Inhibition of Geranylgeranylation Mediates the Effects of 3-Hydroxy-3-methylglutaryl (HMG)-CoA Reductase Inhibitors on Microglia*

Inflammatory responses involving microglia, the resident macrophages of the brain, are thought to contribute importantly to the progression of Alzheimer’s disease (AD) and possibly other neurodegenerative disorders. The present study tested whether the mevalonate-isoprenoid biosynthesis pathway, which affects inflammation in many types of tissues, tonically regulates microglial activation. This question takes on added significance given the potential use of statins, drugs that block the rate-limiting step (3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase)) in mevalonate and cholesterol synthesis, in AD treatment. Both mevastatin and simvastatin caused a concentration- and time-dependent activation of microglia in cultured rat hippocampal slices. This response consisted of a transformation of the cells from a typical resting configuration to an amoeboid, macrophage-like morphology, increased expression of a macrophage antigen, and up-regulation of the cytokine tumor necrosis factor-α. Evidence for proliferation was also obtained. Statin-induced microglial changes were blocked by mevalonate but not by cholesterol, indicating that they were probably due to suppression of isoprenoid synthesis. In accord with this, the statin effects were absent in slices co-incubated with geranylgeranyl pyrophosphate, a mevalonate product that provides for the prenylation of Rho GTPases. Finally, PD98089, a compound that blocks activation of extracellularly regulated kinases1/2, suppressed statin-induced up-regulation of tumor necrosis factor-α but had little effect on microglial transformation. These results suggest that 1) the mevalonate-isoprenoid pathway is involved in regulating microglial morphology and in controlling expression of certain cytokines and 2) statins have the potential for enhancing a component of AD with uncertain relationships to other features of the disease.

High levels of cholesterol, and in particular of cholesterol esters (1), influence the generation and aggregation of β-amyloid peptides in dissociated cell cultures (2–6) and transgenic mice (7). Patients with high plasma cholesterol levels and cardiovascular diseases have increased risk of Alzheimer’s disease (AD) (8–10), and there is evidence that statins, a family of compounds that inhibit the rate-limiting enzyme in cholesterol synthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase: HMG-CoA reductase), decrease the incidence of the disease (11, 12). These observations have led to an extensive and ongoing evaluation of statins as preventive treatments for Alzheimer’s disease (13–16).

Mevalonate, a cholesterol precursor, the synthesis of which is blocked by statins, is converted into several bioactive compounds. Among these, the isoprenoids are of particular importance because, among other functions, they provide for the covalent addition of lipid moieties (prenylation) to regulatory proteins (17) and thereby affect critical cell functions. Prenylation by the mevalonate products farnesyl and geranylgeranyl diphosphate, for example, contributes to the regulation of gene expression and cell migration in various types of tissue (18–21). Several studies have used statins to investigate the roles of cholesterol and protein prenylation in the regulation of immune cell properties, including cell morphology, migration, and secretion of cytokines. The results have not been consistent; although some experiments found that statins induce proinflammatory responses (22), others indicate that the drugs have direct anti-inflammatory (23) and immunomodulatory (24) actions. Whether statins also influence the activation of microglia, the resident cells mediating inflammatory responses in brain, is unknown, although the question is of evident importance for the use of the drugs in AD. Microglial activation, along with classic features of the immune response, including increases in proinflammatory cytokines and the presence of complement proteins, are found in the brains of patients with AD (see Ref. 25 for a review). Possibly related to this, epidemiological studies suggest that non-steroidal anti-inflammatory drugs reduce the risk and slow the progression of the disease. On the other hand, accumulating evidence points to the conclusion that microglia help to clear extracellular amyloidogenic peptides, an activity that would presumably retard the development of AD-related pathology (26). Beneficial activation of microglia has thus been proposed as a rationale for therapeutic vaccination (27, 28).

The present study explored the effects of inhibiting HMG-CoA reductase on microglial morphology and expression of the cytokine TNF-α using a novel cultured brain slice preparation. This preparation has been used previously to study pathologies

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1 The abbreviations used are: AD, Alzheimer’s disease; HMG, 3-hydroxy-3-methylglutaryl; TNF-α, tumor necrosis factor-α; BrdUrd, 2′-deoxyuridine; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase.
associated with the aged human brain (29, 30), including an intense microglial reaction to suspected causes of age-related pathology such as amyloid-β peptide uptake by neurons (31) and partial lysosomal dysfunction (32). Cultured slices thus provide a convenient system with which to test whether protracted exposure to statins triggers brain inflammatory reactions. Our results indicate that statins elicit microglial activation, as evidenced by transformation to macrophage morphology and up-regulation of the cytokine TNF-α, and that this effect is likely due to suppression of the mevalonate pathway and of its geranylgeranylation products.

**EXPERIMENTAL PROCEDURES**

**Preparation and Maintenance of Hippocampal Slice Cultures—**Organo
typic hippocampal cultures were prepared using the technique of Stoppini et al. (33). Briefly, hippocampi were harvested from brains of 9–12-day-old Sprague-Dawley rat pups under sterile conditions. Slices (400-μm thick) were cut perpendicular to the long axis of hippocampus using a McIlwain tissue chopper and collected into a cutting medium consisting of minimum Eagle’s medium with Earle’s salts (Invitrogen), 25 mM HEPES, 10 mM Tris base, 10 mM glucose, and 3 mM MgCl2 (pH 7.2); the media were filtered onto 300-nm cell culture inserts (CM, Millipore, Bedford, MA) that were placed in 6-well culture trays with 1 ml of growth medium/well (growth medium: 50% basal medium eagle, 25% Earle’s balanced salt solution, 25% horse serum, and the following supplements: 136 mM NaCl, 2 mM CaCl2, 2.5 mM MgSO4, 5 mM NaHCO3, 3 mM glutamine, 40 mM glucose, 0.5 mM ascorbic acid, 20 mM HEPES buffer (pH 7.3 at 23°C), 1 mg/liter insulin, 25 mg/liter penicillin, and 50 mg/liter streptomycin). Sections were blocked in 10% normal horse serum (room temperature). Sections were then incubated with the monoclonal anti-CD11b antibody OX-42 (1:500; Sigma) and the medium was changed every other day until use, generally 12–14 days later.

Hippocampal slices maintained in vitro for 12–14 days were exposed to medium containing mevastatin (0.01–50 μM), simvastatin (0.01–10 μM; both were from Calbiochem), or vehicle (Me2SO, 0.1%) for 0–6 days. In some experiments, the aforementioned chemicals were applied in the presence of mevalonate (100 and 500 μM; Sigma) to examine for possible effects of mevalonate on microglial morphological transformation. For assessment of overall cell proliferation, 5-bromo-2′-deoxyuridine (BrdU, 10 μM; Sigma) was added to culture medium 24 h before the slices were fixed.

**Immunocytochemistry—**Following treatment, slices were thoroughly washed with 0.1 M sodium phosphate-buffered saline, fixed for 12–16 h in cold 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde, cryoprotected in 20% sucrose for 1–2 h, and then carefully removed from the tissue membranes. Sections (25-μm thick) were then cut parallel to the broad face of the explant, using a freezing microtome. Immunocytochemistry was performed using the standard avidin-biotin horseradish peroxidase complex (ABC) method using the reagents and instructions of the VECTASTAIN® Elite ABC kit from Vector Laboratories (Burlingame, CA). Briefly, free-floating sections were preincubated with 10% normal horse serum in phosphate buffer for 1 h at room temperature. Sections were then incubated with the monoclonal antiboby ED-1 (1:1000), with the monoclonal anti-CD11b antibody OX-42 (1:1000; both were from Serotec, Oxford), or a polyclonal goat antibody against TNF-α antibody (1:1000; Sigma) in 5% normal horse serum overnight at 4°C. Sections were then washed in phosphate buffer, incubated in biotinylated anti-mouse IgG (for ED-1 and OX-42) or anti-goat IgG (for TNF-α) (both used at 1:400) for 2–3 h, washed in phosphate buffer, incubated in the avidin-biotin complex solution for 45 min, and then processed for dianamino benzidine reaction. After final rinses in phosphate-buffered saline, sections were mounted on SuperFrost Plus slides (Fisher Scientific), air-dried, dehydrated in a series of graded ethanol, and coverslipped (clearing solvent; Seeds Scientific, Kalamazoo, MI) with Permount (Fisher Scientific).

For BrdUrd staining, DNA was denatured with 2 M HCl for 30 min at 37°C, and the antigens were unmasked by incubation with 0.1% pepsin. Sections were blocked in 10% normal horse serum (room temperature for 1 h) followed by incubation with monoclonal mouse anti-BrdUrd (1:200; Sigma). Subsequently, tissues were further processed for immunonastaining following the aforementioned procedures.

**Methods for Quantification of Microglia Structural Features—**Images of ED-1, TNF-α, and BrdUrd immunostaining in the stratum pyramidale of CA1 region and in the polymorph layer of the dentate gyrus of cultured hippocampal slices were visualized using a Zeiss microscope (Axioskop 2; ×10 objective, 0.75 optovar). Images of tissue sections were digitized and scanned with a Zeiss digital photo camera (Axiocam Hrc) and an automated image-scanning program, KS 400 (Zeiss). An automated in-house computer program was used to enhance image contrast, extract ED-1, TNF-α, or BrdUrd immunopositive particles, and measure their relevant parameters. Computed measures consisted of: the number of particles (immunolabeled cells), total area, and average area per particle. Statistical significance was determined by one-way analysis of variance using Prism V. 4.

**Immunoblotting—**Electrophoresis and immunoblotting were carried out following conventional procedures. Control and experimental slices were collected in ice-cold 10 mM Tris-HCl harvest buffer consisting of 10 mM Tris, 0.32 mM sucrose, 2 mM EDTA, 2 mM EGTA, and 0.1 mM leupepin, pH 7.4, centrifuged, and sonicated after resuspension in lysis buffer (10 mM Tris/HC1, 1 mM EDTA, 1 mM EGTA, 0.1% protein inhibitor mixture (Sigma), pH 7.4). Proteins (40–60 μg) from each sample were denatured by boiling for 5 min in sample buffer (2% SDS, 50 mM Tris-HCl (pH 6.8), 10% 2-mercaptoethanol, 10% glycerol, and 0.1% bromophenol blue) and separated by electrophoresis on SDS-polyacrylamide gels (10%), after which the proteins were transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with OX-42 (1:5000) or monoclonal antibodies against TNF-α (1:1000) or 123I-labeled antibodies against diphotosphorylated ERK1/2 (1:10,000; both antibodies are from Sigma) for a 12–16-h incubation at room temperature; the blots were then stained to less than maximum intensity with anti-IgG-alkaline phosphatase conjugates, using a nitro-blue tetrazolium substrate system (Bio-Rad). Images were analyzed by densitometry using the NIH Image system (version 1.60).

**Preparation of Membrane Fractions—**Membrane fractions were prepared as described previously (36). Briefly, hippocampal slices treated with mevastatin (0.1, 1, 5 μM) or vehicle only (0.1% MeSO) were homogenized in lysis buffer and centrifuged at 25,000 × g for 20 min at 4°C. Membrane and cytoplasm proteins were denatured in sample buffer and resolved by SDS-PAGE, immunoblotting for Rho was carried out as described above using an anti-Rho antibody (1:1000; Upstate Biotechnology, Lake Placid, NY).

**Statistical Analysis—**All results are expressed as means ± S.E. analysis of variance and two-tail Student’s t tests were performed for statistical analysis as appropriate. p < 0.05 was considered to be statistically significant.

**RESULTS**

**Statins Activate Microglia in Cultured Hippocampal Slices—**Mevastatin and simvastatin, two inhibitors of the rate-limiting enzyme HMG-CoA reductase, were used to test for interactions between cholesterol metabolism and microglia activation. The antibody ED-1, which predominately labels activated macrophages and microglia (37–39), recognized few cells in untreated cultured slices (Fig. 1A). The immunopositive elements had a thin cell body with twisted fine processes (Fig. 1A, inset), a morphology corresponding to that associated with resting microglia. Six-day treatment with 10 μM mevastatin caused a large increase in the number of ED-1-labeled cells (Fig. 1D), along with pronounced morphological changes. As shown in Fig. 1D (inset), cells in the treated slices were amoeboid, notably devoid of processes, and in general resembled macrophage-like mononuclear cells. These effects could not be detected at 0.1 μM (Fig. 1B), were evident at 1.0 μM (Fig. 1C), and were maximal at 10 μM (Fig. 1D). Higher concentrations (>25 μM) decreased the number of stained cells, with the remainder having features of dying microglia (Fig. 1, E and F).

Typical results obtained with OX-42, a monoclonal antibody against a second microglial marker (Cd11b), are described in Fig. 2. In accord with earlier work (40), this antibody provides a near complete image of resting microglia, as can be seen by the labeling of fine details in the ramified processes (Fig. 2A, arrows). Six-day treatment with 10 μM mevastatin greatly increased the density of labeling, causing the normally unlabeled cell bodies (Fig. 2A, arrowheads) to become almost completely filled with immunoreactive products (Fig. 2B). Moreover, the thin twisted processes found in resting cells were either alto-
Geranylgeranylation and Microglia Activation

FIG. 1. Effects of various mevastatin concentrations on microglia in cultured hippocampal slices. Cultured hippocampal slices were treated for 6 days in the presence of 0 (A), 0.1 (B), 1 (C), 10 (D), 25 (E), or 50 (F) μM mevastatin. At the end of treatment, slices were processed for immunohistochemistry with the ED-1 antibody. Scale bar, 30 μm. Insets in A and D are at a higher magnification to illustrate the changes in individual microglia.

FIG. 2. Effects of mevastatin on microglial morphology using an antibody against the macrophage marker CD11b. Cultured hippocampal slices were treated for 6 days in the presence of 0 (A) or 10 (B) μM mevastatin. At the end of treatment, slices were processed for immunohistochemistry with the OX-42 antibody against the CD11b antigen. Scale bar, 40 μm.

FIG. 3. Effects of various mevastatin concentrations on number, area, and size of ED-1-immunopositive cells in cultured hippocampal slices. Number (A), area (B), and size (C), expressed as ratio of area/number of cells labeled with ED-1 antibody, were determined as described under “Experimental Procedures” and were expressed as -fold of control (treated with vehicle only). Results are means ± S.E. of 8–22 slices prepared from 6 to 8 animals. Results at concentrations of mevastatin at 1 μM or higher are statistically significantly different from control (A–C) (*, analysis of variance followed by Newman-Keuls multiple comparison test, p < 0.005). Results at concentrations of mevastatin from 1 to 10 μM are statistically significantly different from control (except for the 1 μM point in dentate gyrus in C). CA1, CA1 stratum pyramidale; DG, dentate gyrus.

gether absent or reduced to stubby, unramified elements (Fig. 2B, arrows). These effects, although more dramatic, were comparable with those obtained with ED-1.

Fig. 3 summarizes the changes in the number of ED-1-positive cells and the area they occupied in field CA1 and dentate gyrus of statin-treated slices. Mevastatin at 0.1 μM had no significant effect on the number (1.1 ± 0.1 times control, p > 0.1, n = 19) or size (total area) of immunopositive microglia (Fig. 3, A and B). Both measures were significantly increased at 1.0 μM. The number of labeled cells doubled (2.1 ± 0.2 times control, p < 0.001, n = 19), whereas the area they occupied increased by 197 ± 18%. As shown in the figure, the effects of the statin at 10 μM appeared to be larger in the pyramidal cell fields than in the dentate gyrus. This variation was highly significant for both number and area when measured at higher concentrations (at 5 μM, p = 0.003 for area and 0.048 for number; at 10 μM, p < 0.0001 for area and p = 0.0014 for number; Mann-Whitney test). Higher concentrations elicited smaller increases in cell number than were found at 10 μM (data not shown), confirming the impression gained from survey micrographs.

A comparison of Fig. 3, A and B, suggested that statins had a larger effect on the area occupied by activated microglia than they did on cell number. A test of this point is summarized in Fig. 3C. The ratio of the two measures was significantly greater than that found in untreated slices at all mevastatin concentrations greater than 0.1 μM and, as shown, increased in a dose-dependent manner. This provides evidence that mevastatin not only increased the number of immunopositive microglia but also enlarged individual cells. The above-described mor-
Geranylgeranylation and Microglia Activation 48241

This hypothesis was tested by analyzing BrdUrd (10 µM) uptake in control and mevastatin-treated slices. Fig. 4 summarizes the results obtained following a six-day treatment. Significant differences from control slices were not observed with 0.01 or 0.1 µM concentrations, but treatment with 1 µM caused a significant increase in the number of BrdUrd-labeled cells in both CA1 and dentate gyrus (p < 0.01 for both regions). The results for the numbers of BrdUrd-labeled cells were variable, and the increases in this measure appeared to be smaller than those for the numbers of ED-1-positive cells. Unexpectedly, mevastatin at 5 and 10 µM had smaller effects than at 1 µM and did not increase cell numbers above control values (Fig. 4). These results suggest that the increase in ED-1-immunopositive microglia produced by low concentrations of mevastatin is in part due to proliferation and in part to activation, and thus labeling, of the extant population of microglia. Only the latter effect was obtained with higher concentrations of the statin.

Statins Up-regulate TNF-α Expression—Activation of macrophages, including microglia, involves up-regulation of cytokines as well as the morphological changes described above. The release of these toxic agents is generally assumed to mediate the contribution of inflammation to the progression of various diseases, including AD; thus, it was of interest to determine whether statin-induced changes in microglia extended to cytokine expression. Fig. 5 describes results for TNF-α, an important central nervous system cytokine. Very few cells were immunopositive for TNF-α in control slices, but this changed dramatically after a 6-day treatment with mevastatin (10 µM). As shown (Fig. 5A, bottom panels), a substantial proportion of the cells in statin-treated slices were positive for the cytokine, and these cells had the amoeboid morphology associated with activated microglia. Comparison of ED-1 and TNF-α staining on the same sections showed that nearly all TNF-α-positive cells were also labeled by ED-1, and this was confirmed in merged images.

Quantitative analyses indicated that the threshold for statin-induced increases in the cytokine was below 0.1 µM (Fig. 5B). The incidence of labeled cells at this concentration was greater than in control slices (CA1: 4.2 ± 1.6 times control, n = 9 versus n = 13), and this difference was highly significant (p = 0.013, U test, two tails). The effects at 1.0 µM were less variable, but not markedly greater, than at 0.1 µM and highly significant. Higher statin concentrations produced very large (10–25-fold) increases in the number of TNF-α-positive microglia (Fig. 5B).

The number of TNF-α labeled cells in the pyramidal cell region was smaller than that for ED-1 labeled cells, suggesting that only a subset of activated microglia expressed the cytokine, as expected from the micrographs in Fig. 5A. Interestingly, the regional differences in the numbers of ED-1-positive cells were not observed with TNF-α-positive cells, as is evident in the CA1 versus dentate gyrus comparison shown in Fig. 5B. This suggests that field CA1 has a population of statin-sensitive microglia that is not present in the dentate gyrus.

Statin-induced changes in the levels of TNF-α were further analyzed by immunoblotting. Levels of the cytokine in cultured slices maintained for 2–3 weeks under control conditions were undetectable (Fig. 6A, cont), and a similar conclusion was reached using reverse transcription-PCR. Six-day incubations with mevastatin or simvastatin (10 µM) induced a marked increase in TNF-α levels (Fig. 6A, Mev and Sim); quantitative

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**Fig. 4.** Effects of various mevastatin concentrations on number of BrdUrd-immunopositive cells in cultured hippocampal slices. The number of BrdUrd-labeled cells was determined by image analysis and expressed as times of control. Results at concentrations of mevastatin at 1 µM are statistically significantly different from control in CA1 and dentate gyrus (DG) (Student’s t test, p < 0.001).

**Fig. 5.** Effects of mevastatin treatment on TNF-α expression in cultured hippocampal slices. A, cultured hippocampal slices were treated with vehicle (control) or 10 µM mevastatin for 6 days and were processed for double immunostaining with ED-1 and anti-TNF-α antibodies. Shown are images from the dentate gyrus. Scale bar, 30 µm. B, concentration-dependent up-regulation of TNF-α expression in mevastatin-treated hippocampal cultures. Cultured slices treated with mevastatin were processed for immunohistochemistry with anti-TNF-α antibodies, the digitized images were quantitatively analyzed as described under “Experimental Procedures,” and the number of TNF-α immunopositive cells was determined. Results are expressed as -fold of control and represent means ± S.E. of 8–10 experiments. Significant effect was observed at concentrations of 0.1 µM and above (analysis of variance followed by Newman-Keuls multiple comparison test, p < 0.05). CA1, CA1 stratum pyramidale; DG, dentate gyrus.

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2 X. Bi, J. Zhou, and G. Lynch, unpublished observation.
Links between Mevalonate-Isoprenoid Biosynthesis and Microglia—HMG-CoA reductase is an early step in cholesterol synthesis and, as might be expected from this, statins block the synthesis of several intermediary, biologically active steroids, with farnesyl pyrophosphate (3 m) not detectably altering the changes in ED-1-immunopositive microglia produced by a 6-day treatment with mevastatin. The labeled cells were densely labeled and oval-shaped; note also that the twisted dendritic processes typical of resting microglia were almost completely absent. In marked contrast, mevalonate (500 μM) completely blocked microglial transformation (Fig. 7D). Cell bodies were only partially labeled (arrowheads), and their processes (arrows) were evident. Mevalonate is the precursor of farnesyl diphosphate and geranylgeranyl diphosphate, two compounds that provide for protein prenylation. Primary targets of these isoprenoids include the Ras (farnesylation) and Rho (geranylgeranylation) families of small GTPases that together play critical regulatory roles in inflammatory and immune responses. Incubating slices with farnesyl pyrophosphate (3 μM) did not detectably alter the effects of mevastatin (data not shown), but equivalent treatments with geranylgeranyl orthophosphate caused a complete blockade of the microglial changes. Six-day treatment with geranylgeranyl pyrophosphate alone had no evident effect on microglia (Fig. 7E) but prevented the changes normally produced by mevastatin (Fig. 7F; compare with panel B). Geranylgeranyl pyrophosphate also completely blocked the induction of TNF-α by mevastatin (compare Fig. 8, A and B). Geranylgeranylation regulates Rho activity by controlling its attachment to membranes. As shown in Fig. 8C, the statin treatments used in the present study were sufficient to cause a concentration-dependent decrease in the levels of membrane-associated Rho proteins in cultured slices. Taken together, these experiments suggest that the mevalonate pathway, by producing geranylgeranylation, and possibly Rho activation,
Geranylgeranylation and Microglia Activation

by cholesterol. Mevalonate, the concentration of which is rapidly lowered by HMG-CoA reductase inhibitors (43, 44), regulates diverse cellular activities in part by generating farnesyl diprophosphate and geranylgeranyl diprophosphate, compounds that provide for lipid modification (prenylation) of proteins, including those belonging to the Rho and Ras families. Blocking prenylation of these regulatory agents provides a plausible route whereby statins could activate microglia. If this is correct, then substituting the appropriate isoprenoid should offset the effects of reduced mevalonate production; this prediction was confirmed in the present studies with the demonstration that geranylgeranyl pyrophosphate completely blocked the effects of statins. Importantly, farnesyl pyrophosphate was ineffective, a result that significantly narrows the range of prenylated proteins as potential effectors for microglia activation. Ras proteins and nuclear lamins are targets for farnesylation, whereas many members of the Rho family, as well as subunits of heterotrimeric G proteins, become geranylgeranylated (17). As expected from these points, statins in the time frames and concentrations used in the present studies caused Rho to dissociate from membranes in cultured hippocampal slices. In all, then, disruption of tonically active pathways driven by Rho proteins is the most likely route whereby depression of mevalonate synthesis results in microglial activation. This argument is not without precedent. For instance, a recent study showed that statins increased the production of TNF-α in macrophages by blocking geranylgeranylation and membrane attachment of Rho family GTPases (45). Furthermore, inhibition of geranylgeranylation, but not of farnesylation, has been shown to induce cell death in lymphoma (46) and multiple myeloma cells (47).

MAP kinases play important roles in inflammation, and this appears to be the case for the effects reported here. Statins caused a marked increase in active ERK1/2, which was localized to microglia by double immunofluorescent staining. Furthermore, PD98059, a compound that blocks MEK1/2, and thereby prevents activation of the ERK1/2 subtypes of MAP kinases, prevented statin-induced cell proliferation and up-regulation of TNF-α within microglia. An inhibitor of the p38 MAP kinase had no effect. How suppression of the mevalonate pathway and protein prenylation could result in ERK1/2 activation is not clear. Blocking prenylation would have the effect of inhibiting, rather than enhancing, the well described Ras-ERK pathway. However, as noted, infusions of farnesyl pyrophosphate, which should have restored Ras function, did not measurably affect statin-induced changes in microglia. Activation of ERK1/2 during periods of Rho family inhibition, although not widely reported, has been described. Decreases in the GTP-bound forms of Rac1 and Cdc42 are associated with ERK activation in fibroblasts (48), RhoB inhibits constitutive activation of ERK in tumor cells (49), and inhibition of RhoA stimulates ERK in MCF-7 breast cancer cells (50). Particularly relevant to the present results, a recent study found that inhibition of Rho up-regulated TNF-α in macrophages and that this effect was mediated by ERK1/2 activation (43). In short, available evidence indicates that Rho family members, under some circumstances, exert a tonic inhibitory influence on ERK. However, although this could account for the results obtained with PD98059, it remains possible that ERK activation and Rho inhibition are independent events, both of which are needed for the full array of effects induced by statins.

Importantly, treatment with PD98059, although blocking several aspects of microglial activation, did not prevent statin-induced conversion of microglia into an amoeboid configuration. This result, together with the observation that geranylgeranyl pyrophosphate replacement was completely effective in replace-

**Fig. 8.** Statin effects are blocked by geranylgeranyl pyrophosphate and accompanied by displacement of Rho from membranes. **A** and **B**, cultured hippocampal slices were treated with 10 μM mevastatin (Mev) at different concentrations for 6 days and processed for immunostaining with anti-TNF-α antibodies. Shown are representative images from 2 to 3 separate experiments. **Scale bar**, 30 μm. **C**, membrane fractions from hippocampal slices treated with mevastatin (Mev) at different concentrations were processed for Western blots using anti-Rho antibody. Similar results were obtained from two different experiments.

**DISCUSSION**

The present results provide the first evidence that HMG-CoA reductase inhibitors activate microglia. The statin-induced response was intense, concentration-dependent, and appeared within 48 h of treatment onset. Activation included transformation of microglia from thin cells with a few twisted processes to a more macrophage-like morphology consisting of an ovoid cell body with no processes. The anatomical changes were accompanied by increased expression of ED-1 and TNF-α, two markers of activated macrophages; up-regulation of the cytokine was detected at 0.1 μM statin concentration, making it the most sensitive of the measures used in the study. Proliferation was also found at relatively low statin concentrations. Cell division and migration are well recognized components of the microglial response to neurodegeneration, and in *vivo*, they begin rapidly enough (within 18–24 h following brain lesions) to account for the effects obtained in the present study (42).

Statin-induced transformation of microglia and up-regulation of TNF-α expression were blocked by mevalonate but not
ment tests, indicates that events downstream of geranylgeranylation split into gene-versus-morphology-related changes, with the former, but not the latter, being ERK-dependent. This is not unexpected given the prominence of MAPK signaling cascades in regulating gene expression and the presence of other, quite different Rho family effectors that potently affect actin polymerization (e.g. Rho kinase, mDia, p21-activated kinase; see Ref. 51 for a recent review). It is possible that the pathways leading to cytoskeletal versus gene regulation diverge at the very beginning of the isoprenylation step and that different members of the Rho family initiate different cascades (52–61).

Plasma levels for most clinically used statins are reported to be ~50 ng/ml, corresponding to concentrations of about 0.25 \( \mu \)M (43). This value is within the range at which the compounds increased TNF-\( \alpha \) expression and close to that at which other signs of microglial activation became evident. Thus, it is possible that the effects observed in the present study take place in subjects using these drugs as an anti-cholesterol treatment. Whether this would have positive, negative, or incidental effects on AD progression is not clear. Efforts to address the broader issue of whether statins affect AD have yielded ambiguous results. Epidemiologic studies point to an association between chronic statin treatment and low risk of AD (15, 62), but a recent review concluded that, although experimental studies support a link between cholesterol and amyloid-\( \beta \) peptide production, available prospective studies show no benefits for any statin tested (63). The more specific question of whether activated microglia make positive or negative contributions to disease progression is also unresolved. Epidemiological observations suggest that anti-inflammatory drugs have positive effects, but direct tests of this in pharmacological trials have in general been disappointing (64–66). Conversely, there is a growing body of experimental results suggesting that the microglial component of the brain inflammatory response could serve to speed the removal of amyloid and thereby to retard the development of AD symptomatology (27, 28).

In conclusion, we have found that HMG-CoA reductase inhibitors induce microglial transformation and expression of the

![In vivo microglial transformation and expression of the](http://www.jbc.org/)

**Fig. 9.** Activation of ERK1/2 by statins and effects of inhibitor of MAP kinases on mevastatin treatment-induced changes in microglial morphology and TNF-\( \alpha \) levels. A, levels of active ERK1/2 were increased in mevastatin (Mev)-treated hippocampal slices. Cultured hippocampal slices were treated for 6 days with mevastatin at the indicated concentrations and processed for Western blot using anti-diphosphorylated ERK1/2 (p44/p42) antibodies. B, microglial localization of active ERK1/2 in the hilus of dentate gyrus in mevastatin-treated hippocampal slices. Slices treated with vehicle (Cont.) or 5 \( \mu \)M mevastatin for 5 days were double-stained with anti-diphosphorylated ERK1/2 (p44/p42) and ED-1 antibodies. Note the lack of, and the presence of, co-localization in vehicle- and in mevastatin-treated slices, respectively. The arrowhead indicates a neuron, and arrows indicate microglia. C, mevastatin-induced up-regulation of TNF-\( \alpha \) was reduced by inhibition of MEK. Cultured hippocampal slices were incubated for 6 days with vehicle (\( n = 8 \)), 10 \( \mu \)M mevastatin (\( n = 8 \)), or mevastatin plus PD98059 (Mev/PD; \( n = 4 \)). Slices were combined and homogenized, and aliquots of homogenates were processed for Western blots with TNF-\( \alpha \) antibodies; blots were quantified, and optical density values were determined. Values were normalized to control values and represented as means ± S.E. D, mevastatin-induced microglial activation was partially blocked by PD98059 but not by SB233580. Cultured hippocampal slices were treated for 6 days with 10 \( \mu \)M mevastatin only (A) or in the presence of 25 \( \mu \)M PD98059 (Mev/PD) or SB233580 (Mev/SB) before being processed for immunohistochemistry with ED-1 antibody. Shown are stained cells in CA1 stratum pyramidale.
Geranylgeranylation and Microglia Activation

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