Lovastatin and Phenylacetate Inhibit the Induction of Nitric Oxide Synthase and Cytokines in Rat Primary Astrocytes, Microglia, and Macrophages

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Abstract

This study explores the role of mevalonate inhibitors in the activation of NF-kβ and the induction of inducible nitric oxide synthase (iNOS) and cytokines (TNF-α, IL-1β, and IL-6) in rat primary astrocytes, microglia, and macrophages. Lovastatin and sodium phenylacetate (NaPA) were found to inhibit LPS- and cytokine-mediated production of NO and expression of iNOS in rat primary astrocytes; this inhibition was not due to depletion of end products of mevalonate pathway (e.g., cholesterol and ubiquinone). Reversal of the inhibitory effect of lovastatin on LPS-induced iNOS expression by mevalonate and farnesyl pyrophosphate and reversal of the inhibitory effect of NaPA on LPS-induced iNOS expression by farnesyl pyrophosphate, however, suggests a role of farnesylation in the LPS-mediated induction of iNOS. The inhibition of LPS-mediated induction of iNOS by FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, suggests that farnesylation of $p21^{ras}$ or other proteins regulates the induction of iNOS. Inhibition of LPS-mediated activation of NF-kβ by lovastatin, NaPA, and FPT inhibitor II in astrocytes indicates that the observed inhibition of iNOS expression is mediated via inhibition of NF-kβ activation. In addition to iNOS, lovastatin and NaPA also inhibited LPS-induced expression of TNF-α, IL-1β, and IL-6 in rat primary astrocytes, microglia, and macrophages. This study delineates a novel role of the mevalonate pathway in controlling the expression of iNOS and different cytokines in rat astrocytes, microglia, and macrophages that may be important in developing therapeutics against cytokine- and NO-mediated neurodegenerative diseases. (*J. Clin. Invest.* 1997. 100:2671–2679.) Key words: lovastatin • phenylacetate • astrocytes • cytokines • iNOS

Introduction

Nitric oxide (NO),[1] a vascular and neuronal messenger and a cytotoxic and cytostatic agent, is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS) (1). Basically, the NOS are classified into two groups. One type, constitutively expressed (cNOS), is regulated predominantly at the posttranscriptional level in several cell types (e.g., neurons, endothelial cells) by calmodulin in a calcium-dependent manner (1, 2). In contrast, the activity of the inducible isoform (iNOS), expressed in response to different stimuli in various cell types including macrophages, hepatocytes, keratinocytes, neutrophils, and endothelial cells, is independent of calcium. Recently, astrocytes, the predominant glial component of the brain, have also been shown to induce iNOS in response to a series of proinflammatory cytokines, including IL-1β, TNF-α, IFN-γ, and bacterial LPS (3, 4). NO derived from astrocytes, macrophages, and microglia under the influence of proinflamatory cytokines is assumed to contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during neuronal degenerating conditions, including brain trauma (4–8).

LPS or cytokines induce an array of genes in astrocytes, macrophages, or microglia. The signaling pathways leading from LPS to the induction of iNOS and cytokines are only partially understood. Inhibition of LPS-induced expression of iNOS and cytokines by antioxidants and tyrosine kinase inhibitors suggests the possible involvement of reactive oxygen species and tyrosine phosphorylation in the signaling process (9–11). Recent reports have identified the activation of Ras/Raf/MEK/MAP kinase cascade after LPS stimulation (12). Although the direct involvement of this cascade in the induction of iNOS is not established so far, activation of NF-kβ by Raf (13), the presence of NF-kβ binding site in the promoter region of iNOS, and activation of NF-kβ in LPS-induced iNOS induction, suggests a role of Raf/MAP kinase cascade and NF-kβ activation in the LPS-mediated induction of iNOS (14, 15).

Mevalonate metabolites, particularly farnesyl pyrophosphate (FPP), are involved in posttranslational modification of some G-proteins, including Ras (16, 17). The inhibition of isoprenylation of Ras proteins by inhibitors of mevalonate pathway and their membrane association and transduction of signal from Ras to Raf/MAP kinase cascade (18) suggests a role of mevalonate metabolites in the transduction of signal from receptor tyrosine kinases to Raf/MAP kinase cascade. Two enzymes that control the rate-limiting steps of the mevalonate pathway are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the formation of mevalonate from acetyl-CoA, and mevalonate pyrophosphate decarboxylase, which controls the use of mevalonate within the cell by converting 3-phospho-5-pyrophospho-mevalonate to isopentenyl pyrophosphate. Lovastatin, a potent inhibitor of HMG-CoA reductase, and sodium salt of phenylacetic acid (NaPA), an inhibitor of mevalonate pyrophosphate decarboxylase, are

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1. *Abbreviations used in this paper:* iNOS, inducible isoform of nitric oxide synthase; FPP, farnesyl pyrophosphate; FPT, farnesyl protein transferase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; NaPA, sodium salt of phenylacetic acid; NO, nitric oxide.

known to reduce the level of cellular isoprenoids (19, 20). The prerequisite of Ras farnesylation in transduction of signals from receptor tyrosine kinase to Raf/MAP kinase cascade suggests a possible role of metabolites of mevalonate pathway in the modulation of iNOS induction.

This study was undertaken to investigate cellular regulation of the induction of iNOS and cytokines by lovastatin and NaPA in rat primary astrocytes, microglia, and macrophages. We herein report first evidence that the induction of iNOS and cytokines (TNF-α, IL-1β, and IL-6) gene expression is uniquely sensitive to lovastatin and NaPA in astrocytes, glial cells, and macrophages. Reversal of lovastatin-mediated inhibition of iNOS induction by mevalonate and FPP, reversal of inhibitory effect of NaPA by FPP, and inhibition by an inhibitor of Ras farnesyl protein transferase (FPT inhibitor II), demonstrate farnesylation reaction as a key step in the regulation of LPS-mediated induction of iNOS and production of NO and cytokines. A complete understanding of the cellular mechanisms involved in the induction of iNOS and cytokines should be able to identify novel targets for therapeutic intervention of NO-mediated pathophysiology in inflammatory diseases.

Methods

Reagents. Recombinant rat IFN-γ, DMEM/F-12 medium, FBS, and HBSS were from GIBCO-BRL (Gaithersburg, MD). Human IL-1β was from Genzyme Corp. (Boston, MA). Mouse recombinant TNF-α was obtained from Boehringer Mannheim (Mannheim, Germany). Lovastatin, mevastatin, and farnesyl pyrophosphate were from BIOMOL Res. Labs Inc. (Plymouth Meeting, PA). Mevalonate, cholesterol, ubiquinone, arginase and LPS (*Escherichia coli*, serotype 0111:B4) were from Sigma Chemical Co. (St. Louis, MO). N^G-methyl-L-arginine (L-NMA), FPT inhibitor II, and antibodies against mouse macrophage iNOS were obtained from Calbiochem Corp. (La Jolla, CA). Immunoassay kits for TNF-α, IL-1β, and IL-6 were obtained from R&D Systems, Inc. (Minneapolis, MN). NF-kβ DNA binding protein detection kit was from GIBCO-BRL. [γ-^{32}P]ATP (3,000 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). [2-^{14}C]acetate was purchased from ICN Biomedicals Inc. (Irvine, CA). NaPA was prepared from phenylacetic acid (Sigma Chemical Corp.) and NaOH as described (21).

Induction of NO production in rat astrocytes, microglia, and C_6 glial cells. Astrocytes were prepared from rat cerebral tissue as described by McCarthy and DeVellis (22). Cells were maintained in DMEM/F-12 medium containing 10% FBS. After 10 d of culture, astrocytes were separated from microglia and oligodendrocytes by shaking for 24 h in an orbital shaker at 240 rpm. To ensure complete removal of the oligodendrocytes and microglia, the shaking was repeated twice after a gap of 1 or 2 d. Cells were trypsinized, subcultured, and stimulated with LPS or different cytokines in serum-free DMEM/F-12 medium. Microglial cells were isolated from mixed glial cultures according to the procedure of Guilian and Baker (23). In brief, on days 7–9 the mixed glial cultures were washed three times with DMEM/F-12 and subjected to a shake at 240 rpm for 2 h at 37°C on a rotary shaker. The floating cells were washed, seeded onto plastic tissue culture flasks, and incubated at 37°C for 2 h. The attached cells were removed by trypsinization and seeded onto new plates for further studies. 90–95% of this preparation was found to be positive for nonspecific esterase, a marker for macrophages and microglia. For induction of NO production, cells were stimulated with LPS or cytokines in serum-free condition. C6 glial cells obtained from American Type Culture Collection (Rockville, MD) were also maintained and induced with different stimuli as indicated above.

Isolation of rat macrophages and induction of NO production. Resident macrophages were obtained from rat by peritoneal lavage with sterile RPMI 1640 medium containing 1% FBS and 100 μg/ml gentamicin. Cells were washed three times with RPMI 1640 at 4°C, and were maintained at 37°C in a humified incubator containing 5% CO_2 in air. Macrophages at a concentration of 2×10^6/ml in RPMI 1640 medium containing L-glutamine and gentamicin were added in volumes of 800 μl to a 35-mm plate. After 1 h, nonadherent cells were removed by washing, and 800 μl of serum-free RPMI 1640 medium with various stimuli was added to the adherent cells. After 24 h the culture supernatants were transferred to measure NO production.

Cell viability. Cytotoxic effects of the inhibitors were determined by measuring the cell viability by Trypan blue exclusion.

Assay for NO synthesis. Synthesis of NO was determined by assay of culture supernatants for nitrite, a stable reaction product of NO with molecular oxygen. In brief, 400 μl of culture supernatant was allowed to react with 200 μl of Griess reagent (24, 25), and was incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all experiments. Nitrite concentrations were calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay. Protein was measured by the procedure of Bradford (26).

Incorporation of [^{14}C]acetate into cholesterol. Astrocytes grown in 100-mm plates (∼ 80% confluency) and preincubated in serum-free media with lovastatin or NaPA for 8 h received [2-^{14}C]acetate (10 μCi/plate). After 3 h, the cells were washed twice with PBS and scraped off. The lipids were extracted with 1 ml 75% ethanol, and the ethanol extract was saponified with 1 ml of 20% ethanolic KOH at room temperature. To this, 2 ml of water was added, mixed, and extracted twice with 2 ml of hexane. The hexane extracts were dried under nitrogen, dissolved in 50 μl of $CHCl_3$/MeOH (1:1), spotted on a TLC plate along with standard [^{3}H]cholesterol, and run with hexane/ether/acetic acid (70:30:1). The plate was then exposed to a photographic film that was stored at −20°C and developed after 2 d. The lanes corresponding to standard cholesterol were scraped and counted in 5 ml of scintillation fluid.

Immunoblot analysis for iNOS. After a 24-h incubation in the presence or absence of different stimuli, cells were scraped off, washed with Hank's buffer, and homogenized in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). After electrophoresis the proteins were transferred onto a nitrocellulose membrane, and the iNOS band was visualized by immunoblotting with antibodies against mouse macrophage iNOS and [^{125}I]-labeled protein A (25).

RNA isolation and Northern blot analysis. Cells were taken out from culture dishes directly by adding Ultraspec-II RNA reagent (Biotecx Laboratories Inc., Houston, TX), and total RNA was isolated according to the manufacturer's protocol. For Northern blot analyses, 20 μg of total RNA was electrophoresed on 1.2% denaturing formaldehyde–agarose gels, electrotransferred to Hybond-Nylon Membrane (Amersham Corp.), and hybridized at 68°C with ^{32}P-labeled cDNA probe using Express Hyb hybridization solution (Clontech, Palo Alto, CA) as described by the manufacturer. The cDNA fragment for iNOS was amplified by PCR using two primers (forward primer: 5′-CTC CTT CAA AGA GGC AAA AAT A-3′; reverse primer: 5′-CAC TTC CTC CAG GAT GTT GT-3′), and was cloned in pGEM-T vector (25, 27). The clone was confirmed by DNA sequencing, and the insert was used as probe. After hybridization, filters were washed two to three times in solution I (2× SSC, 0.05% SDS) for 1 h at room temperature, followed by solution II (0.1× SSC, 0.1% SDS) at 50°C for another hour. The membranes were then dried and exposed with x-ray films (Eastman Kodak Co., Rochester, NY). Same filters were stripped and rehybridized with probes for GAPDH. The relative mRNA content for iNOS was measured after scanning the bands with a Bio-Rad (Model GS-670; Richmond, CA) imaging densitometer.

Nuclear run-on assay. For the measurement of gene transcription, nuclei were prepared, and in vitro transcriptional activity was measured with nuclei (25×10^6 nuclei per assay) using 30 μCi of [α-^{32}P]-

UTP (400 Ci/mmol) as described by Caira et al. (28). In brief, the filters were prehybridized in 1 ml of hybridization buffer (50% formamide, 5× SSC, 1% SDS, 15% dextran sulfate, 1× Denhardt's solution, and 50 μg/ml heparin). After 24 h of prehybridization in the above buffer, hybridization was carried out with the labeled RNAs (1.3×10^5 cpm) at 42°C for 60 h to 3 μg of the immobilized plasmid pGEM-T as a control, or to plasmids containing inserts of rat glyceraldehyde-3-phosphate dehydrogenase, rat actin, and human iNOS cDNAs. The filters were washed twice in 2× SSC, 0.1% SDS for 15 min at 42°C, and twice in 0.5× SSC, 0.1% SDS for 15 min. Then the filters were treated with RNase buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.4, 40 mM EDTA, 10 μg/ml RNase A, and 350 U/ml RNase T1) at 37°C for 30 min, in the same buffer without RNases for another 30 min, and were then autoradiographed.

Determination of TNF-α, IL-1β, and IL-6 in culture supernatants. Cells were stimulated with LPS in serum-free media for 24 h in the presence or absence of lovastatin or NaPA, and concentrations of TNF-α, IL-1β, and IL-6 were measured in culture supernatants by using high-sensitivity enzyme-linked immunosorbent assay (R&D Systems, Inc.) according to the manufacturer's instructions.

Preparation of nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts from stimulated or unstimulated astrocytes (1×10^7 cells) were prepared using the method of Dignam et al. (29) with slight modifications. Cells were harvested, washed twice with ice-cold PBS, and lysed in 400 μl of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin) containing 0.1% Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 14,000 rpm for 30 s. The pelleted nuclei were resuspended in 40 μl of buffer B (20 mM Hepes, pH 7.9, 25% [vol/vol] glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin). After 30 min on ice, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants containing the nuclear proteins were diluted with 20 μl of modified buffer C (20 mM Hepes, pH 7.9, 20% [vol/vol] glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) and stored at −70°C until use. Nuclear extracts were used for the electrophoretic mobility shift assay using the NF-kβ DNA binding protein detection system kit (GIBCO/BRL) according to the manufacturer's protocol.

Results

Inhibitors of mevalonate pathway inhibit LPS-induced expression of iNOS in primary astrocytes. Both HMG-CoA reductase and mevalonate pyrophosphate decarboxylase are the rate-limiting enzymes of the mevalonate pathway (16, 19). We examined the effect of inhibitors of HMG-CoA reductase (lovastatin and mevastatin) and mevalonate pyrophosphate decarboxylase (NaPA) on the induction of iNOS and production of NO. Results in Table I show that bacterial LPS at a concentration of 1.0 μg/ml induced the production of NO by about eightfold. Inhibition of NO production by arginase, an enzyme that degrades the substrate (L-arginine) of NOS and L-NMA, a competitive inhibitor of NOS, suggests that LPS-induced NO production in astrocytes is dependent on NOS-mediated arginine metabolism (Table I). Lovastatin or mevastatin alone was neither stimulatory nor inhibitory to nitrite production in control astrocytes. Both the inhibitors, however, when added 8 h before the addition of LPS, potentially inhibited LPS-mediated induction of nitrite production in astrocytes. Only 25% inhibition in LPS-induced NO production was found when lovastatin was added to the cells along with LPS, however, the degree of inhibition increased with the increase in time of preincubation, with lovastatin reaching ~ 90% inhibition of NO production within 8–10 h of preincubation (data not shown). Previous studies have shown that treatment of MEL cells with lovastatin for several hours results in accumulation of unmodified (nonisoprenylated) proteins in the cytoplasm (30). Similar to lovastatin, NaPA also inhibited the induction of NO production in rat primary astrocytes (Fig. 1). To understand the mechanism of inhibitory effect of these inhibitors on LPS-mediated nitrite production, we examined the effect on protein and mRNA levels of iNOS. Western blot analysis with antibodies against murine macrophage iNOS and Northern blot analysis for iNOS mRNA analysis of LPS-stimulated astrocytes clearly showed that both lovastatin and NaPA significantly inhibited the LPS-mediated induction of iNOS protein (Fig. 1 *B*) and mRNA (Fig. 1 *C*). A combination of lovastatin and NaPA at a dose lower than the one used individually almost completely inhibited LPS-induced production of NO and expression of iNOS. To gain further insight into the mechanism of the inhibitory effect of lovastatin and NaPA on LPS-mediated expression of iNOS mRNA, we examined the influence of lovastatin and NaPA on the rate of iNOS gene transcription, as measured by nuclear run-on assays. Fig. 2 shows that LPS induced the transcription of the iNOS gene in astrocytes, and that preincubation of cells with lovastatin or NaPA inhibited LPS-induced transcription of the iNOS gene. Consistent with the inhibition of LPS-induced expression of mRNA, protein and activity of iNOS by lovastatin and NaPA, the combination of lovastatin and NaPA completely inhibited the transcription of iNOS gene. These results clearly suggest that lovastatin and NaPA inhibit LPS-induced expression of iNOS mRNA, protein and activity by inhibiting transcription of the iNOS gene.

Sinensky et al. (31) observed no suppression of isoprenylated protein maturation in vitro by lovastatin treatment that produced 50% inhibition of sterol biosynthesis. The IC_{50} for inhibition of sterol synthesis is 10 nM, whereas the IC_{50} for inhibition of conversion of pro-$p21^{ras}$ to mature-$p21^{ras}$ is maximal at 2.6 μM (31). The pharmacologically attainable concentration for NaPA, however, is 1 to 5 mM (32). To determine if the synergistic inhibitory effect of lovastatin and NaPA on LPS-

Table I. Inhibition of LPS-induced NO Production in Rat Primary Astrocytes by Lovastatin and Mevastatin

Stimuli	Nitrite	% Inhibition
	nmol/mg/24 h	
Control	2.9±0.5	—
LPS	25.3±3.2	—
LPS + arginase	5.9±0.8	87
LPS + L-NMA	5.5±0.7	88
Lovastatin	2.9±0.3	—
Mevastatin	2.8±0.4	—
LPS + lovastatin	5.2±0.5	90
LPS + mevastatin	5.5±0.5	88

Astrocytes were cultured for 24 h in serum-free DMEM/F-12 with the listed reagents; nitrite concentration in the supernatants was measured as described in Methods. Arginase (100 U/ml) and L-NMA (0.1 mM) were added to the cells together with LPS (1.0 μg/ml), however, cells preincubated with lovastatin (10 μM) or mevastatin (10 μM) for 8 h received LPS. Data are mean±SD of three different experiments.

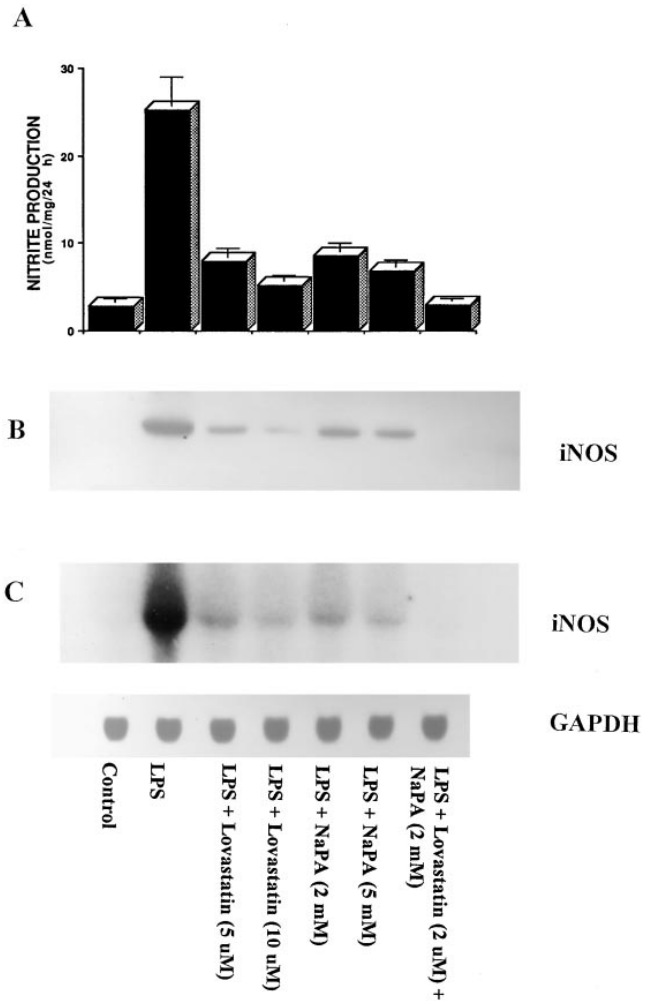

Figure 1. Inhibition of LPS-induced expression iNOS by lovastatin and NaPA in rat primary astrocytes. Cells preincubated in serum-free media with different concentrations of lovastatin (5 or 10 μM) or NaPA (2 or 5 mM) or a combination of 2 μM lovastatin and 2 mM NaPA for 8 h received 1.0 μg/ml of LPS. (*A*) After 24 h, supernatants were used for nitrite assay as mentioned in Methods. Data are mean±SD of three different experiments. (*B*) Cell homogenates were electrophoresed, transferred onto nitrocellulose membranes, and immunoblotted with antibodies against mouse macrophage iNOS as mentioned in Methods. (*C*) After 5 h of incubation, cells were taken out directly by adding ultraspec-II RNA reagent (Biotecx Laboratories Inc.) to the plates for isolation of total RNA, and Northern blot analysis for iNOS mRNA was carried out as described in Methods.

induced iNOS expression in astrocytes could be explained solely by inhibition of the mevalonate pathway, we examined the incorporation of [2-^{14}C]acetate into cholesterol. Cells preincubated with lovastatin or NaPA for 8 h received [2-^{14}C]acetate for 3 h. Lovastatin (10 μM) and NaPA (5 mM) inhibited the synthesis of cholesterol by 73±6.2 and 64±5.3%, respectively. The combination of lovastatin (2 μM) and NaPA (2 mM), however, caused 93±4.2% inhibition, indicating that lovastatin and NaPA affect cholesterol synthesis in an additive fashion. Therefore, absence of complete inhibition of iNOS mRNA or protein by lovastatin or NaPA could be due to the absence of complete inhibtion of the mevalonate pathway and depletion of mevalonate metabolites.

Inhibition of LPS- and cytokine-induced production of NO by lovastatin in rat primary astrocytes. Similar to LPS, different cytokines and their several combinations are known to induce the expression of iNOS (2, 6–9). To examine whether cytokine-induced NO production is also inhibited by lovastatin, primary astrocytes were stimulated with different combinations of LPS, TNF-α, IL-1β, and IFN-γ for 24 h, and the production of NO was measured. All the combinations of LPS and cytokines significantly induced production of NO, however, addition of 10 μM lovastatin to astrocytes potently inhibited NO production (Fig. 3 *A*) and induction of iNOS protein (Fig. 3 *B*), suggesting that similar to LPS, cytokine-mediated expression of iNOS also involves the mevalonate pathway. Under similar conditions, lovastatin was also found to inhibit LPS- and cytokine-induced NO production in rat C6 glial cells (data not shown).

Inhibition of LPS-induced activation of NF-kβ and expression of iNOS by lovastatin and NaPA, and its reversal by farnesyl pyrophosphate in rat primary astrocytes. Since activation of NF-kβ is necessary for induction of iNOS (14, 15, 25), to understand the basis of the inhibition of iNOS, we examined the effect of these inhibitors on LPS-induced activation of NF-kβ in astrocytes by gel-shift DNA-binding assay. Treatment of astrocytes with 1.0 μg/ml of LPS resulted in activation of NF-kβ (Fig. 4). This gel shift assay detected a specific band in response to LPS that was competed off by an unlabelled probe (Fig. 4 *A*). Lovastatin or NaPA alone at different concentrations failed to induce NF-kβ. Both lovastatin and NaPA, however, markedly inhibited LPS-induced activation of NF-kβ (Fig. 4 *B*), suggesting that inhibition of iNOS expression by lovastatin and NaPA is possibly due to inhibition of NF-kβ. We have demonstrated earlier that activation of NF-kβ is neces-

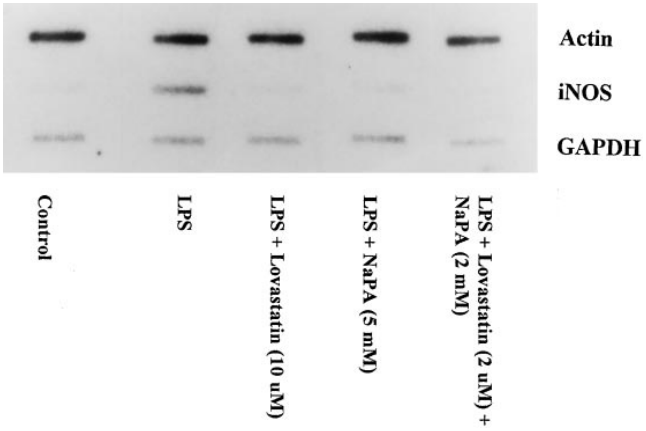

Figure 2. Lovastatin and NaPA inhibit the relative rate of nuclear transcription of the iNOS gene in LPS-stimulated rat primary astrocytes. Cells preincubated in serum-free media with 10 μM lovastatin or 5 mM NaPA, or a combination of 2 μM lovastatin and 2 mM NaPA for 8 h received 1.0 μg/ml of LPS. After 4 h cells were taken out, and nuclei were collected for nuclear run-on assays. ^{32}P-labeled mRNA was transcribed in vitro from isolated nuclei, and 1.3×10^5 cpm of run-on products were hybridized to each blot as described in Methods. The plasmids used were pGEM-T without any insert (negative control) or containing iNOS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or actin cDNA inserts.

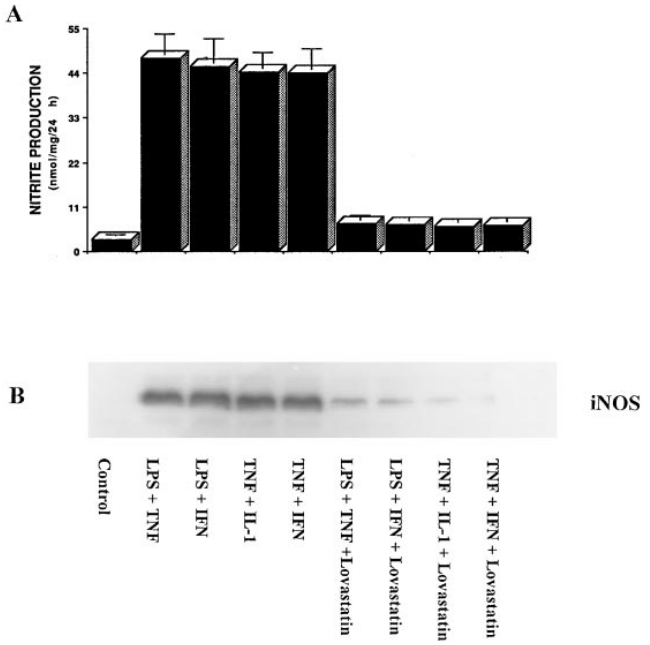

Figure 3. Inhibition of LPS- and cytokine-induced expression of iNOS in rat primary astrocytes by lovastatin. Cells preincubated in serum-free media with 10 μM lovastatin for 8 h received different combinations of LPS and cytokines. After 24 h of incubation, production of nitrite was measured in supernatants (*A*) as mentioned earlier. Data are mean±SD of three different experiments. Cell homogenates were analyzed for iNOS protein by immunoblotting technique (*B*) as described before. Concentration of different stimuli were as follows: LPS, 0.5 μg/ml; TNF-α, 20 ng/ml; IL-1β, 50 ng/ml; IFN-γ, 50 U/ml.

sary for iNOS expression in rat primary astrocytes, and that cAMP derivatives inhibit the expression of iNOS by inhibiting the activation of NF-kβ (25). To evaluate the possible mechanism of the effect of lovastatin and NaPA, or to determine whether reduced concentrations of end products as opposed to intermediate products of the mevalonate pathway were responsible for the effects of lovastatin and NaPA, we performed rescue experiments with cholesterol, ubiquinone, mevalonate, and FPP. Addition of 10 μM ubiquinone or cholesterol to astrocytes did not prevent the inhibitory effect of lovastatin and NaPA (data not shown). These observations support the possibility that depletion of intermediary products rather than end products of mevalonate pathway is responsible for the observed inhibitory effect of lovastatin or NaPA on LPS-induced iNOS expression. On the other hand, mevalonate or FPP substantially reversed the inhibitory effect of lovastatin on iNOS expression and NF-kβ activation (Fig. 5). FPP, however, but not mevalonate, reversed the inhibitory effect of NaPA, suggesting that the use of mevalonate rather than its synthesis is the prime target of the NaPA.

An inhibitor of Ras farnesyl protein transferase (FPT inhibitor II) inhibits LPS-induced expression of iNOS and activation of NF-kβ in rat primary astrocytes. Inhibition of LPS-induced expression of iNOS and activation of NF-kβ by NaPA and its reversal by FPP, but not by mevalonate, suggests a possible involvement of the farnesylation reaction in activation of NF-kβ and induction of iNOS. Since farnesylation is a necessary step

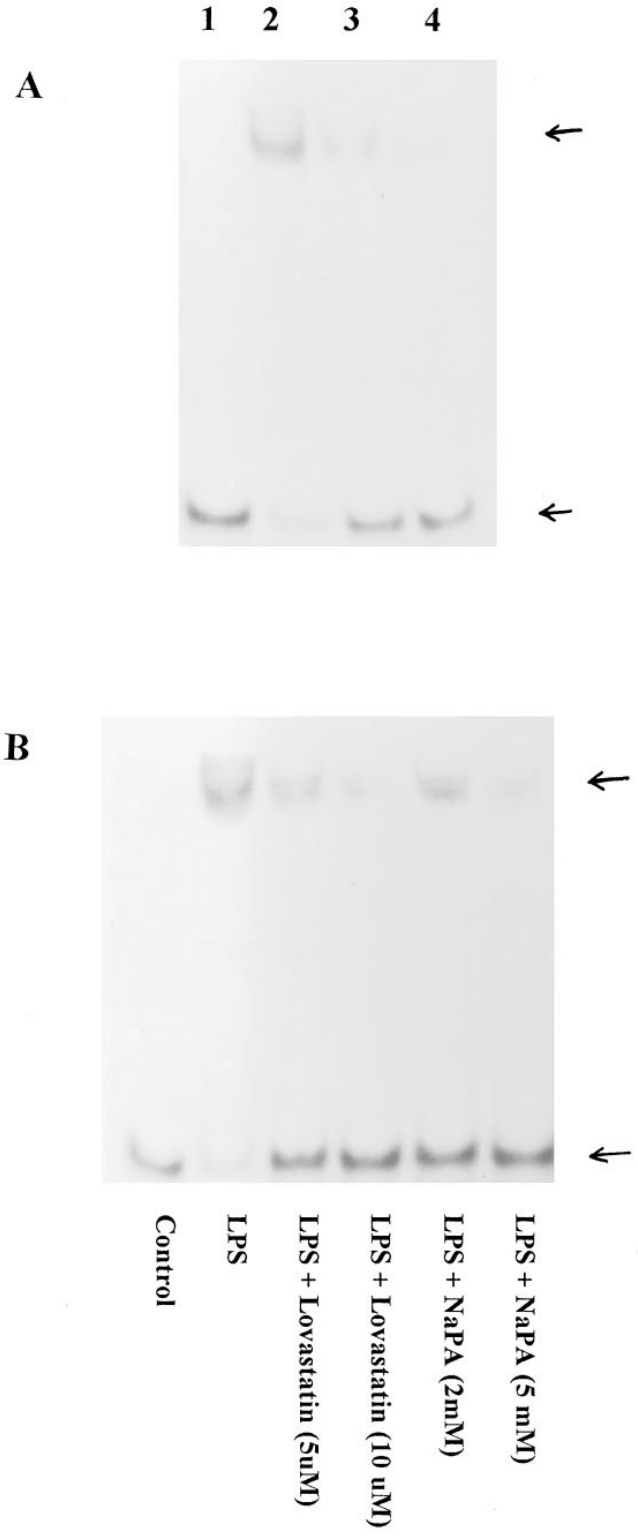

Figure 4. Inhibition of LPS-induced activation of NF-kβ by lovastatin and NaPA in rat primary astrocytes. (*A*) Cells incubated in serum-free media received 1.0 μg/ml of LPS. After 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF-kβ as described in Methods. Lanes *1–4* represent control, LPS, LPS-treated nuclear extract with 25-fold excess of unlabeled probe, and LPS-treated nuclear extract with a 50-fold excess of unlabeled probe, respectively. (*B*) Cells preincubated in serum-free media with 10 μM of lovastatin or 5 mM of NaPA for 8 h received 1.0 μg/ml of LPS. The upper arrow in both figures indicates the induced NF-kβ, whereas the lower arrow in both figures indicates the unbound probe.

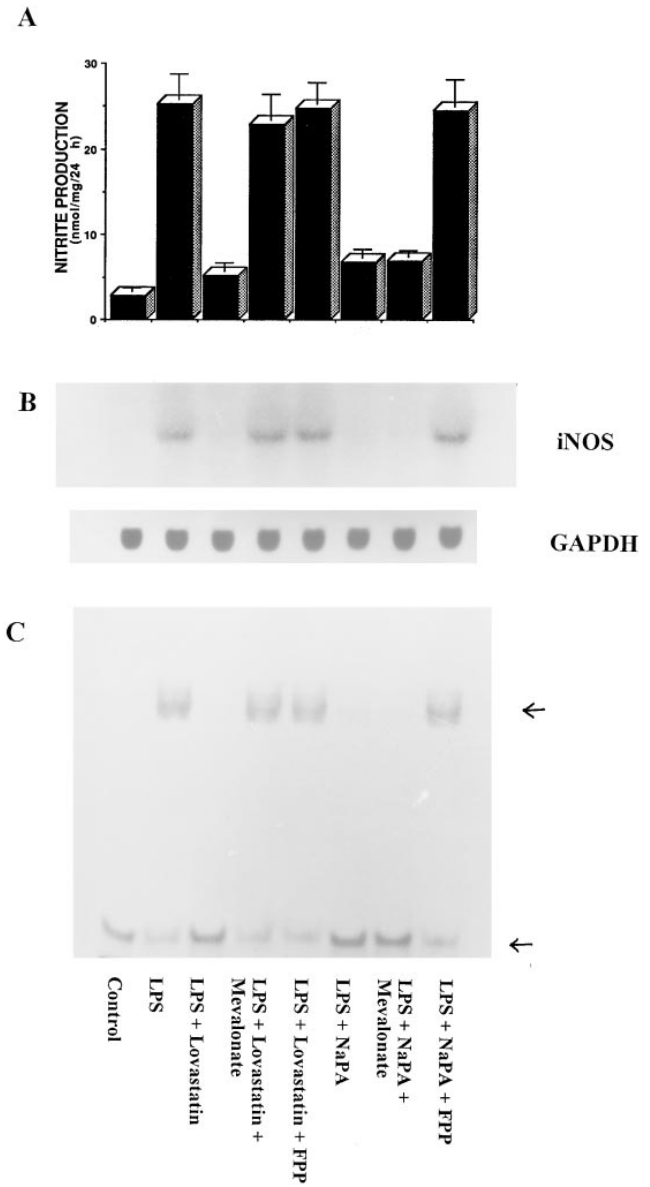

Figure 5. Effect of mevalonate and farnesyl pyrophosphate on the inhibition of lovastatin and NaPA. Cells preincubated in serum-free media with 10 μM of lovastatin or 5 mM of NaPA for 8 h received 1.0 μg/ml of LPS along with 100 μM mevalonate or 200 μM farnesyl pyrophosphate. (*A*) After 24 h, supernatants were used for nitrite assay as mentioned in Methods. Data are mean±SD of three different experiments. (*B*) After 5 h of incubation, cells were analyzed for iNOS mRNA by Northern blotting technique as described earlier. (*C*) After 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF-kβ as described in Methods. The upper arrow indicates the induced NF-kβ, whereas the lower arrow indicates the unbound probe.

for activation of $p21^{ras}$, the central molecule upstream of the Raf/MAP kinase cascade, we examined the effect of FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, on LPS-mediated expression of iNOS and activation of NF-kβ in rat primary astrocytes. FPT inhibitor II selectively inhibits ras farnesyl protein transferase with the IC_{50} of 75 nM. In whole cells, however, 25–250 μM of FPT inhibitor II inhibits farnesylation of $p21^{ras}$ by ~ 90% (33). Fig. 6 shows that preincubation of cells for 1 h with 100 or 200 μM FPT inhibitor II potentially inhibited LPS-induced activation of NF-kβ, expression of iNOS, and production of NO, demonstrating the importance

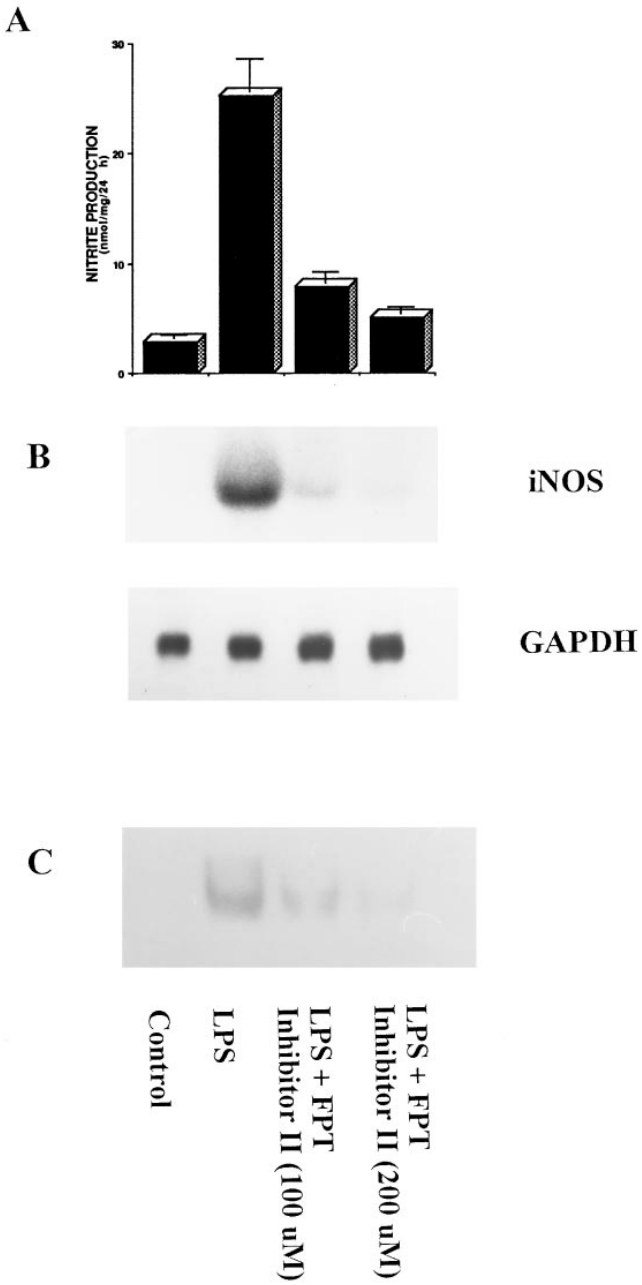

Figure 6. Inhibition of LPS-induced expression of iNOS and activation of NF-kβ by an inhibitor of Ras farnesyl pyrophosphate-S-transferase in rat primary astrocytes. Cells preincubated in serum-free media with 100 μM or 200 μM FPT inhibitor II for 1 h received 1.0 μg/ml of LPS. (*A*) After 24 h of incubation, supernatants were used for nitrite assay as described in Methods. Data are mean±SD of three different experiments. (*B*) After 5 h of incubation, cells were analyzed for iNOS mRNA by Northern blotting technique as described earlier. (*C*) After 1 h of incubation, cells were taken out to prepare nuclear extracts, and nuclear proteins were used for the electrophoretic mobility shift assay of NF-kβ as described in Methods.

Table II. Inhibition of LPS-induced Production of NO, TNF-α, IL-1β, and IL-6 in Rat Primary Astrocytes, Microglia, and Macrophages by Lovastatin and NaPA

Cells	Production of NO or cytokines	Treatments		
		LPS only	LPS + lovastatin	LPS + NaPA
Astrocytes	NO	25.3±3.2	5.2±0.4	5.4±0.6
	TNF-α	5.3±0.8	0.3±0.05	0.4±0.06
	IL-1β	10.4±1.5	0.8±0.1	1.1±0.2
	IL-6	136.5±16.8	6.9±0.9	7.6±0.8
Microglia	NO	81.2±6.9	5.9±0.4	6.9±0.9
	TNF-α	14.5±2.1	0.9±0.1	1.3±0.2
	IL-1β	28.2±3.4	2.1±0.3	2.4±0.2
	IL-6	295.6±33.5	7.8±1.1	9.3±1.2
Macrophages	NO	118.5±12.5	7.2±0.9	9.5±0.7
	TNF-α	18.6±2.3	1.2±0.1	1.7±0.2
	IL-1β	34.6±4.5	2.3±0.3	3.1±0.4
	IL-6	350.0±27.6	8.3±0.6	10.2±1.4

Cells preincubated with 10 μM lovastatin or 5 mM NaPA for 8 h in serum-free condition was stimulated with 1.0 μg/ml of LPS. After 24 h of incubation, concentrations of NO, TNF-α, IL-1β, and IL-6 were measured in supernatants as mentioned in Methods. NO is expressed as nmol/24 h/mg protein whereas TNF-α, IL-1β, and IL-6 are expressed as ng/24 h/mg protein. Data are expressed as the mean±SD of three different experiments.

of $p21^{ras}$ farnesylation in LPS-mediated activation of NF-kβ and induction of iNOS in astrocytes.

Lovastatin and NaPA inhibit the LPS-induced expression of TNF-α, IL-1β, and IL-6 in rat primary astrocytes. Activated astrocytes, the major glial cell population of brain, are reported to secrete TNF-α, IL-1β, and IL-6 (34). Since lovastatin and NaPA inhibited LPS-induced expression of iNOS in astrocytes, we examined the effect of these two inhibitors on LPS-induced expression of TNF-α, IL-1β, and IL-6. Astrocytes preincubated with lovastatin or NaPA were stimulated with LPS. Concentrations of TNF-α, IL-1β, and IL-6 were measured in the supernatants after 24 h of incubation (Table II), and the mRNA expression of these cytokines was examined in the cells after 5 h of LPS stimulation (Fig. 7). Bacterial LPS markedly induced the mRNA expression and production of respective cytokines in astrocytes. Although lovastatin or NaPA alone had no effect on the production of cytokines, however, these two compounds strongly inhibited LPS-induced production of TNF-α, IL-1β, and IL-6 in the supernatants (Table II). The decrease in cytokine production was also accompanied by an inhibition of their mRNA expression (Fig. 7), demonstrating that lovastatin and NaPA downregulate expression of all the inflamatory mediators (iNOS, TNF-α, IL-1β, and IL-6) in astrocytes.

Inhibition of LPS-induced production of NO, TNF-α, IL-1β, and IL-6 in rat primary microglia and macrophages by lovastatin. Both macrophages and microglia, important sources of NO and cytokines, actively participate in the pathophysiologies of different inflamatory disorders. Since lovastatin and NaPA inhibited the LPS-induced production of NO, TNF-α, IL-1β, and IL-6 in astrocytes, we also examined the effect of these two compounds on LPS-stimulated production of NO, TNF-α, IL-1β, and IL-6 in rat primary microglia and macrophages (Table II). The rate of production of NO and cytokines after LPS stimulation was much higher in both macrophages and microglia than in astrocytes. Similar to astrocytes, lovastatin or NaPA alone had no effect on the production of NO and cytokines in macrophages and microglia (data not shown). Both of these compounds, however, strongly inhibited the LPS-induced production of NO, TNF-α, IL-1β, and IL-6 in macrophages and microglia (Table II). These studies demonstrate the importance of the mevalonate pathway in the LPS-induced production of NO, TNF-α, IL-1β, and IL-6 in astrocytes as well as in microglia and macrophages (Table II).

The inhibitors (lovastatin, mevastatin, or NaPA), cytokines (TNF-α, IL-1β, and IFN-γ), or LPS used under these experimental conditions had no effect on the viability of astrocytes, microglia, or macrophages, measured by Trypan blue exclu-

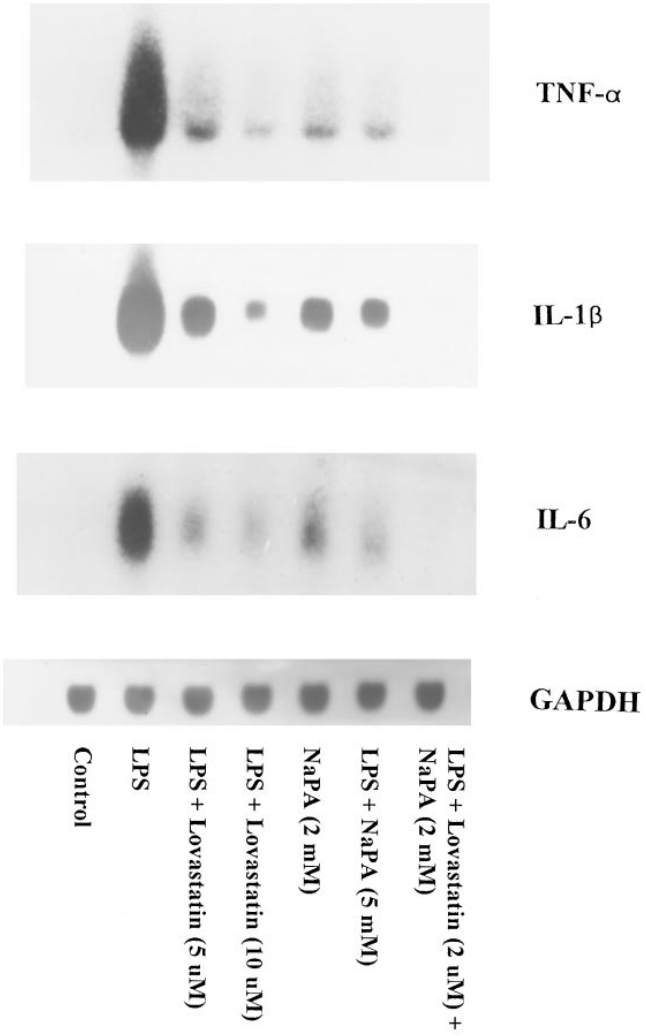

Figure 7. Inhibition of LPS-induced expression of TNF-α, IL-1β, and IL-6 by lovastatin and NaPA in rat primary astrocytes. Cells preincubated in serum-free media with different concentrations of lovastatin (5 or 10 μM) or NaPA (2 or 5 mM), or a combination of 2 μM of lovastatin and 2 mM of NaPA for 8 h, received 1.0 μg/ml of LPS. After 5 h of incubation, cells were analyzed for TNF-α, IL-1β, and IL-6 mRNAs by Northern blotting technique as described earlier.

sion. Therefore, the conclusion drawn in this study is not due to any change in viability of the cells.

Discussion

Several lines of evidence presented in this manuscript clearly support the conclusion that inhibitors of HMG-CoA reductase (lovastatin or mevastatin) and NaPA reduce the induction of inflammatory mediators (iNOS, TNF-α, IL-1β, and IL-6) in rat astrocytes, microglia, and macrophages, demonstrating the involvement of mevalonate metabolite(s) and farnesyl pyrophosphate in the induction of inflammatory mediators. This conclusion was based on the following observations: first, LPS-induced expression of iNOS, TNF-α, IL-1β, and IL-6, and activation of NF-kβ, was inhibited by lovastatin and NaPA; second, inhibitory effects of lovastatin and NaPA on LPS-mediated induction of iNOS and cytokines was not reversed by cholesterol and ubiquinone, end products of mevalonate pathway, suggesting that this inhibitory effect of lovastatin was not due to depletion of end products of mevalonate pathway; third, the reversal of inhibitory effect of lovastatin by mevalonate and FPP and that of NaPA by only FPP, but not by mevalonate, indicates that mevalonate and FPP are necessary compounds for LPS signal transduction; fourth, inhibition of LPS-induced activation of NF-kβ and induction of iNOS by FPT inhibitor II, an inhibitor of Ras farnesyl protein transferase, suggests that farnesylation of $p21^{ras}$ or other proteins is required for signal transduction in the LPS-induced expression of iNOS. Since iNOS, TNF-α, IL-1β, and IL-6 have been implicated in the pathogenesis of demyelinating and neurodegenerative diseases (6–8), our results provide a potentially important mechanism whereby inhibitors of HMG-CoA reductase and mevalonate pyrophosphate decarboxylase may ameliorate neural injury.

The LPS-induced signal transduction pathways are not very well understood so far. LPS is shown to bind cell-surface receptor (CD 14) (35), and LPS-responsive elements (LRE) have also been identified in promoter regions of several immediate early genes, including MuRantes and crg-2 (36). Although the LRE has not been identified in the promoter region of iNOS, several evidences clearly indicate that LPS induces iNOS via activation of NF-kβ (14, 15, 25). Inhibition of LPS-induced NF-kβ activation and iNOS expression by lovastatin, NaPA, and FPT inhibitor II indicates that the observed inhibition of iNOS expression is due to inhibition of NF-kB activation. Previous studies of Law et al. (37) demonstrating the inhibition of NF-kβ activation by mevinolin and 5′-methylthioadenosine suggested a role of protein farnesylation and carboxyl methylation reactions in the activation of NF-kβ. The Ras protooncogene proteins, a family of GTP-binding proteins, function by binding to the cytoplasmic surface of the plasma membrane. This membrane localization of $p21^{ras}$ involves prenylation of cysteine in the CAAX motif present at the COOH terminus, proteolytic removal of AAX tripeptide, and then carboxymethylation of COOH-terminal cysteine (38). The activation of $p21^{ras}$ by receptor tyrosine kinase occurs through conversion of the GDP-bound inactive form to the GTP-bound active form by Sos and Grb2, and then through transduction of signal to downstream effector molecules (39). One such candidate is Raf-1 (serine-threonine kinase). $p21^{ras}$ interacts directly with Raf-1, and is believed to function by positioning Raf-1 at the plasma membrane in the vicinity of its activator; tyrosine phosphorylation of Raf-1 seems to be essential for $p21^{ras}$-induced activation of Raf-1 (40). Raf-1 in turn phosphorylates and activates MEKs and ERKs (members of MAP kinase cascade). Since mevalonate availability regulates the posttranslational isoprenylation of many intracellular signaling proteins including $p21^{ras}$ (16), the observed inhibition of NF-kβ activation and induction of iNOS by lovastatin and NaPA may be due to the decrease or lack of the isoprenylation of $p21^{ras}$, that in turn leads to the lack of or abnormal signal transmission from receptor tyrosine kinase to Raf/MAP kinase cascade, activation of NF-kβ, and induction of iNOS.

NO, a diffusible free radical, plays many roles as a signaling and as an effector molecule in diverse biological systems including neuronal messenger, vasodilation, and antimicrobial and antitumor activities (1, 2). In the nervous system, NO appears to have both neurotoxic and neuroprotective effects, and may have a role in the pathogenesis of stroke and other neurodegenerative diseases, and in demyelinating conditions (e.g., multiple sclerosis, experimental allergic encephalopathy, X-adrenoleukodystrophy) associated with infiltrating macrophages and production of proinflamatory cytokines (6–8, 41). NO and peroxynitrite (reaction product of NO and O_2^-) are potentially toxic molecules to neurons and oligodendrocytes that may mediate toxicity through the formation of iron–NO complexes of iron-containing enzyme systems (42), oxidation of protein sulfhydryl groups (43), nitration of proteins, and nitrosylation of nucleic acids and DNA strand breaks (44). Although monocytes/macrophages are the primary source of iNOS in inflammation, LPS and other cytokines induce a similar response in astrocytes and microglia (3, 4). NO derived from macrophages, microglia, and astrocytes has been implicated in the damage of myelin-producing oligodendrocytes in demyelinating disorders like multiple sclerosis and neuronal death during neuronal degenerating conditions including brain trauma (3–8). The studies described in this manuscript suggest that lovastatin and NaPA, alone or in combination, may represent a possible avenue of research for therapeutics directed against cytokine- and nitric oxide–mediated brain disorders, particularly in demyelinating conditions.

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References

1. Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 6:3051–3064.

2. Jaffrey, S.R., and S.H. Snyder. 1995. Nitric oxide: a neural messenger. *Annu. Rev. Cell Dev. Biol.* 11:417–440.

3. Hu, S.X., W.S. Sheng, P.K. Peterson, and C.C. Chao. 1995. Differential regulation by cytokines of human astrocyte nitric oxide production. *Glia.* 15: 491–494.

4. Galea, E., D.L. Feinstein, and D.J. Reis. 1992. Induction of calcium-independent nitric oxide synthase activity in primary rat glial cultures. *Proc. Natl. Acad. Sci. USA.* 89:10945–10949.

5. Koprowski, H., Y.M. Zheng, E. Heber-Katz, N. Fraser, L. Rorke, Z.F. Fu, C. Hanlon, and B. Dietzshold. 1993. In vivo expression of inducible nitric oxide synthase in experimentally induced neurologic diseases. *Proc. Natl. Acad. Sci. USA.* 90:3024–3027.

6. Mitrovic, B., L.J. Ignarro, S. Montestruque, A. Smoll, and J.E. Merril. 1994. Nitric oxide as apotential pathological mechanism in demyelination: its effects on primary glial cells in vivo. *Neuroscience.* 61:575–585.

7. Bo, L., T.M. Dawson, S. Wesselingh, S. Mork, S. Choi, P.A. Kong, D.

Hanley, and B.D. Trapp. 1994. Induction of nitric oxide synthase in demyelinating regions of multiple sclerosis brains. *Ann. Neurol.* 36:778–786.

8. Merrill, J.E., L.J. Ignarro, M.P. Sherman, J. Melinek, and T.E. Lane. 1993. Microglial cell cytotoxicity of oligodendrocytes is mediated through nitric oxide. *J. Immunol.* 151:2132–2141.

9. Pahan, K., F.G. Sheikh, A.M.S. Namboodiri, and I. Singh. 1997. *N*-Acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. *Free Radical Biol. Med.* 24:39–48.

10. Feinstein, D.L., E. Galea, J. Cermak, P. Chugh, L. Lyandvert, and D.J. Reis. 1994. Nitric oxide synthase expression in glial cells: suppression by tyrosine kinase inhibitors. *J. Neurochem.* 62:811–814.

11. Nishiya, T., T. Uehara, and Y. Nomura. 1995. Herbimycin A suppresses NF-kβ activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells. *FEBS Lett.* 371:333–336.

12. Reimann et al. 1994. *J. Immunol.* 153:5740–5749.

13. Li, S., and J.M. Sedivy. 1993. Raf-1 protein kinase activates the NF-kβ transcription factor by dissociating the cytoplasmic NF-kβ-Ikβ complex. *Proc. Natl. Acad. Sci. USA.* 90:9247–9251.

14. Xie, Q.-W., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-kβ/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* 269:4705–4708.

15. Kwon, G., J.A. Corbett, C.P. Rodi, P. Sullivan, and M.L. McDaniel. 1995. Interleukin-1 beta-induced nitric oxide synthase expression by rat pancreatic beta-cells: evidence for the involvement of NF-kβ in the signaling mechanism. *Endocrinology.* 136:4790–4795.

16. Goldstein, J.L., and M.S. Brown. 1990. Regulation of the mevalonate pathway. *Nature (Lond.).* 343:425–430.

17. Casey, P.J., P.A. Solski, C.J. Der, and J.E. Buss. 1989. P21ras is modified by a farnesyl isoprenoid. *Proc. Natl. Acad. Sci. USA.* 86:8323–8327.

18. Kikuchi, A., and L.T. Williams. 1994. The post-translational modification of ras p21 is important for Raf-1 activation. *J. Biol. Chem.* 269:20054–20059.

19. Castillo, M., M. Martinez-Cayuela, M.F. Zafra, and E. Garcia-Peregrin. 1991. Effect of phenylalanine derivatives on the main regulatory enzymes of cholesterogenesis. *Mol. Cell. Biochem.* 105:21–25.

20. Samid, D., Z. Ram, W.R. Hudgins, S. Shack, L. Liu, S. Walbridge, E.H. Oldfield, and C.E. Meyers. 1994. Selective activity of phenylacetate against malignant gliomas: resemblance to fetal brain damage in phenylketonuria. *Cancer Res.* 54:891–895.

21. Samid, D., S. Shack, and L.T. Sherman. 1992. Phenylacetate: a novel nontoxic inducer of tumor cell differentiation. *Cancer Res.* 52:1988–1992.

22. McCarthy, K., and J. DeVellis. 1980. Preparation of separate astroglial and oligodendroglial cultures from rat cerebral tissue. *J. Cell Biol.* 85:890–902.

23. Giulian, D., and T.J. Baker. 1986. Characterization of amoebid microglia isolated from developing mammalian brain. *J. Neurosci.* 6:2163–2178.

24. Feinstein, D.L., E. Galea, S. Roberts, H. Berquist, H. Wang, and D.J. Reis. 1994. Induction of nitric oxide synthase in rat C6 glial cells. *J. Neurochem.* 62:315–321.

25. Pahan, K., A.M.S. Namboodiri, F.G. Sheikh, B.T. Smith, and I. Singh. 1997. Increasing cAMP attenuates induction of inducible nitric oxide synthase in rat primary astrocytes. *J. Biol. Chem.* 272:7786–7791.

26. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. *Anal. Biochem.* 72:248–254.

27. Geller, D.A., C.J. Lowenstein, R.A. Shapiro, A.K. Nussler, M. Di Silvio, S.C. Wang, D.K. Nakayama, R.L. Simmons, S.H. Snyder, and T.R. Billiar. 1993. Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. USA.* 90:3491–3495.

28. Caira, F., C. Pacot, O. Bardot, M.C. Malki, and N. Latruffe. 1995. Transcriptional and post-transcriptional analysis of peroxisomal protein encoding genes from rat treated with an hypolipemic agent, ciprofibrate. *Biochem. Pharmacol.* 49:611–619.

29. Dignam, J.D., R.M. Lebovitz, and R.G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucl. Acids Res.* 11:1475–1489.

30. Repko, E.M., and W.A. Maltese. 1989. Post-translational isoprenylational of cellular proteins is altered in response to mevalonate availability. *J. Biol. Chem.* 264:9945–9952.

31. Sinensky, M., L.A. Beck, S. Leonard, and R. Evans. 1991. Differential inhibitory effects of lovastatin on protein isoprenylation and sterol synthesis. *J. Biol. Chem.* 265:19937–19941.

32. Thibault, A., D. Samid, M.R. Cooper, W.D. Figg, A.C. Tompkins, N. Patronas, D.J. Headlee, D.R. Kohler, D.J. Venzon, and C.E. Myers. 1995. Phase I study of phenylacetate administration twice daily to patients with cancer. *Cancer.* 75:2932–2938.

33. Manne, V., C.S. Ricca, J.G. Brown, A.V. Tuomori, N. Yan, et al. 1995. Ras farnesylation as a target for novel antitumor agent: potent and selective farnesyl diphosphate analog inhibitors of farnesyl transferase. *Drug Dev. Res.* 34:121–137.

34. Sharif, S.F., R.J. Hariri, V.A. Chang, P.S. Barie, R.S. Wang, and J.B. Ghajar. 1993. Human astrocyte production of tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 following exposure to lipopolysaccharide endotoxin. *Neurol. Res.* 15:109–112.

35. Stefanova, I., M.L. Corcoran, E.M. Horak, L.M. Wahl, J.B. Bolen, and I.D. Horak. 1993. Lipopolysaccharide induces activation of CD14-associated protein tyrosine kinase p53/56lyn. *J. Biol. Chem.* 268:20725–20728.

36. Shin, H.S., B.E. Drysdale, M.L. Shin, P.W. Noble, S.N. Fisher, and W.A. Paznekas. 1994. Definition of a lipopolysaccharide-responsive element in the 5′-flanking regions of MuRantes and crg-2. *Mol. Cell. Biol.* 14:2914–2925.

37. Law, R.E., J.B. Stimmel, M.A. Damore, C. Carter, S. Clarke, and R. Wall. 1992. Lipopolysaccharide-induced NF-kB activation in mouse 70Z/3 pre-B lymphocytes is inhibited by mevilonin and 5′-methyladenosine: roles of protein isoprenylation and carboxylation reactions. *Mol. Cell. Biol.* 12:103–111.

38. Hancock, J.F., K. Cadwaller, and C.J. Marshall. 1991. Methylation and proteolysis are essential for efficient membrane binding of prenylated $p21^{k\text{-}ras(B)}$. *EMBO J.* 10:641–646.

39. Kikuchi, A., and L.T. Williams. 1994. The post-translational modification of ras p21 is important for raf-1 activation. *J. Biol. Chem.* 269:20054–20059.

40. Jelinek, T., P. Dent, T.W. Sturgill, and M.J. Weber. 1996. Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation. *Mol. Cell. Biol.* 16:1027–1034.

41. Dawson, V.L., T.M. Dawson, E.D. London, D.T. Bredt, and S.H. Snyder. 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. *Proc. Natl. Acad. Sci. USA.* 88:6368–6371.

42. Drapier, J.-C., and J.B. Hibbs. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophages effector cells. 1988. *J. Immunol.* 140:2829–2838.

43. Radi, R., J.S. Beckman, K.M. Bush, and B.A. Freeman. 1991. Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *J. Biol. Chem.* 266:4244–4250.

44. Wink, D.A., K.S. Kasprazak, C.M. Maragos, R.K. Elespuru, M. Misra, T.M. Dunams, T.A. Cebula, W.H. Koch et al. 1991. DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. *Science (Wash. DC).* 254: 1001–1003.