Fluvastatin Reverses Endothelial Dysfunction and Increased Vascular Oxidative Stress in Rat Adjuvant-Induced Arthritis

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Objective. To investigate the effect of statins on vascular dysfunction in rat adjuvant-induced arthritis (AIA).

Methods. Fluvastatin (5 mg/kg/day) was administered orally to rats with AIA, for 21 days after the onset of arthritis. The vasodilatory response to acetylcholine of aortic rings isolated from rats with AIA that were not treated or were treated with fluvastatin and from normal rats was determined. The amounts of 4-hydroxy-2-nonenal (HNE) and nitrotyrosine in aortas were measured by Western blotting. In vitro and in situ superoxide production in aortas was evaluated based on fluorogenic oxidation of dihydroethidium to ethidium. Expression of NAD(P)H components and endothelial nitric oxide synthase (eNOS) in aortas was examined by real-time reverse transcriptase–polymerase chain reaction and Western blotting. Serum levels of tetrahydrobiopterin, a critical eNOS cofactor, were determined by high-performance liquid chromatography.

Results. Fluvastatin reversed endothelial dysfunction in AIA without affecting the clinical severity of arthritis or serum cholesterol concentration. Fluvastatin reduced the amounts of HNE and nitrotyrosine in the aorta, and the levels of superoxide expressed in endothelial cells and smooth muscle cells in the tissue, in rats with AIA. NADH- or L-arginine–induced superoxide production was not observed in the aortic samples from fluvastatin-treated rats with AIA. Fluvastatin decreased the levels of expression of messenger RNA for p22phox, a NAD(P)H oxidase component, in the aortas of rats with AIA, but did not affect the expression of eNOS. Serum levels of tetrahydrobiopterin were significantly reduced in rats with AIA, and were increased by administration of fluvastatin.

Conclusion. Our findings demonstrate that fluvastatin has potent vascular protective effects in AIA and provide additional scientific rationale for the use of statins to reduce cardiovascular mortality in patients with rheumatoid arthritis.

Endothelial dysfunction, which represents the earliest stage of atherosclerosis, has been observed in patients with rheumatoid arthritis (RA) with high inflammatory activity, suggesting that this dysfunction contributes to the increased cardiovascular mortality in this disease (1–3). Although the pathophysiologic mechanism is not fully understood, mounting evidence of an association between atherosclerosis and inflammation suggests that systemic inflammation in RA may be involved in the early stages of vascular damage (4–6). We recently provided additional evidence to support this hypothesis. We found that endothelial function is depressed in rat adjuvant-induced arthritis (AIA), which has been widely used as a model of RA (7). Reactive oxygen species (ROS), which likely contribute to the pathophysiology of endothelial dysfunction caused by systemic inflammation, were found to be overproduced in the aortas of rats with AIA (7). The study results indicated that vascular damage in RA can be partially modeled in animals and that it occurs early in the course of disease. It prompted us to consider utilizing this model as a tool to examine therapeutic options to improve endothelial dysfunction caused by systemic inflammation.

One class of molecules that may improve vascular
dysfunction is the statins, hydroxymethylglutaryl-coenzyme A reductase inhibitors. Statins have been demonstrated to reduce both cardiovascular morbidity and mortality (8–10). It has been shown that hypercholesterolemia impairs endothelial function, which is restored by statin treatment (11–13). Although the beneficial effect generally has been attributed to the decrement in plasma cholesterol levels, much evidence suggests that statins exert effects that are independent of cholesterol-lowering actions. Statins restore endothelial function before serum cholesterol levels have been significantly reduced (14). It is reported that statins up-regulate expression of endothelial nitric oxide synthase (eNOS), which is a key enzyme in the synthesis of vascular NO, a major endothelium-dependent vasodilator (15,16). Furthermore, statins induce the phosphorylation and activation of eNOS via the protein kinase Akt pathway (17). Statins have also been shown to have antioxidant effects. They inhibit angiotensin II–induced free radical production by vascular smooth muscle cells in vitro and reduce aortic superoxide (O$_2^-$) production in hypertensive rats (18).

It has recently been demonstrated that statins have various antiinflammatory and immunomodulatory effects, and the unique properties of statins have motivated rheumatologists to study this drug for the treatment of arthritis (19). In a randomized placebo-controlled study, RA patients who received atorvastatin exhibited a modest, but statistically significant, improvement of arthritis (19). In a randomized, placebo-controlled study, artemether (Tanabe Pharmaceutical, Osaka, Japan) dissolved in drinking water, daily for 21 days after the onset of arthritis. Limbs were individually scored every other day on a scale of 0–3 (maximum possible score 12). Scores were assigned based on the amount of erythema, swelling, or joint rigidity in each limb. Systolic arterial blood pressure was measured by the tail cuff method with a pulse transducer (model BP98-A; Softron, Tokyo, Japan). Twenty-one days after the onset of arthritis, rats were killed and thoracic aortas and serum samples were isolated for further experiments. Mice without AIA were studied as normal controls. All procedures were performed in accordance with our institutional guidelines for animal research.

**MATERIALS AND METHODS**

**Induction and treatment of rat AIA.** Six-week-old male Lewis rats were obtained from Charles River Japan (Kanagawa, Japan). Freund’s complete adjuvant was prepared by suspending heat-killed Mycobacterium butyricum (Difco, Detroit, MI) in mineral oil at 10 mg/ml. Rats were injected intradermally with 100 μl of the adjuvant at the base of the tail. Arthritis developed by day 10 post–adjuvant injection. Rats with AIA were subsequently left untreated or were adminis-
(mRNA), which were normalized to the quantity of GAPDH mRNA as described previously (24).

Measurement of fluvastatin, adiponectin, and BH4. Serum samples were collected 21 days after the onset of arthritis in rats with AIA that were left untreated or treated with fluvastatin and from normal rats. Serum concentrations of fluvastatin were measured by high-performance liquid chromatography (HPLC) (25) at Tanabe R&D Service (Osaka, Japan). Serum levels of adiponectin were determined by quantitative sandwich enzyme-linked immunosorbent assay (Otsuka Pharmaceutical, Tokushima, Japan). Concentrations of BH4 in serum were determined by HPLC as described elsewhere (26,27). Briefly, serum samples were mixed 1:1 with a solution of 0.5 M perchloric acid containing 0.1 mM Na2-EDTA and 0.1 mM Na2S2O3 for protein separation. After filtration, BH4 concentrations in the samples were measured by HPLC. BH4 was detected fluorometrically at wavelengths of 350 nm for excitation and 440 nm for emission, by postcolumn NaNO2 oxidation with a reverse-phase ion-pair LC system (LC-10 series; Shimadzu, Kyoto, Japan).

Statistical analysis. Data were expressed as the mean ± SEM. The Mann-Whitney U test was used to compare group means. P values less than 0.05 were considered significant.

RESULTS

Fluvastatin-induced reversal of endothelial dysfunction in AIA. We first evaluated endothelium-dependent vasodilatory responses in rats with AIA that were left untreated or treated with fluvastatin. Consistent with findings in our previous study (7), the endothelium-dependent relaxation of the aortic ring was significantly depressed in rats with AIA compared with that in normal rats. We further found in the present study that administration of fluvastatin reversed the endothelial dysfunction in AIA (Figure 1).

Physical and metabolic features of the rats 21 days after arthritis onset are summarized in Table 1. Of note, despite the apparent vascular protective effect, fluvastatin did not affect the severity of clinical arthritis during the course of disease. The mean body weight of untreated rats with AIA was 83% of that in control rats,

| Table 1. Physical and metabolic features of the rats* |
|---------------------------------|-----------------|-----------------|
|                                  | Normal          | AIA, untreated  | AIA, fluvastatin-treated |
| Weight, gm                      | 328 ± 8         | 273 ± 6†        | 239 ± 4†                  |
| Arthritis score                 | 0               | 11.5 ± 0.3      | 11.8 ± 0.3                |
| Systolic blood pressure, mm Hg  | 112 ± 5         | 105 ± 2         | 109 ± 2                   |
| Total cholesterol, mg/dl        | 89.0 ± 1.5      | 83.0 ± 1.7      | 90.7 ± 2.4                |
| Triglycerides, mg/dl            | 139.0 ± 13.9    | 89.0 ± 6.7†     | 71.2 ± 6.7†               |
| Aspartate aminotransferase, IU/liter | 86.7 ± 13.4  | 70.2 ± 4.0     | 73.4 ± 4.8                |
| Alanine aminotransferase, IU/liter | 25.3 ± 1.6     | 18.8 ± 0.9     | 23.8 ± 2.7                |
| Adiponectin, µg/ml              | 10.8 ± 11.0     | 6.3 ± 0.5†     | 10.2 ± 0.6‡               |
| Tetrahydrobiopterin, ng/ml      | 2.3 ± 0.2       | 1.5 ± 0.2‡     | 3.1 ± 0.3§                |

* Rats with adjuvant-induced arthritis (AIA) were left untreated or were administered fluvastatin at 5 mg/kg/day orally for 21 days after arthritis onset. Arthritis score was determined as described in Materials and Methods; serum concentrations of adiponectin and tetrahydrobiopterin were determined by enzyme-linked immunosorbent assay and high-performance liquid chromatography, respectively. Values are the mean ± SEM (6 rats per group).
† P < 0.01 versus normal rats.
‡ P < 0.05 versus untreated rats with AIA.
and this was not affected by treatment with fluvastatin. There was no difference between normal rats, untreated rats with AIA, and fluvastatin-treated rats with AIA in terms of blood pressure, serum total cholesterol levels, and liver enzyme levels. The serum triglyceride level was significantly lower in untreated rats with AIA than in normal rats, and this was not affected by fluvastatin treatment. Interestingly, serum levels of adiponectin, an antiatherogenic protein (28), were significantly reduced in untreated rats with AIA ($P < 0.01$ versus normal rats), and were increased by administration of fluvastatin ($P < 0.05$ versus untreated rats with AIA). The mean ± SEM serum concentration of fluvastatin in rats with AIA that were administered the drug ($n = 6$) was $76.8 ± 13.0$ ng/ml.

**Effect of fluvastatin on enhanced oxidative stress in the aortas of rats with AIA.** We next examined whether fluvastatin affects vascular oxidative stress in AIA. Western blot analysis revealed that the amounts of HNE-modified or nitrotyrosine-containing proteins, indicators of oxidative stress, were significantly increased ($P < 0.01$) in the aortas of rats with AIA compared with those of normal rats. We found that fluvastatin significantly ($P < 0.05$) reduced the amounts of HNE-modified proteins (1.19-fold increase over normal, versus 2.73-fold in rats with AIA that were not treated with fluvastatin) (Figure 2A) and nitrotyrosine-containing proteins (1.29-fold increase over normal, versus 1.82-fold in rats with AIA not treated with fluvastatin) (Figure 2B).

**Figure 2.** Reduction of oxidative stress in the aortas of rats with AIA treated with fluvastatin. A and B, Western blot analyses of oxidatively modified proteins containing 4-hydroxy-2-nonenal (A) and proteins containing nitrotyrosine (B) in aortas isolated from normal control rats and rats with AIA that were left untreated or treated with fluvastatin ($n = 6$ per group). Representative blots are shown. The bands were normalized to the quantity of actin protein, using NIH image analysis software. Quantitative values (relative to control) were determined and are shown as the mean and SEM. $* = P < 0.01$ versus normal rats; $# = P < 0.05$ versus untreated rats with AIA. C, Superoxide ($O_2^-$) production in aortic tissue from a normal control rat, an untreated rat with AIA, and a fluvastatin-treated rat with AIA. In situ $O_2^-$ generation was evaluated based on fluorogenic oxidation of dihydroethidium to ethidium and imaged by confocal laser scanning microscopy (original magnification × 200). See Figure 1 for definitions.

**Figure 3.** Fluvastatin-induced reduction in the activity of NAD(P)H oxidase and nitric oxide synthase, which are responsible for superoxide ($O_2^-$) production in the aorta of rats with AIA. Homogenates of aortas from normal control rats, untreated rats with AIA, and fluvastatin-treated rats with AIA were incubated with NADH or L-arginine, and $O_2^-$ production was measured by fluorogenic oxidation of dihydroethidium (DHE) to ethidium. Diphenylene iodonium chloride (DPI) was added to inhibit NAD(P)H oxidase. The effect of tetrahydrobiopterin (BH$_4$) on L-arginine–induced $O_2^-$ production was also examined. Values are the mean and SEM ($n = 4$ rats per group) and are expressed relative to values in normal controls. $* = P < 0.05$ versus normal control rat aortas; $# = P < 0.05$ versus untreated rats with AIA; $## = P < 0.05$ versus aortas from untreated rats with AIA incubated with NADH; $### = P < 0.05$ versus aortas from untreated rats with AIA not treated with fluvastatin. See Figure 1 for other definitions.
Vascular $O_2^−$ production was evaluated based on dihydroethidium conversion to ethidium and imaged by confocal laser-scanning microscopy (Figure 2C). The results revealed that $O_2^−$ was highly expressed in endothelial cells as well as smooth muscle cells in aortic tissue of rats with AIA. Fluvastatin was found to decrease the levels of $O_2^−$ expressed in both cell types in the tissue.

**Fluvastatin-induced inhibition of NAD(P)H oxidase in the aortas of rats with AIA.** To examine the mechanisms underlying the decreased vascular ROS production in rats with AIA treated with fluvastatin, we assessed the activity and expression of NAD(P)H oxidases. First, homogenates of the isolated aortas were incubated with or without NADH, and $O_2^−$ production was measured by fluorescence spectrometric assay (Figure 3). In accordance with the data shown in Figure 2, spontaneous production of $O_2^−$ was significantly ($P < 0.05$) greater in the aortas of rats with AIA than in those of normal rats, whereas this increase was not observed in the aortas of rats with AIA treated with fluvastatin. Incubation of aortas of rats with AIA with NADH resulted in a significant increase in $O_2^−$ production compared with that in aortas incubated without substrate ($P < 0.05$). The NADH-induced increase in $O_2^−$ production was not observed in the samples from rats with AIA treated with fluvastatin. These results indicate that functional activity of NAD(P)H oxidase in the aortas of rats with AIA was inhibited by fluvastatin treatment.

NAD(P)H oxidase is a multicomponent enzyme. We investigated levels of expression of mRNA for NAD(P)H oxidase components in rat aortas, by real-time RT-PCR (Figure 4). The membrane-associated components p22phox and gp91phox and the cytosolic component p47phox (29) were examined. Expression of mRNA for all NAD(P)H oxidase components was significantly higher ($P < 0.05$ for each) in the aortas of rats with AIA than in normal rat aortas. Administration of fluvastatin to rats with AIA significantly ($P < 0.05$) reduced the level of expression of p22phox mRNA (1.7-fold increase over the level in normal rats, versus 6.9-fold increase in untreated rats with AIA). Expression of gp91phox and p47phox mRNA in the aortas of rats with AIA tended to be decreased by treatment with fluvastatin, but the reductions were not statistically significant (for gp91phox, 1.9-fold increase over normal in fluvastatin-treated rats with AIA versus 3.7-fold in untreated rats with AIA; for p47phox, 4.0-fold increase in fluvastatin-treated rats with AIA versus 5.9-fold in untreated rats with AIA).

Fluvastatin-induced amelioration of eNOS dysfunction in the aortas of rats with AIA. Another important source of vascular $O_2^−$ excess in AIA is eNOS (7). Consistent with results in our previous study (7), we found in the present study that both mRNA and protein levels of eNOS in the aortas of rats with AIA were higher ($P < 0.05$) than levels in normal rat aortas (Figure 5). Fluvastatin did not affect either mRNA or protein expression of eNOS in the aortas of rats with AIA (Figure 5).

When homogenates of aortas from fluvastatin-treated rats with AIA were incubated with l-arginine, a substrate of eNOS, there was no increase in $O_2^−$ production (Figure 3). These results suggest that eNOS dysfunction in the aortas of rats with AIA is ameliorated by fluvastatin. We measured serum levels of BH$_4$, an essential cofactor of eNOS, in each group, by HPLC. As shown in Table 1, serum BH$_4$ levels in rats with AIA
were significantly \((P < 0.01)\) lower than those in normal rats. Of interest, serum \(\text{BH}_4\) levels were increased by administration of fluvastatin \((P < 0.05)\).

### DISCUSSION

RA is associated with increased mortality due to cardiovascular events \((30–33)\). The frequency of carotid atherosclerosis has been shown to be increased among patients with RA independent of traditional risk factors \((34)\), although the precise mechanism remains unknown. Further studies are needed to identify interventional strategies that will prevent the development of clinical cardiovascular diseases in RA patients. Statins are a promising class of drug for this purpose. However, clinical or basic science evidence of the protective effect of statins against vascular damage in RA is currently very limited. In a randomized double-blind placebo-controlled study, 4-week treatment with simvastatin was shown to improve endothelial function in patients with active RA, without affecting systemic inflammation or clinical arthritis \((35)\).

To our knowledge, no basic science study on the effect of statins on vascular damage in animal models of RA has been reported previously. In the current study, we demonstrated that oral treatment with fluvastatin reverses endothelial dysfunction in rat AIA. This effect is accompanied by decreased ROS production in the aorta. Our results may provide insight into the basis of the effects of statins on vascular functions, and an additional scientific rationale for using these drugs to reduce cardiovascular mortality in patients with RA. We selected a 5-mg/kg daily dosage of fluvastatin because 5–10 mg/kg of this drug does not affect serum lipid levels in other disease models in rats \((36–38)\). Indeed, levels of serum lipids were not changed by treatment with fluvastatin in our arthritis model.

Although it is now well recognized that statins have antiinflammatory and immunomodulatory effects \((19)\), fluvastatin at 5 mg/kg/day in the present study exerted no influence on the clinical severity of arthritis or body weight in rat AIA. To examine whether other compounds of the statin family suppress arthritis, we have administered pravastatin \((10 \text{ mg/kg/day})\) or pitavastatin \((3 \text{ mg/kg/day})\) to rats with AIA, and no significant effects were observed \((\text{Haruna Y, Morita Y: unpublished observations})\). A limited number of animal studies have investigated whether statins exert antiarthritic effects. In murine collagen-induced arthritis, simvastatin administered intraperitoneally at a daily dosage of 40 mg/kg was shown to suppress arthritis, whereas lower doses of this drug had no significant effect \((10 \text{ or } 20 \text{ mg/kg/day})\) \((39)\). A more recent study showed no preventive or curative effects of atorvastatin, rosuvastatin, or simvastatin in murine collagen-induced arthritis \((40)\). In a rat model of AIA, atorvastatin administered orally at a daily dosage of 1–10 mg/kg was shown to inhibit arthritis \((41)\).

These discrepancies could be explained by the differences in models, drugs, and doses. Our results suggest that the vascular protective effect of statins in the arthritis model can be independent of antiarthritic or cholesterol-lowering effects, and may occur with the use of lower doses.

NAD(P)H oxidases have been shown to have a
Major role in oxidative excess in the vasculature (27,42). In rat AIA, NAD(P)H oxidases are responsible for vascular overproduction of $O_2^-$ (7). We showed in the present study that fluvastatin inhibits activity of vascular NAD(P)H oxidases in AIA. Levels of expression of mRNA for p22phox, a membrane-associated component of NAD(P)H oxidase, were found to be decreased by treatment with fluvastatin. It has been demonstrated that modulation of p22phox expression is closely connected with NAD(P)H oxidase activity (43,44). The inhibitory effect of statins on the expression of NAD(P)H oxidase components has been shown in other atherogenic conditions. Treatment with atorvastatin reduces vascular expression of p22phox mRNA in spontaneously hypertensive rats (45). Our results indicate that the down-regulation of vascular NAD(P)H oxidases by fluvastatin contributes to the decreased ROS production in the vasculature in this arthritis model.

Previously, we found that eNOS expression in the aortas of rats with AIA is increased at the mRNA and protein levels, and this enzyme also contributes to vascular ROS production (7). Since statins have been described to up-regulate eNOS expression by stabilizing its mRNA (15,16), we investigated whether fluvastatin alters expression of eNOS in the aorta in AIA. The data revealed that fluvastatin does not affect eNOS levels in AIA. However, it was also shown that l-arginine does not induce an increase in $O_2^-$ in isolated aortas from fluvastatin-treated rats with AIA. These results suggest that fluvastatin alters the functional nature of vascular eNOS in rats with AIA.

Of note, serum levels of BH$_4$, an essential cofactor of eNOS, were increased upon administration of fluvastatin. Deficiency of BH$_4$ due to impaired synthesis or increased catabolism is known to be associated with the formation of uncoupled, dysfunctional eNOS, contributing to generation of $O_2^-$ instead of NO (46,47). Treatment of AIA with BH$_4$ was previously shown to reverse the endothelial dysfunction (7). We thus speculate that the decrease in vascular ROS production in AIA due to treatment with fluvastatin results in increased BH$_4$ availability, leading to the prevention of formation of the uncoupled form of eNOS.

Another interesting observation in the present study was that serum levels of adiponectin, an adipocyte-specific secretory protein, were increased in rats with AIA that were treated with fluvastatin. Since body weight was not affected by the treatment, we conclude that fluvastatin alters adiponectin levels independent of adiposity.

There is accumulating evidence suggesting that adiponectin has antiatherogenic properties. Reduced adiponectin levels are reported to be linked to development of insulin resistance and increased risk of coronary artery disease (48,49). Hypoadiponectinemia in hypertensive patients has been shown to be associated with endothelial dysfunction (50). Moreover, endothelium-dependent vasodilation is impaired in adiponectin-knockout mice (50). It has also been demonstrated that adiponectin stimulates in vitro production of NO in vascular endothelial cells (51). Thus, the fluvastatin-induced increase in adiponectin levels may in part mediate the improved endothelial function in AIA. Elevated adiponectin levels in the circulation have been shown to suppress the development of atherosclerosis in apolipoprotein E–knockout mice (52). Although the mechanism by which statins increase adiponectin levels remains unclear, this effect could be another benefit of use of statins to protect vascular function.

The finding of early vascular dysfunction in rats with AIA is consistent with a previous report (53) that the risk of coronary heart disease in RA patients precedes fulfillment of the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (54). Physicians who care for RA patients should be aware of the increased risk of cardiovascular disease in these patients. The present study demonstrates that fluvastatin has potent vascular protective effects in an animal model of RA. Statins may have clinical benefit beyond lipid lowering in improving endothelial function in RA patients (35). However, supporting data from large clinical trials are needed before statin therapy for RA can be recommended.

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AUTHOR CONTRIBUTIONS

Dr. Morita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Morita.

Acquisition of data. Haruna, Yada.

Analysis and interpretation of data. Haruna, Morita, Satoh, Fox, Kashihara.

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