Fluvastatin Upregulates Inducible Nitric Oxide Synthase Expression in Cytokine-Stimulated Vascular Smooth Muscle Cells

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Abstract—Nitric oxide (NO) production by inducible NO synthase (iNOS) may play an important role in the pathogenesis of atherosclerosis. Although fluvastatin has been shown to reduce progression of atherosclerosis, it is not known whether it regulates iNOS expression. We investigated the effects of fluvastatin on iNOS expression and subsequent NO synthesis in vascular smooth muscle cells (VSMCs) and the mechanism by which fluvastatin exerts its effects. Fluvastatin significantly increased interleukin-1β (IL-1β)–induced nitrite production by VSMCs in a time-dependent (0 to 24 hours) and dose-dependent (10−8 to 10−5 mol/L) manner. Increased nitrite production by fluvastatin was accompanied by increased iNOS mRNA and protein accumulation. IL-1β induced nuclear factor-κB activation in VSMCs, which was not affected by fluvastatin. Exogenous mevalonate significantly prevented the stimulatory effect of fluvastatin on nitrite production. Cotreatment with geranylgeranyl-pyrophosphate also reversed the effect of fluvastatin. Furthermore, both Rho inhibitor C3 exoenzyme and Rho kinase inhibitor Y-27632 significantly increased IL-1β–induced nitrite accumulation in VSMCs. These results demonstrated that fluvastatin upregulates iNOS expression and subsequent NO formation in rat VSMCs through inhibition of Rho. (Hypertension. 2000;36:923-928.)

Key Words: nitric oxide • atherosclerosis • interleukins • muscle, smooth

Statins are specific inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. Large intervention clinical trials have shown significant improvements in outcome of coronary heart disease after statin therapy in primary as well as secondary prevention.1,2 Similar to other statins (lovastatin, simvastatin, and pravastatin), fluvastatin shows antiatherosclerotic effects.3 Although the improvements in atherosclerosis by this agent are associated with the lowering of serum cholesterol level, recent studies suggested that fluvastatin also has direct effects on the vascular wall; it inhibits proliferation of vascular smooth muscle cells (VSMCs) in vitro4 and intimal thickening of rabbit carotid and femoral arteries in vivo.5 The above effects of fluvastatin are not related to cholesterol levels.

Nitric oxide (NO) is formed during the metabolism of L-arginine by NO synthase (NOS). There are 3 distinct types of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). eNOS and nNOS are constitutive enzymes and are calcium dependent. In contrast, iNOS is inducible and calcium independent. After exposure to bacterial endotoxin or inflammatory cytokines such as interleukin-1β (IL-1β), iNOS can be induced in various types of cell, including VSMCs. The observation that iNOS expression increased in atherosclerotic lesions suggested that iNOS plays some important roles in the atherosclerotic process.6 Despite extensive studies on iNOS, the effects of statins on iNOS expression by VSMCs remain unclear. The purpose of this study was to determine whether fluvastatin regulates iNOS expression and subsequent NO synthesis in cultured rat VSMCs and to investigate the mechanism by which fluvastatin affects iNOS expression.

Methods

Reagents
Fluvastatin was kindly provided by Tanabe Pharmaceutical Co. Recombinant human IL-1β was provided by Otsuka Pharmaceutical Co. Y-27632 was a gift from Yoshitomi Pharmaceutical Industries, Ltd. A monoclonal anti-mouse iNOS antibody, which cross-reacts with rat iNOS, was obtained from Transduction Laboratory. Mevalonate, squalene, farnesyl-pyrophosphate (FPP), and geranylgeranyl-pyrophosphate (GGPP) were obtained from Sigma. Clostridium botulinum C3 exoenzyme was purchased from List Biological Laboratories, Inc.

Cell Culture
Primary cultures of VSMCs were obtained from the media of the thoracic aorta of Sprague-Dawley rats, as described previously.7 Cells were plated in 24-well dishes in DMEM and allowed to grow to subconfluence for 24 to 48 hours, after which they were preincu-
bated in 0.5% fetal calf serum–containing culture medium for 24 hours and used for the experiments described below.

**Measurement of Nitrite**

No production by the cultured cells was determined by measurement of the nitrite contents of the culture medium. Nitrite levels were corrected by protein measurement (data are shown as nmol/mg protein).

**Western Blotting Analysis**

The expression of iNOS protein was analyzed by immunoblotting with an anti-iNOS antibody as described previously. Peroxidase-labeled proteins were detected with the enhanced chemiluminescence detection system (Amersham International) on x-ray film, and the results were quantified by densitometry.

**Assay for iNOS mRNA**

The expression of iNOS mRNA was analyzed by quantitative reverse transcription–polymerase chain reaction (RT-PCR) as reported previously. The PCR products were visualized and digitally photographed with a Luminescent Image Analyzer LAS-1000 (Fuji Photo Film Co and quantified with Image Gauge version 3.0 (Fiji Photo Film).

**Gel Retardation Assay**

The levels of nuclear factor (NF)-κB proteins in nuclear extracts from VSMCs were analyzed by gel retardation assay as described previously. NF-κB consensus oligonucleotides (5′-AGTTGAGGCGACTTTCCCAGGC-3′, Santa Cruz Biotechnology Inc) were used as competitors. An anti-NF-κB p65 antibody (goat polyclonal IgG, 2 mg/mL) was used for supershift assay.

**Statistical Analysis**

The data are expressed as mean±SEM. Differences were statistically analyzed by ANOVA. A value of P<0.05 was considered statistically significant. All experiments were repeated at least 3 times.

**Results**

**Effects of Fluvastatin on NO Synthesis**

First, we analyzed the time course of the effect of IL-1β and fluvastatin on nitrite accumulation in the culture medium of VSMCs (Figure 1A). Unstimulated cells produced no nitrite during 48 hours of incubation. Addition of IL-1β (10 ng/mL) induced a significant increase in nitrite accumulation in a time-dependent manner, and cotreatment with fluvastatin (10−5 mol/L) further increased IL-1β–induced nitrite accumulation.

We then investigated the effects of fluvastatin at different concentrations on nitrite accumulation in the culture medium. VSMCs were exposed to fluvastatin (10−5 to 10−3 mol/L) for 24 hours with or without IL-1β. As shown in Figure 1B, nitrite production in IL-1β–stimulated cells was dose-dependently increased by fluvastatin, beginning at a concentration of 10−4 mol/L. Fluvastatin at a concentration of 10−3 mol/L increased IL-1β–induced nitrite production by ∼100%. No effect of fluvastatin was observed in IL-1β–unstimulated cells.

**Effect of Fluvastatin on iNOS Protein and mRNA Expression**

The increased NO production by fluvastatin could be due to direct improvement in iNOS enzymatic activity or induction of iNOS protein. To investigate whether fluvastatin upregulates expression of iNOS protein, Western blotting was performed (Figure 2). No immunoreactive band of iNOS was detected in unstimulated VSMCs. The iNOS protein band with a molecular mass of 125 kDa appeared after exposure to IL-1β for 24 hours. This IL-1β–induced iNOS protein expression was increased by fluvastatin in a dose-dependent manner (10−7 to 10−3 mol/L).

We further examined whether fluvastatin enhanced IL-1β–induced nitrite production at a transcriptional level. Unstimulated VSMCs did not show any detectable iNOS mRNA expression (Figure 3A). Incubation with IL-1β increased expression of iNOS mRNA, and its expression was further enhanced in the presence of fluvastatin. The half-life of iNOS mRNA induced by IL-1β was ∼4 hours (Figure 3B). Fluvastatin did not affect stability of iNOS mRNA.

**Effects of Fluvastatin on NF-κB Activation**

To verify the effects of fluvastatin on NF-κB, NF-κB activation was evaluated by gel retardation assay (Figure 4A). NF-κB proteins were visualized in a concentration-dependent manner, indicating that fluvastatin enhanced NF-κB activation. However, the time course of the effect of IL-1β on NF-κB activation was different from that on nitrite accumulation (Figure 4B). IL-1β–stimulated NF-κB activation was maximal 24 hours after stimulation, whereas fluvastatin induced increase in nitrite accumulation and expression of iNOS protein started at 12 hours. Thus, the NF-κB activation induced by fluvastatin would not be a cause of enhanced nitrite production.

**Effects of Fluvastatin on iNOS mRNA Expression**

Fluvastatin treatment caused a dose-dependent increase in iNOS mRNA expression (Figure 5A). The mRNA expression was increased to almost 100% of mRNA level in unstimulated cells. The half-life of iNOS mRNA was ∼4 hours (Figure 5B). Fluvastatin treatment did not affect stability of iNOS mRNA.
4). No NF-κB activation was detected in unstimulated control VSMCs (lane 1), whereas stimulation with IL-1β led to strong activation of NF-κB (lane 2). Cotreatment with fluvastatin did not affect IL-1β-induced NF-κB activation (lane 3).

Effects of Isoprenoids on the Action of Fluvastatin
To assess whether the effects of fluvastatin described above were associated with inhibition of HMG-CoA reductase and subsequent reduced mevalonate levels in VSMCs, the cells were exposed to IL-1β and fluvastatin in the presence of mevalonate (10⁻⁴ mol/L). As shown in Figure 5, mevalonate significantly reduced the stimulatory effect of fluvastatin on nitrite production after a 24-hour incubation. This demonstrated that mevalonate was involved in nitrite production by fluvastatin.

To further identify the product of HMG-CoA reductase reaction necessary for the effect of fluvastatin, we incubated the cells with squalene, FPP, or GGPP in the presence of IL-1β and fluvastatin. Squalene is a cholesterol precursor, and FPP and GGPP are involved in farnesylation and geranylgeranylation of proteins, respectively.¹¹ Squalene (10⁻⁵ mol/L) did not affect nitrite production induced by fluvastatin, suggesting that the effect of fluvastatin is independent of its lipid-lowering properties. Similarly, FPP (15 µmol/L) did not block the effect of fluvastatin, whereas cotreatment with GGPP (15 µmol/L) completely reversed the fluvastatin-induced increase in nitrite production. Mevalonate, FPP, GGPP, and squalene alone did not affect the basal or IL-1β–induced nitrite production (data not shown). These observations indicated that the effect of fluvastatin on iNOS expression is related to changes in protein geranylgeranylation but not to changes in protein farnesylation.

Effects of Rho Inhibition on NO Synthesis
Rho is an important geranylgeranylated protein. To determine whether inhibition of Rho is related to upregulation of iNOS by fluvastatin, we incubated cells with C3 exoenzyme, which ADP-ribosylates and inactivates Rho. As shown in Figure 6, C3 exoenzyme increased IL-1β–induced nitrite production in a dose-dependent manner (1 to 50 µg/mL). Neither GGPP nor mevalonate prevented this stimulatory effect of C3 exoenzyme on nitrite production (data not shown).

We also used Y-27632, a specific inhibitor of Rho-dependent kinase.¹² As shown in Figure 7, treatment with Y-27632 significantly increased nitrite production by IL-1β–stimulated VSMCs, also suggesting that the effect of fluvastatin on iNOS expression is related to inhibition of Rho.

Discussion
Previous studies have indicated that statins regulate eNOS expression and subsequent NO synthesis in endothelial cells¹³,¹⁴ and improve NO-mediated endothelium-dependent relaxation.¹⁵ In this study, we demonstrated that fluvastatin significantly upregulates iNOS expression and NO synthesis in IL-1β–stimulated VSMCs at concentrations of 10⁻⁶ to 10⁻⁵ mol/L. The peak concentration (Cmax) of fluvastatin in the plasma after administration of multiple doses is ~10⁻⁶ mol/L.¹⁶ Thus, clinical concentrations of fluvastatin upregulate iNOS expression. This effect is related to an inhibition of the geranylgeranylated protein Rho.

HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate metabolism yields a series of isoprenoid compounds including FPP, GGPP, and squalene. The mevalonate pathway plays an important role in cell growth and signal transduction.¹¹ Previous studies demon-
strated that exogenous mevalonate completely prevented the inhibition of proliferation and migration of VSMCs induced by fluvastatin and the inhibition of preproendothelin-1 transcription induced by simvastatin or atrovastatin. It has also been demonstrated that cotreatment with mevalonate completely reverses eNOS upregulation induced by simvastatin. In this study, we observed that addition of mevalonate prevented the stimulatory effect of fluvastatin on IL-1β–induced NO production. Furthermore, we identified complete reversion of the effect of fluvastatin by GGPP. These results raise the possibility that the decrease in GGPP levels secondary to depletion of mevalonate is related to the stimulatory effect of fluvastatin on iNOS expression.

Among the geranylgeranylated small GTP-binding proteins, Rho is the most important and is linked to the contraction, proliferation, and migration of VSMCs. It is unique small GTP-binding proteins that can be inhibited by C3 exoenzyme. GTP-bound Rho has been shown to bind to and activate Rho kinase. In the present study, both C3 exoenzyme and the Rho kinase inhibitor Y-27632 increased nitrite production by IL-1β–stimulated VSMCs. Guijarro et al. observed that lipophilic statins (lovastatin, simvastatin, atrovastatin) but not pravastatin inhibit Rho in VSMCs. Lovastatin was reported to inhibit geranylgeranylated proteins, among which Rho is the principal species, and this effect was associated with some effects of lovastatin, including regulation of eNOS expression. It was also reported that inhibition of Rho increased IL-1β–induced iNOS expression in rat pulmonary artery smooth muscle cells. Although we have not demonstrated a direct relation between inhibition of Rho and induction of iNOS by fluvastatin in the present study, these findings suggest that fluvastatin upregulates iNOS expression in VSMCs through inhibition of Rho.

The mechanism by which inhibition of Rho enhances iNOS expression is not clear. Previous actinomycin D studies indicated that statins prolonged the half-life of eNOS mRNA. Moreover, it was demonstrated that statin-induced inhibition of Rho was responsible for an increase in eNOS mRNA stability. In the present study, fluvastatin did not interfere with the half-life of iNOS mRNA induced by IL-1β. Therefore, it is unlikely that fluvastatin-mediated inhibition of Rho increases iNOS expression by improving iNOS mRNA stability. In addition, fluvastatin did not interfere with NF-κB activation induced by IL-1β.

The different effect of statins on NO production was also observed in the present study; lovastatin as well as fluvastatin increased IL-1β–induced NO synthesis by VSMCs, whereas pravastatin did not (data not shown). A previous study revealed that pravastatin inhibited sterol synthesis in hepatocytes with potency equivalent to other statins. Therefore, the differences between pravastatin and other statins, lovastatin and fluvastatin, may be related to the former’s hydrophilic nature and lack of a specific carrier for pravastatin on the extracellular cell membrane, making diffuse of pravastatin through the plasma membrane difficult. The specific carrier for pravastatin may exist only in hepatocytes.

Atherosclerosis is a complex multifactorial process and associated with many risk factors. Hence, there are a number of possible targets for pharmacological intervention to regress, stabilize, or slow the progression of existing lesions.
and prevent the formation of new lesions. The NO system is one of these targets. Under most physiological conditions, the vasculature is regulated by NO synthesized by eNOS present in the vascular endothelium. The endothelium-derived NO exerts important antiatherosclerotic effects. However, in certain pathological conditions such as atherosclerosis, NO may also be synthesized by iNOS expressed in VSMCs. iNOS is distributed not only in the intima but also in the media in atherosclerotic arteries. Moreover, an elevated cGMP content was found in deendothelialized atherosclerotic aortas, which could be due to the presence of iNOS in these tissues. All of these observations indicate that iNOS is not only induced but is functional in atherosclerotic lesions. Although the contribution of iNOS to atherosclerotic pathophysiological changes is still a matter of debate, several lines of evidence indicate that iNOS expression is beneficial in the treatment or limitation of atherosclerosis. It was reported that NO production by iNOS inhibited platelet aggregation. Aji et al demonstrated that inhibition of NOS, including iNOS, abrogated the beneficial effects of arginine on reducing xanthoma formation. Furthermore, it was demonstrated that cGMP content was found in deendothelialized atherosclerotic aortas, which could be due to the presence of iNOS in these tissues. All of these observations indicate that iNOS is not only induced but is functional in atherosclerotic lesions.
NO produced by iNOS suppressed the development of allograft atherosclerosis in rats in vivo. In addition, it has been shown that enlargement of carotid artery segments caused by increased NO synthesized from iNOS compensated for arterial narrowing in the early stage of atherosclerosis.

On the basis of these findings and the observation that fluvastatin is effective for prevention of atherosclerotic diseases, we assume that the increase in NO synthesis induced by fluvastatin may compensate for lack of endothelium-derived NO in atherosclerotic lesions and act as a defense reaction against progression of atherosclerosis lesions. However, NO also has toxic and cytolytic effects, and increased expression of iNOS may promote the process of atherogenesis by increasing cell death and necrosis. Further studies are required to prove our premise.

In conclusion, we demonstrated that fluvastatin enhances iNOS expression and subsequent NO synthesis in cytokine-stimulated VSMCs through inhibition of Rho activity.

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References

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