastatin-treated; $3.42 \pm 10^{-3} \times 0.02 \times 10^{3}$, n=12 each). In the 3-week hypoxic control arteries, many endothelial cells were blocked together very tightly and directed along the blood flow (longitudinally), and these changes may reflect the high blood pressure. The number of endothelial cells per unit area of the 3-week hypoxic control arteries increased from $3.35 \pm 10^{-3} \times 0.07 \times 10^{3}$ (normoxic control) to $4.71 \times 10^{-3} \times 0.12 \times 10^{3} / \mathrm{mm}^2$ (hypoxic control, n=5, P<0.01).

In the 3-week hypoxic fluvastatin-treated (1 mg/kg daily), the endothelial cells showed intermediate changes between 3-week normoxic and hypoxic control pulmonary arteries, and the number of endothelial cells per unit area of the 3-week hypoxic control arteries increased to $3.89 \times 10^{-3} \times 0.16 \times 10^{3} / \mathrm{mm}^2$ (n=4).

**Endothelium-Dependent Relaxation in Rat Pulmonary Artery**

In the pulmonary arteries with endothelium isolated from 1- or 3-week normoxic controls, hypoxic controls, fluvastatin-treated (1 mg/kg daily) normoxic rats, and fluvastatin-treated (1 mg/kg daily) hypoxic rats, the cumulative addition of phenylephrine (1 nmol/L-10 mol/L) induced similar concentration-dependent contractions (n=4 each; data not shown).

In these arteries, carbachol (0.01 to 30 μmol/L) caused relaxation of the contraction elicited by 100 nmol/L phenylephrine in a concentration-dependent manner. In the 1- or 3-week hypoxic control pulmonary arteries, however, the relaxation induced by 30 μmol/L carbachol was significantly smaller than that in the age-matched normoxic control pulmonary arteries (Figure 6A and 6B). Fluvastatin treatment (1 mg/kg daily) significantly restored these impairments of relaxation (n=5 each).

**Discussion**

In the present experiments, we found that daily oral administration of fluvastatin (1 mg/kg daily) prevented hypoxia-induced right ventricular hypertrophy, hypertrophy, increase in R-wave amplitude in ECG, and medial layer hypertrophy of the distal pulmonary artery. Systemic hypotension is a severe side effect of long-term pulmonary hypertension. Rhabdomyolysis and chemically induced liver disease are well known, severe side effects of long-term fluvastatin treatment. At the 1 mg/kg daily dosage, a 3-week treatment with fluvastatin attenuated the increase in right ventricular pressure without alteration in systemic arterial pressure. Also serum CPK levels, an indicator of rhabdomyolysis, and serum AST and ALT, the indicators of liver disease either in hypoxic or normoxic rats remained normal (Table I, available online at http://atvb.ahajournals.org). Consequently, we decided to further investigate the mechanism of the effects of fluvastatin on pulmonary hypertension.

The beneficial effects of statins are predominantly attributed to their lipid-lowering effects. In the present study, however, the total serum cholesterol level was not changed by hypoxic exposure and/or fluvastatin treatment (Table I, online data). Therefore we can recognize that its beneficial effects on hypoxia-induced pulmonary hypertension is independent of cholesterol lowering, as other reports have suggested. Moreover, elevated HCT indicates hypoxia-induced erythropoiesis and polycythemia, and increased blood viscosity was reported to elevate the Ppa. It was reported that simvastatin treatment over the course of 2 weeks (20 mg/kg daily intraperitoneally) partially inhibited the polycythemia normally seen in hypoxia-induced pulmonary hypertensive rats. However, in the present study, the hypoxia-induced increase in HCT was completely unaffected by fluvastatin treatment (Table I, online data). This indicates that the therapeutic effects of fluvastatin on hypoxia-induced pulmonary hypertension are associated with other mechanisms.

Endothelium-derived NO contributes not only to the local regulation of vascular smooth muscle tone but also to smooth muscle cell proliferation and inflammation. Several reports have suggested that some of the beneficial effects of statins are caused by their independent, cholesterol-lowering effects on endothelial cells. Thus, in this study, we focused on the effects of fluvastatin on the eNOS-activating pathway in hypoxia-induced pulmonary hypertension. There are 3 subtypes of NOS (iNOS, eNOS, and nNOS), which synthesize NO. We did not confirm which subtype of NOS is mainly contributing for NO production in whole lung. But, recently, Alyson et al showed that eNOS has the most important role to keep pulmonary circulation comparing eNOS<sup>-/-</sup>, nNOS<sup>-/-</sup>, and iNOS<sup>-/-</sup> mice. And our current results from whole lung cGMP measurement are consistent...
with the data that we got intrapulmonary through eNOS activation previously.\textsuperscript{2,9} Thus, our results that showed that fluvastatin recovered the cGMP decrease by chronic hypoxia may reflect eNOS activity in lung; further investigation is needed to clarify this point.

We next examined whether the beneficial effects of fluvastatin on NO generation are caused by the effect on eNOS mRNA transcription and/or stability (Figure I, available online at http://atvb.ahajournals.org). In the 1-week hypoxic control rat lung, a slight increase in eNOS mRNA expression was observed with no change in protein expression. Several previous studies using various models reported that hypoxia either increased or decreased eNOS mRNA expression.\textsuperscript{27,28} This discrepancy is not yet clarified. It is well recognized that statins protect the vascular endothelium by stabilizing eNOS mRNA\textsuperscript{22,29} and causing an increase in circulating endothelial progenitor cells.\textsuperscript{30} In this study, however, the 1-week fluvastatin administration did not increase the eNOS mRNA/protein expression or endothelial cell number, when compared with the hypoxic control. Girgis et al also reported that simvastatin treatment did not change eNOS protein expression in chronic hypoxia-induced pulmonary hypertensive rats.\textsuperscript{15} Taken together, these results suggest that neither eNOS mRNA/protein expression nor endothelial progenitor cells are essential for the therapeutic effect of fluvastatin on hypoxia-induced pulmonary hypertension. Especially in vivo experiments, there are various complex mechanisms regulating eNOS expression, such as hemodynamic change and varying levels of expression of physiologically active substances such as vascular endothelial growth factor. Those factors may affect the phenomena to be confusing in the eNOS expression. Further detail investigation is required in each model to reveal these discrepancies.

We previously reported that chronic hypoxia (10% O\textsubscript{2}, 1-week) induced the atrophy of endothelial cells and tight coupling of caveolin-1 to eNOS under both resting and carbachol-stimulated conditions. These changes blocked the eNOS activation process through binding of Ca\textsuperscript{2+}–calmodulin, and HSP90 to eNOS, and eNOS Ser\textsuperscript{1177} phosphorylation by serine/threonine kinase Akt in the pulmonary arterial endothelium.\textsuperscript{2,9} Based on these data, we examined the effects of fluvastatin on the eNOS–caveolin-1 and eNOS–HSP90 interaction and eNOS Ser\textsuperscript{1177}–phosphorylation in the lung and localization of eNOS in pulmonary endothelial cells. We found that 1- and 3-week fluvastatin treatments prevented the hypoxia-induced tight coupling between eNOS and caveolin-1, dissociation of HSP90 from eNOS, and decreased the eNOS Ser\textsuperscript{1177}–phosphorylation in the rat lung. An increased level of Akt phosphorylation has been reported after the statin treatment using isolated endothelial cells.\textsuperscript{31} However, we found the Akt Ser\textsuperscript{273}–phosphorylation level in lung homogenates from hypoxic pulmonary hypertensive rats with or without fluvastatin treatment. These results suggested that fluvastatin improved eNOS phosphorylation by some mechanism other than Akt activation. Except for the result of fluvastatin on Akt phosphorylation, we found inconsistent results in Akt phosphorylation without fluvastatin treatment after hypoxia from our previous study. There we reported a decrease in Akt phosphorylation after hypoxic exposure using rabbit organ-cultured pulmonary artery.\textsuperscript{2} Akt is expressed to express in various cell types including smooth muscle cell and endothelial cells. Thus, there is possibility that these differences in Akt phosphorylation are caused by differences in tissue, experimental model, and/or treatment time course. Again, further experiments are needed to clarify this point. Immunohistochamical study further revealed that fluvastatin inhibited the hypoxia-induced eNOS protein condensation with caveolin-1. This is especially prominent in 1-week hypoxic pulmonary endothelium. In addition, fluvastatin treatment improved the hypoxia-induced impairments of endothelium-dependent relaxation stimulated with carbachol. These findings suggest that fluvastatin prevents the hypoxia-induced abnormal interaction between eNOS and caveolin-1. In whole-mount immunostaining, it was found that a 3-week period of hypoxia increased the number of endothelial cells per unit area, and increased eNOS mRNA and protein expression. In addition, accompanying these compensatory responses to chronic hypoxia, the cGMP content, eNOS Ser\textsuperscript{1177}–phosphorylation, and carbachol-induced endothelium-dependent relaxation partially recovered in the 3-week hypoxic rats, as was reported in our previous study.\textsuperscript{9} These phenomena strongly indicate the possibility that the body may compensate for the hypoxia-induced pulmonary endothelial dysfunction by increasing eNOS expression and the number of endothelial cells via angiogenesis and vasculogenesis. Further investigation is needed to clarify the mechanisms involved.

In a previous study, we revealed that chronic hypoxia impairs endothelium-dependent relaxation without changing eNOS mRNA or protein expression, and specifically causes atrophy of endothelial cells and the condensation of eNOS protein into caveolae in the organ-cultured pulmonary artery.\textsuperscript{2,17} These results were consistent with those of our in vivo study using chronic hypoxia-induced pulmonary hypertensive rats.\textsuperscript{9} These results suggest that the hypoxic organ-culture method can duplicate the endothelial dysfunction in hypoxia-induced pulmonary hypertension in vitro. As shown in the online data (Figure II), fluvastatin also improved the endothelial dysfunctions induced by chronic hypoxia in organ-cultured pulmonary arteries. In addition, the fluvastatin treatment prevented the condensation of eNOS into caveolin-1 (data not shown; n=4 each). These results strongly indicate that fluvastatin directly acts on pulmonary endothelium to prevent endothelial dysfunction in the hypoxia-induced hypertensive pulmonary artery.

Because endothelial NO production have been shown to play a key role in vascular reactivity and modulation of smooth muscle cell proliferation as well as inflammation,\textsuperscript{4,5,25} at least, in part, the protective effects of statins on endothelium contribute to recover pulmonary hypertension. However, statins have been reported to have other mechanisms with therapeutic effects, such as the direct inhibition of smooth muscle cell proliferation and the reduction of platelet aggregation.\textsuperscript{14,18} Statins may prevent pulmonary wall thickness by direct inhibition of smooth muscle proliferation. All of them are of interest for their potential use in therapies for pulmonary hypertension.
In this study, we investigated the effects of fluvastatin on pulmonary hypertension, but other water-soluble and lipid-soluble statins may have additional effects on pulmonary hypertension. Further investigation will be needed to clarify these effects.

In summary, fluvastatin has a significant inhibitory effect on hypoxia-induced pulmonary hypertension without toxicity. Its beneficial effect is through protection of the eNOS–NO pathway from impairment by chronic hypoxia. A HMG-CoA reductase inhibitor could be a potential therapeutic agent for the treatment of patients with pulmonary hypertension.

Acknowledgments
This work was partly supported by a grant-in-aid for scientific research from the Ministry of Education (Japan), and by research fellowships from the Japan Society for the Promotion of Science for Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.

References
1. Ghofrani HA, Schermuly RT, Rose F, Wiedemann R, Kohstall MG, Kishimoto (Teijin Limited, Japan), Tomohiro Kawaguti (The University of Tokyo, Japan), and Masahito Kasai (Kasai Animal Young Scientists. The authors gratefully acknowledge Dr. Tadashi Kishimoto, Tomohiro Kawaguti, and Masahito Kasai for technical assistance in measuring the right ventricular pressure, the low-oxygen chamber for donation of the low-oxygen chamber and expert technical assistance in measuring the right ventricular pressure, the systemic blood pressure, and blood chemistry.
Statin Protects Endothelial Nitric Oxide Synthase Activity in Hypoxia-Induced Pulmonary Hypertension

Takahisa Murata, Kazuya Kinoshita, Masatoshi Hori, Masayoshi Kuwahara, Hirokazu Tsubone, Hideaki Karaki and Hiroshi Ozaki

Arterioscler Thromb Vasc Biol. 2005;25:2335-2342; originally published online September 15, 2005;
doi: 10.1161/01.ATV.0000186184.33537.48

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/25/11/2335

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/09/19/01.ATV.0000186184.33537.48.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Objective

We examined the effects of fluvastatin on eNOS mRNA expression.

Materials and methods

RT-PCR

The total RNA (1 μg/μl) was extracted from the rat lungs and used. PCR amplification (28, 33, and 38 cycles) was performed using the forward primers and reverse primers for the eNOS and GAPDH as below: ATA GAA TTC ACC AGC ACC TTT GGG AAT GGC GAT (forward primer for eNOS), ATA GAA TTC GGA TTC ACT GTC TGT GTT GCT GGA CTC CTT (reverse primer for eNOS), TCC CTC AAG ATT GTC AGC AA (forward primer for GAPDH), and AGA TCC ACA ACG GAT ACA TT (reverse primer for GAPDH). After the electrophoresis, the densitometric intensity of 260 base pairs for eNOS and of 308 base pairs for GAPDH at 33 cycles was quantified using an image-processing program, NIH Image 1.55. The results are expressed as the ratio of the optical density of eNOS to that of GAPDH.

Results

eNOS mRNA expression

We examined the effect of the 1- or 3-week treatments with 1 mg/kg daily fluvastatin on eNOS mRNA expression using semiquantitative RT-PCR (Figure 1). Amplification at 28-38 cycles showed a step-wise and similar increases in mRNA signals for GAPDH in the normoxic control, hypoxic control, normoxic fluvastatin-treated and hypoxic fluvastatin-treated rat lung (n=5 each). One- and 3-week hypoxia increased eNOS mRNA expression relative to GAPDH mRNA expression in an exposure-time dependent manner (1-week; from 0.33 ± 0.04 to 0.40 ± 0.02, Figure 1, n=5 each, 3-week; from 0.32 ± 0.01 to 0.47 ± 0.03, Figure 2B-2, n=5 each). One- and 3-week fluvastatin-treatments inhibited the increases in eNOS mRNA expression (1-week; from 0.40 ± 0.02 to 0.34 ± 0.02, 3-week; from 0.47 ± 0.03 to 0.38 ± 0.02, at 33 cycles of amplification, n=5 each).
Figure 1

The effect of fluvastatin on the eNOS mRNA expression in 1-week (A) or 3-week (B) hypoxic lung. Upper panel: typical trace of agarose-gel electrophoresis of RT-PCR products; lower panel: quantitative graph showing eNOS mRNA expression level at 33 cycles amplification. * and **: significantly different from the normoxic control with $P < 0.05$ and $0.01$, respectively. §: significantly different from the hypoxic control with $P < 0.01$. 
Objective

We confirmed that 3 μmol/L fluvastatin directly prevented the hypoxia (5% O₂, 7 days)-induced impairment of pulmonary endothelium, using organ-cultured rabbit pulmonary arteries.

Materials and methods

Organ-culture procedure

The organ-culture procedure was performed as described previously.²⁻¹⁷ Briefly, each intrapulmonary artery isolated from male Japanese White rabbits (2-3 kg) was cut into rings approximately 1.5 mm wide. The arterial rings were then placed in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) in the presence or absence of 0.3 or 3 μmol/L fluvastatin. The muscle rings were maintained at 37°C under an atmosphere of 90% N₂, 5% O₂ and 5% CO₂ (hypoxia) or 95% air and 5% CO₂ (normoxia) for 7 days.

Muscle tension

The vascular muscle tension was recorded as in the previous paper.⁹ Briefly, the muscle tension was recorded isometrically with a force-displacement transducer under a resting tension of 10 mN. At the end of the experiment, 100 μmol/L papaverine was added to determine the basal tone. Data are shown as the percent relaxation of the steady-state precontraction.

Results

In the normoxic cultured pulmonary arteries with endothelium, substance P (0.1-10 nmol/L) caused vasorelaxation of the muscle contraction elicited by 1 μmol/L prostaglandin F₂α in a concentration-dependent manner (n=5). Although, in the hypoxic cultured pulmonary arteries, the substance P-induced endothelium-dependent relaxation was significantly attenuated (n=5). Treatment of tissue with 3 μmol/L fluvastatin under the hypoxic condition
significantly restored these impairments of relaxation: At the lower concentration (0.3 μmol/L), fluvastatin had no such significant protective effects against the hypoxia-induced endothelial dysfunction.

Figure II

Effects of fluvastatin on substance P-induced relaxation in rabbit pulmonary arteries cultured under normoxic or hypoxic conditions. ** and §: significantly different from the normoxic control and hypoxic control with P < 0.01, respectively.
<table>
<thead>
<tr>
<th></th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Normoxia +Fluva</th>
<th>Hypoxia +Fluva</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUN (mg/dl)</td>
<td>17.1 ± 2.4</td>
<td>16.1 ± 3.8</td>
<td>19.9 ± 0.7</td>
<td>20.2 ± 2.6</td>
</tr>
<tr>
<td>CRE (mg/dl)</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>TCHO (mg/dl)</td>
<td>54.2 ± 3.7</td>
<td>52.0 ± 15.6</td>
<td>60.3 ± 9.0</td>
<td>54.7 ± 4.5</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>124.7 ± 50.1</td>
<td>99.0 ± 23.6</td>
<td>141.3 ± 71.1</td>
<td>126.3 ± 56.5</td>
</tr>
<tr>
<td>TBIL (mg/dl)</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>102.3 ± 23.2</td>
<td>104.7 ± 25.1</td>
<td>96.3 ± 33.9</td>
<td>115.7 ± 43.8</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>42.3 ± 8.2</td>
<td>34.0 ± 10.5</td>
<td>36.6 ± 18.1</td>
<td>50.3 ± 4.9</td>
</tr>
<tr>
<td>CPK (U/l)</td>
<td>421 ± 124.3</td>
<td>731.7 ± 161.5</td>
<td>377.0 ± 209.8</td>
<td>598.3 ± 172.5</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>44.3 ± 5.2</td>
<td>67.3 ± 9.5 §</td>
<td>46.1 ± 6.2</td>
<td>65.8 ± 6.3 §</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>13.5 ± 1.0</td>
<td>13.8 ± 1.2</td>
<td>13.6 ± 1.2</td>
<td>13.1 ± 1.0</td>
</tr>
</tbody>
</table>

Blood was extracted from 3-week normoxic and hypoxic rats, after the centrifugation, blood urea nitrogen (BUN), creatine kinase (CRE), total cholesterol (TCHO), triglyceride (TG), total bilirubin (TBIL), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine phosphokinase (CPK), and hematocrit (HCT) were measured. §: significantly different from the normoxic arteries with P < 0.01.
Objective

We examined the side effects of fluvastatin in liver weight and blood chemistry.

Materials and methods

Blood procession

Blood was centrifuged at 2000 rpm at 4 °C for 20 minutes. Hematocrit (HCT, packet cell volume/blood volume) was determined, and a blood chemistry test was performed.

Results

Serum chemistry and liver weight

To assess the toxicity of the chronic administration of 1 mg/kg daily fluvastatin, we measured liver weight and performed serum chemistry (Table 1). Fluvastatin-treatment (1 mg/kg daily) did not affect the test results (n=5 each) and liver weights at 3-week normoxia and hypoxia (n=6 each). We also confirmed that there was no morphological change related to liver damage (n=4, data not shown). HCT (Table 1; 3-week) was significantly increased in 3-week hypoxic control rats over normoxic control rats (HCT; from 44.3% to 67.3%, n=5 each). Fluvastatin (1 mg/kg daily) treatment did not affect the level of HCT increased by hypoxia (n=5). In serum chemistry, fluvastatin-treatment (1 mg/kg daily) did not affect the test results (n=5 each) and liver weights at 3-week normoxia and hypoxia (n=6 each).