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降血脂藥物 lovastatin 及 pravastatin 對巨噬細胞誘導 iNOS 作用之影響

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HMG-CoA Reductase Inhibitors Inhibit Inducible Nitric Oxide Synthase Gene Expression in Macrophages

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Abstract

The 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors, statins, are potent inhibitors of cholesterol synthesis and have wide therapeutic use in cardiovascular diseases. Recent evidence, however, suggests that the beneficial effects of statins may extend beyond their action on serum cholesterol levels. In this study, we investigated the effects of lovastatin, pravastatin, atorvastatin, and fluvastatin on macrophage formation of nitric oxide (NO) in murine RAW 264.7 cells. Stimulation of macrophages with lipopolysaccharide (LPS) and interferon-ã (IFN-ã) resulted in inducible NO synthase (iNOS) expression, which was accompanied by a large amount of NO formation. Within 0.1-30 ì *M*, statins can inhibit stimuli-induced NO formation and iNOS induction to different extents. This inhibition occurs at the transcriptional level, and displays potency in the order of lovastatin > atorvastatin > fluvastatin >> pravastatin. We found that LPS-induced IKK and NF-êB activation, and IFN-ã-induced STAT1 phosphorylation were reduced by lovastatin. Moreover, lovastatin inhibition of NO production and êB activation were reversed by mevalonate, geranylgeranyl-PP and farnesyl-PP. All these results suggest that inhibition of iNOS gene expression by statins is attributed to interference with protein isoprenylation, which mediates both NF-êB and STAT1 activation in the upstream signaling pathways for iNOS gene transcription.

Introduction

Clinical benefits of cholesterol reduction have been established in large-scale primary and secondary intervention trials with statins. Statins including lovastatin, pravastatin, atorvastatin, fluvastatin, simvastatin, and cerivastatin are widely used agents for lowering cholesterol and reducing heart attacks. Treatment with statins results in decreased morbidity and mortality related to hyperlipidemia and arteriosclerosis [29, 67]. These orally prescribed statin drugs, some prescribed for over 10 years for lowering cholesterol, appear to have relatively good safety profiles. In large trials involving patients with hypercholesterolemia, the in vivo efficacy of the ability of these statins to reduce total cholesterol, LDL-cholesterol, apolipoprotein B, and triglyceride levels was in the order of cerivastatin > atorvastatin > simvastatin > lovastatin, pravastatin, and fluvastatin [42].

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting step in cholesterol synthesis [1, 45]. HMG-CoA is not only involved in cholesterol biosynthesis, but also controls the cellular level of its direct product mevalonate, which is a precursor to a number of non-sterol compounds vital to a variety of cellular functions. Reports have evidenced that mevalonate itself and mevalonate-derived isoprenoids (farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP)) are involved in posttranslational modification,

i.e., isoprenylation, of several proteins in the signal transduction pathway, such as Rho, Rac, Cdc42, Ras, Rap, and Rab [21-23, 46, 55]. Some of the clinical benefits attributed to HMG-CoA reductase inhibitors result from their ability to interrupt the isoprenylation of small G proteins by decreasing FPP and GGPP levels, leading to accumulation of inactive small G proteins in the cytoplasm [27, 41].

Nitric oxide (NO), regardless of whether it serves as a key messenger for cell function or as a cytotoxic agent in disease progression, is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) [49, 54]. Three isoforms of NOS have been identified: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) isoforms. Upregulation of eNOS expression from vascular endothelial cells [3, 15, 40], prevention of oxidized LDL [30] and tumor necrosis factor-á-induced eNOS downregulation [24] all contribute to the benefits of statins. Until now, although more attention has been focused on eNOS, the effect of statins on iNOS expression and the underlying mechanisms of action still remain controversial and seem to be dependent on cell types. In astrocytes, microglia, and macrophages, lovastatin was shown to block iNOS induction by lipopolysaccharide (LPS) through an inhibitory step at farnesylation [56]. In contrast, consequent studies demonstrated that statins could upregulate cytokine-induced iNOS expression through inhibition of small G proteins of the Rho family in vascular smooth muscle cells [12, 51], airway epithelial cells [27,

36], fibroblasts [27], and cardiac myocytes [32]. In alveolar epithelial cells, the potentiation of the iNOS response by statin might be due to enhancement of iNOS promoter activity by removing isoprenoid precursors, which would regulate iNOS promoter activity by geranylgeranylation events [36]. Rho signaling mediated through ROCK suppresses iNOS production downstream of the promoter function at the mRNA and protein levels [36]. Moreover, in native endothelial cells, atorvastatin, cerivastatin, and pravastatin decreased tumor necrosis factor-á plus interferon-ã (IFN-ã)-stimulated iNOS expression via a mechanism irrespective of HMG-CoA reductase inhibition [69]. Based on these inconsistent findings with statins on iNOS induction, and knowing that the high amount of NO production from macrophages is a key mediator in the inflammatory stage of many diseases, it is crucial to understand the effect of several statins on iNOS induction from macrophages. In the present study, we investigate the possibility in cellular regulation that atorvastatin, fluvastatin, lovastatin, and pravastatin affect the induction of iNOS by LPS and IFN-ã in murine RAW 264.7 cells.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Rabbit polyclonal Abs against active (Y701 phosphorylated) signal transducer and activator of transcription 1 (STAT1) were purchased from New England Biolabs (Beverly, MA). Horseradish peroxidase-coupled anti-mouse and anti-rabbit Abs, and the ECL detection agent were purchased from Amersham Biosciences (Piscataway, NJ). Rabbit polyclonal antibodies specific for iNOS, IêB kinase á (IKKá), IKKâ, and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Murine IFN-ã was purchased from R&D (Minneapolis, MN). The oligonucleotide sequence specific for nuclear factor-êB (NF-êB) binding was used as previously reported [26]. [á-^{32}P]dATP (3000 Ci/mmol) and [ã-^{32}P]ATP (5000 Ci/mmol) were obtained from NEN (Boston, MA). The plasmid of pGEX-IêBá (amino acids 5-55) was provided by Dr. Frank S. Lee (Pennsylvania Medical Center, philadelphia, PA). Lovastatin, phenol-extracted LPS (L8274) from *E. coli*, mevalonate, FPP, GGPP, and other chemicals were purchased from Sigma Aldrich (St. Louis, MO). Atorvastatin, fluvastatin, and pravastatin were respectively provided by Pflizer (New York, NY), Novartis (Basel, Switzerland), and Sankyo (Tokyo, Japan). All materials for SDS-PAGE were obtained from Bio-Rad Laboratories (Hercules, CA). Lovastatin acid was prepared as

previously described [53] by hydrolyzing lovastatin in a 0.05 *N* NaOH solution with stirring at 20 ^{o}C for 30 min. The hydrolyzed solutions were adjusted to pH 7.4 with 0.2 *N* HCl and then stored at 4 ^{o}C until use.

Cell Culture

Murine RAW 264.7 macrophages obtained from American Type Culture Collection (Manassas, VA) were grown at 37 ^{o}C in 5% CO_2 using DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Nitrite Measurement

Nitrite production was measured in RAW 264.7 macrophage supernatants. Briefly, cells were cultured in 24-well plates in 500 ìl of culture medium until confluence. Cells were treated with LPS or IFN-ã for the time indicated, then the culture media were collected. Nitrite was measured by adding 100 ìl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100-ìl samples of culture medium. The optical density at 550 nm (OD_{550}) was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the OD_{550} produced using standard solutions of sodium nitrite in the culture medium.

Immunoblotting Analysis

After stimulation, cells were rinsed twice with ice-cold PBS, and 100 ì l of cell lysis buffer (20 m*M* Tris-HCl, pH 7.5, 125 m*M* NaCl, 1% Triton X-100, 1 m*M* $MgCl_2$, 25 m*M* β-glycerophosphate, 50 m*M* NaF, 100 μ*M* Na_3VO_4, 1 m*M* PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) was then added to each plate. Protein was denatured in SDS, electrophoresed on a 10% SDS/polyacrylamide gel, and transferred to a nitrocellulose membrane. Nonspecific binding was blocked with TBST (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.1% Tween 20) containing 5% non-fat milk for 1 h at room temperature. After incubation with the appropriate first antibodies, membranes were washed 3 times with TBST. The secondary antibody was incubated for 1 h. Following 3 washes with TBST, the protein bands were detected with the ECL reagent.

Reverse-transcription Polymerase Chain Reaction (RT-PCR)

To amplify iNOS mRNA, the specific primers for RT-PCR analysis were synthesized. Macrophages treated with indicated agents were homogenized in 1 ml of RNAzol B reagent (Gibco), and total RNA was extracted by an acid guanidinium thiocyanate-phenol-chloroform extraction. RT was performed using a StrataScript

RT-PCR kit, and 10 μg of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding iNOS and β-actin genes were amplified using PCR. The oligonucleotide primers used corresponded to mouse macrophage iNOS (5'-CCC TTC CGA AGT TTC TGG CAG CAG C-3' and 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3') and mouse β-actin (5'-GAC TAC CTC ATG AAG ATC CT-3' and 5'-CCA CAT CTG CTG GAA GGT GG-3'). PCR was performed in a final volume of 50 μl containing Taq DNA polymerase buffer, all 4 dNTPs, oligonucleotide primers, Taq DNA polymerase, and RT products. After initial denaturing for 2 min at 94 ^{o}C, 35 cycles of amplification (94 ^{o}C for 45 s, 65 ^{o}C for 45 s, and 72 ^{o}C for 2 min) were performed followed by a 10-min extension at 72 ^{o}C. PCR products were analyzed on 2% agarose gels. The mRNA of β-actin served as an internal control for sample loading and mRNA integrity.

Transfection and Reporter Gene Assay

Using electroporation (280 V, 1070 ì F, 0.4-ms time constant), RAW 264.7 cells (2 x 10^{7} cells/cuvette) were cotransfected with 1 ì g pGL2-ELAM-Luc (êB-Luc) and 1 ì g â-galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden). The êB-Luc plasmid was constructed under the control of 3 NF-êB binding sites. After

electroporation, cells were cultured in 24-well plates at 2 x 10^6 cells/well. After a 24-h incubation, cells were incubated with the indicated concentrations of agents. After another 24-h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 µl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 30 s. Aliquots of cell lysates (5 µl) containing equal amounts of protein (10-20 µg) were placed into the wells of an opaque, black 96-well microplate. An equal volume of luciferase substrate (Promega) was added to all samples, and the luminescence was measured in a microplate luminometer (Packard, Meriden, CT). Luciferase activity values were normalized to transfection efficiency monitored by â-galactosidase expression, and was presented as the percentage of luciferase activity measured with LPS stimulation alone and in the absence of statins.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)

Nuclear extracts from stimulated or non-stimulated macrophages were prepared by cell lysis followed by nuclear lysis; cells were suspended in 30 µl of buffer containing 10 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* dithiothreitol, and 0.2 m*M* phenylmethylsulfonyl fluoride; vigorously vortexed for 15

s; allowed to stand at 4 °C for 10 min; and centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in buffer containing 20 m*M* HEPES (pH 7.9), 25% glycerol, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol, and 0.2 m*M* phenylmethylsulfonyl fluoride for 20 min on ice, and then the lysates were centrifuged at 15,000 rpm for 2 min. The supernatants containing the solubilized nuclear proteins were stored at -70 °C until used for EMSAs. The binding reaction mixture (15 µl) contained 0.25 µg of poly(dI-dC) (Amersham Biosciences) and 20,000 dpm of a ^{32}P-labeled DNA probe in binding buffer consisting of 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 4% Ficoll, 1 m*M* dithiothreitol, and 75 m*M* KCl; the binding reaction was initiated by the addition of cell extracts and continued for 1 h. Samples were analyzed on native 5% polyacrylamide gels. For supershift experiments, 5 µg of the p65 antibody was mixed with nuclear extract proteins.

Immunoprecipitation and IKK Assay

To determine the effects on IKK, anti-IKKá, IKKâ (each at 1 ì g), and protein A/G-agarose beads were added to the prepared cell extracts as mentioned above. Immunoprecipitation proceeded at 4 °C overnight. The precipitated beads were washed 3 times with 1 ml of ice-cold cell lysis buffer and twice with kinase buffer (25 m*M* HEPES, pH 7.4, 20 m*M* $MgCl_2$, 0.1 m*M* Na_3VO_4, 2 m*M* dithiothreitol). The

immune-complex kinase assay of one-half of the immunoprecipitates was performed at 30 °C for 30 min in 20 ì l of kinase reaction buffer containing 1 ì g GST-IκBα, 25 μ*M* ATP, and 3 μCi [γ-^{32}P] ATP. The reaction was terminated with 5X Laemmli sample buffer, and the products were resolved by 12% SDS-PAGE gel electrophoresis. The phosphorylated IκBα was visualized by autoradiography. The other half of the immunoprecipitates was subjected to SDS-PAGE and immunoblotting to verify that an equal amount of kinase was undergoing the kinase reaction.

Statistical Evaluation

Values were expressed as the mean ± S.E.M. of at least 3 experiments, which were performed in duplicate. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a p value of less than 0.05 was considered statistically significant.

Results

Statins Inhibit iNOS/NO Induction by LPS and IFN-ã

When cells were stimulated with LPS (1 ì g/ml) for 24 h, the nitrite level increased from the basal value of 5 ± 1 ì *M* to 42 ± 5 ì *M* (n = 12). Simultaneous addition of various statins at a concentration ranging from 0.1 to 30 ì *M* together with

LPS led to reduced NO production. Examining the inhibitory efficacies achieved at 30 ì *M* (shown in parentheses), the potency among the 4 statins was in the order of lovastatin (57% ± 5%) > atorvastatin (38% ± 7%) > fluvastatin (24% ± 3%) >> pravastatin (9% ± 3%) (Fig. 1A). In agreement with the extents for NO reduction, iNOS protein induced by LPS was accordingly affected by these statins (Fig. 1B).

The lipophilicity of statins can determine their transport characteristics and functions in a variety of cells. Accumulating evidence has shown that hydrophilic pravastatin is the weakest statin which permeates into nonhepatic cells and regulates cell responses compared to other lipophilic statins [32-34, 39, 62, 63, 66, 72]. In contrast, the lacton form of lipholic statins is necessary for cell function [66, 74]. To assess this point, we tested the effects of lovastatin acid and higher concentrations of pravastatin. As shown in Fig. 1A, lovastatin acid and pravastatin did not inhibit NO production at concentrations of up to 300 ì *M*. Since statins have been reported to cause cell toxicity at higher concentrations [38, 52, 70], we were concerned whether their inhibitory effect on NO generation is associated with this action. Using the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as an index for mitochondria function, we found that incubation with these statins at 30 ì *M* for 40 h induced no significant cell toxicity. Increasing the concentration to 100 ì *M* and the incubation time to 24 h, a slight reduction in MTT activity by lovastatin (18%

± 4%, *n* = 4), but not fluvastatin or atorvastatin, was observed. Pravastatin and lovastatin acid had no toxicity at 300 ì *M* (data not shown). When cells were analyzed by flow cytometry with propidium iodide staining, none of the statins at 30 ì *M* affected the cycle progress (data not shown).

Following our findings showing that statins can inhibit iNOS induction by LPS, it was interesting to further understand their effect on IFN-ã-induced iNOS expression. Upon 3 ng/ml IFN-ã stimulation for 24 h, RAW 264.7 macrophages increased NO release to 28 ± 5 ì *M* (*n* = 10). In the presence of lovastatin, the NO increase caused by IFN-ã was diminished in a concentration-dependent manner within 0.1-30 ì *M* (Fig. 2A). Compared to the more obvious inhibition reaching 80% by lovastatin, atorvastatin and fluvastatin at 30 ì *M* induced only weak inhibition by 30% and 20%, respectively. In contrast, pravastatin did not affect the IFN-ã response. The NO effect of these statins was accompanied by changes in iNOS protein expression (Fig. 2B). In order to assess the mechanism attributed to iNOS inhibition, lovastatin was chosen in the following experiments, and its action in some cases was compared.

Transcriptional Inhibition of iNOS Gene Expression by Statins

Since iNOS is an inducible gene product, we determined whether the effect of lovastatin results from decreased gene transcription. Using RT-PCR analysis, we

found that, as shown in Fig. 3A and C, LPS- and IFN-ã-induced upregulation of iNOS mRNA was time- and concentration-dependently inhibited by lovastatin (at 10 and 30 ì *M*). In contrast, no inhibition was seen with 30 ì *M* pravastatin.

In another experiment to confirm the iNOS reduction resulting from transcriptional inhibition, we treated cells with lovastatin (10 or 30 ì *M*) at different time intervals after LPS stimulation. Results indicated that the inhibitory effect on iNOS/NO production by lovastatin displayed a time-dependency. A gradually diminished inhibition was detected, as lovastatin treatment was delayed by up to 12 h after LPS stimulation (Fig. 3B). This result further suggests that the inhibitory action of lovastatin occurs at the gene transcription level.

NO Inhibition by Lovastatin is Dependent on Protein Isoprenylation

To explore whether cholesterol deficiency resulting from HMG-CoA reductase inhibition might contribute to NO reduction, we supplied cells with the cholesterol precursor substrates, mevalonate, FPP, and GGPP. Results indicated that in the presence of each substrate, lovastatin-induced NO inhibition was significantly restored (Fig. 4). This suggests that an isoprenylated protein mediates LPS induction of iNOS gene expression, and that this action is suppressed by statins.

Lovastatin Inhibits Signal Pathways Required for iNOS Induction

It has been demonstrated that the DNA binding activity of NF-êB transcription factor is a prerequisite for the activation of iNOS gene expression [9, 73]. To understand the effect of statins on NF-êB activation, analysis of the nuclear extract was carried out by EMSA. Figure 5A, as reflected from the specific binding by the shift assay with p65 NF-êB antibody, indicates that after LPS treatment for 1 h, the nuclear translocation and DNA binding activity of NF-êB in RAW 264.7 cells was inhibited by the presence of lovastatin (10 and 30 ì*M*), but was unaltered by pravastatin (30 ì*M*). To support the inhibitory action on NF-êB, the upstream signaling effector IKK was investigated. Figure 5B shows that the activity of IKK, which is a convergent element for NF-êB activation in response to many stimuli, including LPS [10, 14], was inhibited by lovastatin. In contrast, pravastatin had no such effect. To confirm this finding, NF-êB activity as assessed by a êB luciferase assay was suppressed by lovastatin, and this inhibition was again reversed by the presence of mevalonate, FPP, and GGPP (Fig. 5C).

Since Tyr 701 phosphorylation of STAT1 following IFN-γ stimulation is associated with the stimulating effect on iNOS expression [43, 28], we examined the effects on STAT1 phosphorylation. As shown in Fig. 5D, upon treatment of RAW 264.7 macrophages with IFN-ã, a dramatic STAT1 phosphorylation at Tyr 701 was

observed, and this action was attenuated by lovastatin, but not by pravastatin.

Discussion

Statins through HMG-CoA reductase inhibition are potent inhibitors of cholesterol synthesis and have wide therapeutic use in cardiovascular diseases. Effects other than cholesterol reduction have been proposed and might contribute to the numerous pleiotropic effects related to statin uses [35]. These effects of statins include decreasing smooth muscle cell migration and proliferation [6], inhibition of tumor growth and metastasis [47], increase in BMP-2 gene expression and bone formation [50], reduction of extracellular matrix expression [61], reduction of LDL oxidation via antioxidative activity [4, 30], upregulation of eNOS, and prevention of tumor necrosis factor-á-induced eNOS downregulation, which contributes to the beneficial effects on endothelial dysfunction associated with cardiovascular diseases [3, 15, 24].

In this study, we confirm the inhibitory ability of statins on LPS-induced iNOS expression in macrophages as previously observed [56], and demonstrate a similar inhibitory effect upon iNOS induction by IFN-ã. Evidence further suggests that the NO inhibitory action on LPS and IFN-ã involves inhibition of upstream signaling by IKK/NF-êB and STAT1, which respectively mediate LPS- [9] and IFN-ã- [28] induced iNOS gene expression. Furthermore, in LPS-mediated NF-ãB signaling, we

found that mevalonate, FPP, and GGPP all have the ability to restore lovastatin inhibition. This effect is like previous studies of statins in regulating various functions, including antiproliferation and anti-migration of smooth muscle cells [60], increased eNOS expression [15], inhibition of osteoclast formation [17], and reduced LDL oxidation [20]. This finding suggests that isoprenylation is a necessary step in LPS- and IFN-ã-mediated IKK and STAT1 activation, and in turn induces the proinflammatory process.

Several lines of evidence have indicated the activation or inhibition of small G proteins by LPS controls its inflammatory responses. Ras activation in smooth muscle cells [44], astrocytes [57], monocytes [25], Rap and Rac activation in macrophages [7, 64, 65], and Rem inhibition [16] have all been reported by LPS stimulation. In this context, Ras has been shown to mediate NF-êB activation [8, 48, 57] and iNOS induction [57], possibly through PKC and ERK intermediate signaling events [10, 13, 68]. In addition, Rac activation in macrophages by LPS [64] can lead to IKK activation. Except for Ras and Rac in LPS signaling, the contribution of other Rho family members, cdc42 and Rho, which can also transmit interleukin-1 and tumor necrosis factor-á signals to activate NF-êB by the IKK-dependent pathway [18, 59], needs further investigation. In this respect, although Rho-associated kinase (ROCK) is involved in regulation of iNOS expression in airway epithelial cells [36], our

preliminary data exclude this possibility, as the ROCK inhibitor (Y27632) did not alter NO production induced by LPS (data not shown). Until now, information with respect to the GTP binding protein in IFN-ã signaling has been quite limited. Although recent studies showed that INF-á can induce an isoprenoid-modified GTP binding protein, GBP1, in murine macrophages [26], its role in iNOS induction remains undefined. In addition, we rule out ERK and PKC activity in the signaling cascades leading to iNOS expression by IFN-ã [11].

Despite lovastatin having been reported to induce apoptosis in some cell types [58, 70], this action related to iNOS inhibition can be ruled out in our study. We detected no cell arrest, apoptosis (from cell cycle measurement), or functional loss of mitochondria activity (from the MTT assay) following incubation of cells with 30 ì *M* lovastatin for 48 h. In addition, L-arginine (10 m*M*) supplementation in culture medium did not restore NO reduction caused by lovastatin, indicating that a possible substrate deficiency in NO synthesis does not seem to exist. Moreover, in NO inhibition, our data again suggest that it is the higher lipophilicity of lovastatin which explains its high efficacy, as the hydrophilic metabolite lovastatin acid can induce no inhibitory effect.

Previous studies have shown that statins can suppress many aspects of macrophage functions related to the development of atherosclerosis. These include

inhibition of cell adhesion to endothelium [71], cell growth induced by oxidized LDL [63], cell expression of matrix metalloproteinase [19] and scavenger receptors [31], and LDL oxidation and uptake [5]. All these cellular events may slow down cholesterol accumulation in macrophages, and reduce plaque stability and atherosclerotic development. Besides these events, since it is known that a macrophage-derived high amount of NO production is a key mediator for the atherosclerosis process [2, 37]**,** the inhibitory effect on iNOS induction by lovastatin observed in this study strengthens the pleiotropic mechanism of statins in anti-atherosclerosis.

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Legends

Fig. 1. Concentration-dependent inhibition of LPS-induced NO release and iNOS expression by statins. RAW264.7 macrophages were treated with LPS (1 ì g/ml), statins, and/or lovastatin acid at the concentrations indicated for 24 h. After incubation, the culture medium was collected for NO assay (A), and cell lysates were subjected to SDS-PAGE for iNOS measurement (B). The data in (A) represent the mean ± S.E.M. from at least 3 independent experiments. The results shown in (B) are representative of 3 separate experiments. The data in parentheses indicate the percentages of iNOS protein induction as compared to the control responses without statin treatment.

Fig. 2. Concentration-dependent inhibition of IFN-ã-induced NO release and iNOS gene expression by statins. RAW264.7 macrophages were treated with IFN-γ (3 ng/ml) and/or statins at the concentrations indicated for 24 h. After incubation, the culture medium was collected for NO assay (A), and cell lysates were subjected to SDS-PAGE for iNOS measurement (B). The data in (A) represent the mean ± S.E.M. from at least 3 independent experiments. The data in parentheses indicate the percentages of iNOS protein induction as compared to the control responses without statin treatment.

Fig. 3. Transcriptional inhibition of LPS-induced iNOS gene expression by lovastatin. Cells were treated with LPS (1 ì g/ml) (A) or IFN-γ (3 ng/ml) (C), either in the presence or absence of lovastatin or pravastatin at the concentrations indicated for different periods (8 or 12 h). Total RNA was prepared and subjected to RT-PCR analysis for the iNOS mRNA level. The â-actin mRNA level was considered an internal control. Data on iNOS mRNA levels, which were measured by densitometry and calculated as percentages of the control response without statin treatment, and then normalized to the level of â-actin mRNA, are expressed as the mean ± S.E.M. from 3-5 independent experiments. *$p < 0.05$ as compared with the control LPS response. In (B), lovastatin was added to the cell cultures at the same time as, or different periods after, LPS (1 ì g/ml) treatment. Twenty-four hours after LPS addition, nitrite production in the medium and the iNOS immunoreactivity were determined. The NO production data are presented as the mean ± S.E.M. from 3 independent experiments.

Fig. 4. NO inhibition by lovastatin was dependent on protein isoprenylation. Mevalonate (200 ì *M*), FPP (50 ì *M*), or GGPP (50 ì *M*) was treated together with LPS (1 ì g/ml), either in the absence or presence of lovastatin (30 ì *M*), for 24 h. Then the culture media were collected for NO assay. The data represent the mean ± S.E.M.

from at least 3 independent experiments. *p < 0.05, indicating the abilities of mevalonate, FPP, and GGPP to reverse the reduction in lovastatin inhibition of LPS-induced NO production.

Fig. 5. Lovastatin inhibition of NF-κB activation, IKK kinase activity, and STAT1 phosphorylation. In (A), cells were treated with 1 ì g/ml LPS and/or statins at the concentrations indicated for 1 h. Nuclear extracts from cell lysates were extracted and assayed for binding activity with specific oligonucleotides containing respective binding sequences for NF-êB. In some experiments, a specific antibody for the p65 subunit of NF-êB was included in the binding mixture to analyze the binding specificity. In (B), cells were treated with 1 ì g/ml LPS and/or statins at the concentrations indicated for 30 min. Total cell lysates were immunoprecipitated overnight with IKKá and IKKâ antibodies together with protein A/G-agarose beads. The immunoprecipitates were then equally divided into 2 parts; one was used for the kinase assay (upper panel), with GST-IêBá as a substrate, and the other was subjected to SDS-PAGE for immunoblotting of IKKá (lower panel). In (C), cells transfected with the êB reporter gene and â-gal-lacZ plasmid were treated with LPS (1 ì g/ml) and/or lovastatin (30 ì *M*), either in the absence or presence of mevalonate (200 ì *M*), FPP (50 ì *M*), and GGPP (50 ì *M*). In each experiment, the luciferase activity derived

from êB activation was normalized to the transfection efficiency with â-gal-lacZ. The data represent the mean ± S.E.M. from at least 3 independent experiments. *$p < 0.05$, indicating the abilities of mevalonate, FPP, and GGPP to reverse the reduction in lovasltatin inhibition of LPS-induced êB activation. In (D), cells were treated with IFN-ã (3 ng/ml) and/or statins at the concentrations indicated for 30 min. After incubation, STAT1 tyrosine phosphorylation was measured by a specific antibody. Results are representative of 3 different experiments.