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Lovastatin-Induced Apoptosis in Macrophages through the Rac1/Cdc42/JNK Pathway

Shu-Ling Liang, Hongli Liu, and Aimin Zhou

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, have been used successfully in the treatment of hypercholesterolemia for more than a decade. To date, they are still the most powerful drugs for lowering cholesterol levels in blood (1–3). In addition to their potential role in lowering serum cholesterol levels, clinical trials and in vitro studies have shown that statins have pleiotropic effects on a wide range of cell functions and exhibit overall clinical benefits on cardiovascular diseases. Statin therapy significantly reduces ischemic stroke for patients with established coronary artery diseases (4–8), and is associated with improved patency of autogenous infrainguinal bypass grafts (9).

Atherosclerosis as a chronic inflammatory disease leads to a variety of cardiovascular disorders, such as myocardial infarction, stroke, peripheral vascular disease, and aortic aneurysm in aged people. Clinically, statins slow plaque progression and promote regression of atherosclerotic lesions (10–15). These beneficial effects of statin therapy are believed to be due, at least in part, to the anti-inflammatory and immunomodulatory roles of statins. Statin treatment reduces the levels of inflammatory markers, decreases the activation and recruitment of immune cells, and delays the progression of atherosclerosis, a chronic inflammatory disease. However, little is known about the direct impact of statins on immune cells, particularly on macrophages. We report that lovastatin, a member of the statin family, effectively induces apoptosis in macrophages. Further investigation of the molecular mechanism has revealed that Rac1 and Cdc42, the small GTPase family members, may play an important role in lovastatin-induced macrophage apoptosis. Moreover, the activation of the JNK pathway may contribute to this event. Our findings provide a better understanding of the molecular basis underlying the anti-inflammatory clinical benefits of statin therapy in cardiovascular diseases. The Journal of Immunology, 2006, 177: 651–656.

The statin family of drugs has been used successfully in the treatment of hypercholesterolemia for more than a decade. To date, they are still the most powerful drugs for lowering cholesterol levels in blood (1–3). In addition to their potential role in lowering serum cholesterol levels, clinical trials and in vitro studies have shown that statins have pleiotropic effects on a wide range of cell functions and exhibit overall clinical benefits on cardiovascular diseases. Statin therapy significantly reduces ischemic stroke for patients with established coronary artery diseases (4–8), and is associated with improved patency of autogenous infrainguinal bypass grafts (9).

Atherosclerosis as a chronic inflammatory disease leads to a variety of cardiovascular disorders, such as myocardial infarction, stroke, peripheral vascular disease, and aortic aneurysm in aged people. Clinically, statins slow plaque progression and promote regression of atherosclerotic lesions (10–15). These beneficial effects of statin therapy are believed to be due, at least in part, to the anti-inflammatory and immunomodulatory properties of statins. Treatment with statins in patients with acute coronary syndromes significantly reduces the levels of inflammatory markers, such as C-reactive protein and serum amyloid A (16–20). Statins have been found to decrease the activation and recruitment of immune cells, an immune response that contributes to the progression of atherosclerotic development (21, 22). However, the direct impact of statins on immune cells, particularly on macrophages, remains largely unexplored.

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of the mevalonate pathway. The mevalonate pathway produces isoprenoids that are critical for diverse cellular functions, ranging from cholesterol synthesis to growth control. Farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), the isoprenoid intermediates of the mevalonate pathway, are also important for the posttranslational modification of a variety of proteins, including Ras and small GTPases, such as RhoA, Rac, and Cdc42. Prenylation of these proteins with the isoprenoids is required for their translocation to the membrane and for cellular functions (23).

Rho, Rac, and Cdc42 are among the best characterized small GTPases. Although they display similar biological activities in most cases, each of the GTPases can also mediate distinct cellular functions through interaction with its own downstream effector proteins in different cell types. For example, the serum response element of the c-fos promoter is activated by Rho A, but not Rac1 and Cdc42 (24). Rac1 regulates the activation of NADPH oxidase for the purposes of innate immunity in phagocytes. However, Cdc42 acts as an antagonist in the formation of reactive oxygen species in these cells (25, 26). Evidence for extensive cross talks and cooperation between GTPases and other signal transduction pathways is well documented. Rac1 and Cdc42 can synergize with Raf to activate ERK (27–29). Furthermore, Rac1 cross talks with PI3K in controlling cell migration and polarity (30). Interestingly, these small GTPases are found to mediate apoptosis in a wide range of cell types. Overexpression of the active form of Cdc42 in Jurkat T lymphocytes induces an increase of ceramide levels, resulting in cell death (31, 32). Both Rac1 and Cdc42 mediate apoptosis induced by diverse stimuli (33). It seems that the contribution of Rac1 and Cdc42 to apoptosis is thought to mainly regulate the activation of the JNK pathways (34–36).

In this study, we investigated the direct impact of lovastatin on macrophages. Our data have shown that lovastatin induces apoptosis in macrophages through enhancing the expression of Rac1 and Cdc42, resulting in the activation of the JNK pathway.
Materials and Methods

Reagents and Abs
Lovastatin (Mevinolin), FPP, GGPP, cycloheximide, and SP600125 were from Sigma-Aldrich. Ab specific to the apoptotically cleaved form (86 kDa) of poly(ADP-ribose) polymerase (PARP) was purchased from Cell Signaling Technology. Abs to total PARP, Ras, RhoA, Rac1, Cdc42, phosho-c-Jun, and β-actin were from Santa Cruz Biotechnology. Activation of lovastatin was performed, as described previously (37). Briefly, lovastatin (25 mg) was dissolved in 0.625 ml of absolute ethanol. Freshly made 0.1 M lovastatin was performed, as described previously (37). Briefly, lovastatin was aliquoted and stored at -20°C.

Cell culture
Raw 264.7 (Raw) (American Type Culture Collection) cells were grown in DMEM (Cleveland Clinic Foundation Core Facility) supplemented with 10% cosmic calf serum (HyClone) and antibiotics in a humidified atmosphere of 5% CO2 at 37°C. Mouse bone marrow cells were isolated from C57Bl/6 mice (The Jackson Laboratory) and grown in RPMI 1640 supplemented with 15% FBS (BioSource International) and 15% L cell medium plus antibiotics for 1 wk to be differentiated into macrophages.

Assay for cell viability
The viability of Raw cells was determined using the colorimetric CellTiter 96 aqueous cell proliferation assay (MTT), according to the instructions provided by the manufacturer (Promega). Briefly, cells (1 x 105 cells/well) were seeded in 96-well plates. One day after seeding, they were treated with or without 10 μM lovastatin in the presence or absence of 5 μM GGPP or 5 μM FPP for 22 h. At the end of incubation, 50 μl of CellTiter 96 aqueous reagent (40% v/v dilution in 1× PBS) was added to each well. Plates were incubated at 37°C for 2 h, and absorbance was measured at 490 nm with a 96-well plate reader (model Spectra Max 340; Molecular Devices).

Caspase assays
The activities of caspase 3 and 9 in the cells treated with lovastatin were examined by using the Caspase-GloTM 3/7 and 9 assay kits (Promega). In these assays, caspase cleaves the prolinesmeins substrate linked with the tetrapeptide DEVD (for caspase 3/7) or LEHD (for caspase 9), resulting in the generation of a glow-type luminescent signal, produced by luciferase. In brief, cells were treated as indicated, and cytosolic extracts were prepared by suspension of cell pellets in Nonidet P-40 lysis buffer (10 mM Tris-HCl (pH 8.0), 5 mM Mg(OAc)2, 90 mM KCl, 0.2 mM PMSF, 100 μM aprotinin, 10 μg/ml leupeptin, and 2% Nonidet P-40). After centrifugation at 10,000 g for 5 min, cell extracts containing 40 μg of proteins were transferred into a 96-well plate, and 50 μl of Caspase-Glo3/7 or Caspase-Glo9 Reagents was added to the wells. After incubation for 1 h at 37°C, caspase activities were determined by a fluorescent plate reader (MictroTiter Plate Lumimeter; Dynex Technologies).

Determination of DNA fragmentation
DNA in the cells, after the indicated treatment, was isolated using an apoptotic DNA ladder kit (Roche). Briefly, 200 μl of cell suspensions in PBS was mixed with 200 μl of binding buffer (Roche). After incubation for 10 min at room temperature, 100 μl of isopropanol was added to the sample and mixed by vortexing. DNA was then purified by using glass fibers. DNA samples were separated on a 2% agarose gel and visualized with ethidium bromide staining under UV light.

TUNEL assay
TUNEL for DNA degradation was performed using an apo-BrdU kit (BD Biosciences). Briefly, cells were scraped using a scraper with medium containing floating cells after the indicated treatments. The cells were fixed in 1% paraformaldehyde for 30 min and stored in 70% ethanol at -20°C until staining and analysis. DNA fragmentation was examined by incorporating BrdU and staining with a labeled anti-bromodeoxyuridine mAb. The total DNA content was determined with propidium iodide, and the labeled cells were sorted by flow cytometry in a FACS.

Western blot analysis
After treatments, cells were washed twice with ice-cold PBS and collected with a scraper. Cytoplasmic extracts were prepared by suspension of cell pellets in Nonidet P-40 lysis buffer, as described above. After centrifugation at 10,000 x g in a microcentrifuge at 4°C for 10 min, cell extracts (100 μg/sample) were fractionated on SDS-10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% nonfat milk in PBS containing 0.02% sodium azide and 0.2% (v/v) Tween 20, and incubated with different primary Abs for 1 h at room temperature. The membranes were then washed with PBS containing 0.2% (v/v) Tween 20 and incubated with specific secondary Abs conjugated with HRP (Cell Signaling Technology) for 1 h at room temperature. After washing, these proteins were detected by a chemiluminescence method, according to the manufacturer’s specification (Amersham).

Results
Lovastatin-induced decrease of macrophage viability was prevented by GGPP and FPP
To determine the direct effect of statins on immune cells, we treated Raw cells, a mouse macrophage cell line, with varied concentrations of lovastatin, a member of the statin family. Cell viability was analyzed using the MTT assay. As shown in Fig. 1A, lovastatin at 10 μM reduced cell viability to 25.6% of that of untreated cells in 24 h. To determine whether lovastatin-induced macrophage apoptosis is cell type specific, we treated Ba/F3 cells, a mouse pro-B cell line, and Jurkat cells, a human T cell line, with lovastatin. Although lovastatin was able to induce apoptosis in these cells, a higher concentration (30 – 50 μM) and a prolonged incubation time (48 – 72 h) were required to achieve the same level of apoptosis (data not shown). These results are consistent with the previous reports that lovastatin at a higher concentration is needed to induce apoptosis in smooth muscle cells, endothelial cells, and plasma cells (38 – 40), suggesting that macrophages are particularly vulnerable to lovastatin-induced apoptosis.

Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase by statins results in the depletion of several intermediate products in the mevalonate pathway. These intermediates, including FPP and GGPP, are isoprenoids that serve as the substrates for the prenylation of Ras and small GTPases, such as RhoA, Rac, and Cdc42. To determine whether supplementation of GGPP or FPP is able to prevent cells from death, Raw cells were coincubated with 10 μM lovastatin and 5 μM GGPP or FPP. Treatment of Raw cells with GGPP or FPP almost completely prevented cell death induced by lovastatin, suggesting that inhibition of protein prenylation accounts for lovastatin-induced macrophage death (Fig. 1B). The lovastatin-induced Raw cell death was observed by phase contrast microscopy (Fig. 1, C-I). To rule out the possibility that this result is cell line specific, we treated J774 cells (data not shown), another murine macrophage cell line, and primary mouse bone marrow-derived macrophages (BMM) (Fig. 1, C-II) with lovastatin. Similar results were obtained. Therefore, our data with all macrophage cells tested indicated that lovastatin is able to induce macrophage death in both cell lines and primary cells.

Lovastatin induces macrophage apoptosis
To determine whether the decreased viability of Raw cells is due to lovastatin-induced apoptosis, the PARP cleavage was measured after the cells were treated with lovastatin. PARP is an enzyme responsible for DNA damage surveillance, and PARP cleavage is considered to be one of the classical characteristics of apoptosis. During apoptosis, PARP (116 kDa) is cleaved by the activated caspase family members, such as caspase 3, into 25- and 86-kDa fragments. In this experiment, Raw cells were treated with 10 μM Lovastatin for different times, and PARP cleavage in the cells was determined by Western blot analysis using a mAb recognizing 86-kDa PARP. As shown in Fig. 1D, PARP cleavage was detected in a time-dependent fashion. To further characterize lovastatin-induced macrophage apoptosis, we determined the activity of...
caspases in Raw cells after being treated with lovastatin. The activity of caspase 3 and 9 in the cells treated with lovastatin was examined by using the Caspase-Glo™ 3/7 and 9 assay kits (Promega). In these assays, caspase cleaves the proluminescent substrate linked with the tetrapeptide DEVD (for caspase 3/7) or LEHD (for caspase 9), resulting in the generation of a glow-type luminescent signal, produced by luciferase. Luminescence is proportional to the amount of caspase activity present. Clearly, lovastatin could greatly induce the activity of caspase 3 in Raw cells. However, the supplementation of GGPP and FPP was able to suppress the caspase 3 activity induced by lovastatin, implicating that the blockage of the mevalonate pathway triggers the apoptotic cascade (Fig. 2A). Notably, caspase 9 is not activated in lovastatin-induced macrophage apoptosis (Fig. 2B).

DNA fragmentation is another typical feature of cell apoptosis. The genomic DNA extracted from Raw cells treated with lovastatin was analyzed by agarose electrophoresis. The degree of laddering was related to the concentration of lovastatin (Fig. 3A), and this event can be blocked by adding GGPP or FPP (Fig. 3B). We also performed the TUNEL to determine apoptotic cells. The TUNEL assay detects DNA strand breaks induced during the process of apoptosis. Treatment of Raw cells with 10 μM lovastatin for 24 h produced 68% TUNEL-positive cells, indicating that Raw cells are very sensitive to lovastatin. To determine

FIGURE 1. Effect of lovastatin on macrophage viability. Raw cells were treated with different amounts of lovastatin (A) or 10 μM lovastatin for 24 h in the presence or absence of 5 μM GGPP or FPP (B). The percentage of viable cells was measured by using MTT assay (Promega). Experiments were performed three times in triplicates. Data are presented as mean ± SD. C, Photomicrographs of Raw cells and BMM after lovastatin treatment. Photographs of the unstained cells were taken at 30 h for Raw cells (C-I) and 48 h for BMM (C-II) afterLovastatin was added under Olympus model CKX31 at ×100 magnification: a, untreated; b, treated with 10 μM lovastatin; c, treated with 10 μM lovastatin plus 5 μM GGPP; d, treated with 10 μM lovastatin plus 5 μM FPP. D, Effect ofLovastatin on the PARP cleavage in Raw cells. Raw cells were treated with 10 μM lovastatin for the times indicated, and the PARP cleavage was determined by Western blot analysis using a specific mAb to the 86-kDa fragment (Cell Signaling Technology).
whether inhibition of protein synthesis impacts lovastatin-induced apoptosis, we treated Raw cells with lovastatin in the presence or absence of cycloheximide, a common inhibitor of protein synthesis. Cycloheximide at a concentration of 0.5 \text{M} remarkably inhibited lovastatin-induced apoptosis in Raw cells, suggesting that a novel protein synthesis is required for the apoptotic process (Fig. 4).

Lovastatin induces the expression of Rac1 and Cdc42 in macrophages

Blockage of the mevalonate pathway by statins results in the depletion of isoprenoids, which are required for the posttranslational modification of Ras and Ras-like GTPase family, such as RhoA, Rac, and Cdc42. These small GTPases act as molecular switches to regulate diverse biochemical functions in all eukaryotic cells through mediating gene expressions. Therefore, we investigated the effect of lovastatin on the expression of certain proteins, including the members of small GTPase family. Intriguingly, we found that lovastatin significantly induced the expression of Rac1 and Cdc42, but not RhoA, in a dose- and time-dependent manner (Fig. 5, A and B). Lovastatin treatment did not affect the level of Ras in the cells (data not shown). The role of Rac1 and Cdc42 in activation of JNK has been well established (34–36). Thus, we examined the activation of JNK pathway. Interestingly, we found that the high levels of Rac1 and Cdc42 proteins in the cells appeared along with the phosphorylation of c-Jun (Fig. 5B) and the cleavage of PARP (Fig. 5, A and B). These data suggest that an increase of the expression of both proteins may be the cause to trigger the apoptotic cascade through the activation of JNK. The supplementation of GGPP and FPP prevented apoptosis in Raw cells induced by lovastatin while suppressing the expression of Rac1 and Cdc42 (Fig. 5C). To determine whether there was a causal relationship between JNK activation and macrophage apoptosis induced by lovastatin, we treated Raw cells with 10 \text{M} lovastatin in the presence or absence of 10 \text{M} SP600125, an inhibitor of JNK. Cell viability was measured by MTT assays. The presence of SP600125 prevented 60% cell death compared with lovastatin alone. This result suggests that JNK activity is responsible for lovastatin-induced apoptosis, although SP600125 was not as effective as GGPP and FPP in preventing cell death. Western blot analysis indicated that SP600125, GGPP, and FPP prevented apoptosis in Raw cells through suppressing c-Jun phosphorylation (Fig. 5D).

Discussion

Our findings demonstrate that lovastatin induces apoptosis in macrophages, correlating with enhanced expression of Rac1 and Cdc42 and activation of the JNK pathway. Although it has been

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**FIGURE 2.** Induction of caspase 3 activity by lovastatin. Raw cells were treated with 10 \text{M} lovastatin in the presence or absence of 5 \text{M} GGPP, 5 \text{M} FPP, 10 \text{M} caspase 3 inhibitor (Ac-DEVD-CHO), and 10 \text{M} caspase 9 inhibitor (LEHD-CHO) for 22 h. The activity of caspase 3 (A) and caspase 9 (B) was measured using Caspase-GloTM 3/7 and 9 assay kits (Promega). Experiments were performed two times in triplicates. Data are presented as mean ± SD.

**FIGURE 3.** Induction of DNA fragmentation in Raw cells by lovastatin. Raw cells were treated for 24 h with different concentration of lovastatin (A) or with 10 \text{M} lovastatin plus 5 \text{M} GGPP or FPP (B). The DNA fragments were purified using an apoptotic DNA ladder kit (Roche), and separated by 2% agarose gel electrophoresis and stained by ethidium bromide.
reported that statin therapy decreases inflammatory markers in blood and modulates the functions of immune cells, our results provide the first evidence that statins may reduce inflammation via elimination of immune cells. Apparently, the mevalonate pathway plays a pivotal role in macrophage survival because supplementation of GGPP or FPP can effectively rescue cells from lovastatin-induced apoptosis. Lovastatin also induces apoptosis in smooth muscle cells, endothelial cells, and plasma cells (38–40). In contrast to the observation by others that FPP is ineffective in preventing cells from apoptosis (38, 41), both GGPP and FPP were able to prevent lovastatin-induced macrophage apoptosis in our experiments. GGPP and FPP are the intermediates in the mevalonate pathway that leads to the synthesis of sterols and ubiquinone (42). It has been well established that GGPP and FPP are very important for the posttranslational modification of a variety of proteins, such as Ras and other GTPase family members. Small GTPases display their biological function through binding GTP, which leads to the interaction with specific molecular targets in the membrane, and subsequently initiates the signaling cascade that results in gene expression. Prenylation of the GTPases is a critical step for them to translocate into cellular membrane. As other groups noted, GGPP only partially restores the translocation of RhoA, but it can completely prevent lovastatin-induced apoptosis, suggesting that this event is unrelated to inhibition of membrane translocation of these GTPases (43, 44). Furthermore, inhibition of protein synthesis by cycloheximide, which does not have any effect on the prenylation of GTPases, efficiently blocks lovastatin-induced macrophage apoptosis. This finding suggests that the induction of cell apoptosis by lovastatin is not due to the depletion of isoprenoids, but that other pathways are involved.

The role of Rac1 and Cdc42 in cell apoptosis has been demonstrated, although the molecular mechanism remains elusive. Both Rac1 and Cdc42 can activate the JNK pathway, resulting in cell apoptosis (34, 35). Our data are consistent with these observations. In our experiments, lovastatin induced the expression of Rac1 and Cdc42 along with the phosphorylation of c-Jun and PARP cleavage (Fig. 5, A and B). GGPP and FPP prevent cells from death through suppressing the expression of Rac1 and Cdc42 (Fig. 5C). The basal level of Rac1 and Cdc42 in Raw cells is very low in comparison with Ras and RhoA. Interestingly, lovastatin selectively induces the expression of Rac1 and Cdc42, but not Ras (data not shown) and RhoA, in the cells and subsequently triggers the JNK pathway, resulting in cell apoptosis. It is conceivable that lovastatin may activate a specific pathway that is responsible for the expression of Rac1 and Cdc42 in macrophages. The specific pathway involved has not been determined, but is under investigation in our laboratory.

A major function of JNKs is to regulate the AP-1 transcription factor through phosphorylation of c-Jun and related proteins. JNKs play a dual role in apoptotic signaling. In some cell types, activation of JNK/AP-1 prevents cells death (45). In other cells, JNKs regulate the activities of pre-existing Bcl-2 family proteins that mediate mitochondrial release of cytochrome c, resulting in caspase activation (46). For example, UV-stimulated proteolytic activation of Bid, a proapoptotic BH3-only Bcl-2-related protein,
is suppressed in similarly treated JNK1/2−/− mouse embryonic fibroblasts. Furthermore, JNK activation failed to induce apoptosis in cells deficient in the proapoptotic proteins, Bax and Bak (47). The effect of lovastatin on the activity of Bcl-2 family members in macrophages is the subject of future investigations in our laboratory.

Statin therapy exerts beneficial pleiotropic effects on a wide range of cardiovascular disorders, including delaying the progression of atherosclerosis independent of serum cholesterol levels in patients. Our present findings provide a better understanding of the molecular basis underlying the clinical benefits of statin therapy in cardiovascular diseases and its role in anti-inflammation. Furthermore, these results could have implication in the design of novel therapeutic strategies for other inflammatory diseases, such as cancer, diabetes, and Alzheimer’s disease.

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Disclosures
The authors have no financial conflict of interest.

References

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