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Lovastatin Enhances Clearance of Apoptotic Cells (Efferocytosis) with Implications for Chronic Obstructive Pulmonary Disease

Konosuke Morimoto,* William J. Janssen,† Michael B. Fessler,† Kathleen A. McPhillips,‡ Valeria M. Borges,‡ Russell P. Bowler,‡ Yi-Qun Xiao,‡ Jennifer A. Kench,‡ Peter M. Henson,‡ and R. William Vandivier2*

Statins are potent, cholesterol-lowering agents with newly appreciated, broad anti-inflammatory properties, largely based upon their ability to block the prenylation of Rho GTPases, including RhoA. Because phagocytosis of apoptotic cells (efferocytosis) is a pivotal regulator of inflammation, which is inhibited by RhoA, we sought to determine whether statins enhanced efferocytosis. The effect of lovastatin on efferocytosis was investigated in primary human macrophages, in the murine lung, and in human alveolar macrophages taken from patients with chronic obstructive pulmonary disease. In this study, we show that lovastatin increased efferocytosis in vitro in an 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase-dependent manner. Lovastatin acted by inhibiting both geranylgeranylation and farnesylation, and not by altering expression of key uptake receptors or by increasing binding of apoptotic cells to phagocytes. Lovastatin appeared to exert its positive effect on efferocytosis by inhibiting RhoA, because it 1) decreased membrane localization of RhoA, to a greater extent than Rac-1, and 2) prevented impaired efferocytosis by lysosphatidic acid, a potent inducer of RhoA. Finally, lovastatin increased efferocytosis in the naive murine lung and ex vivo in chronic obstructive pulmonary disease alveolar macrophages in an HMG-CoA reductase-dependent manner. These findings indicate that statins enhance efferocytosis in vitro and in vivo, and suggest that they may play an important therapeutic role in diseases where efferocytosis is impaired and inflammation is dysregulated. The Journal of Immunology, 2006, 176: 7657–7665.

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3 Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; HMG-CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; FPP, farnesyl pyrophosphate; GPP, geranylgeranyl pyrophosphate; PL, phosphatidylycerine; LDL, low-density lipoprotein; LPA, lysosphatidic acid.
because it is defective in systemic lupus erythematosus, rheumatoid arthritis, cystic fibrosis, bronchiectasis, asthma, and chronic obstructive pulmonary disease (COPD) (31–37). The regulation of efferocytosis is tightly controlled by the Rho family GTPases, in that RhoA inhibits (38–40) and Rac-1/Cdc42/RhoG promotes (41–43) the process. Because statins robustly inhibit RhoA, a negative regulator of efferocytosis, we hypothesized that statins would enhance efferocytosis, and sought to determine whether statins might have therapeutic potential in COPD, a disease of impaired efferocytosis (31, 32).

Indeed, our data demonstrate that statins are potent inducers of efferocytosis in vitro and in vivo in an HMG CoA-reductase dependent manner. Statins appear to exert their effect, not by suppressing cholesterol, but by disproportionately suppressing the prenylation and membrane localization of RhoA. Finally, these data may have therapeutic implications for the treatment of chronic inflammatory diseases of the lung, because statins augment efferocytosis in the naïve murine lung and in alveolar macrophages obtained from patients with COPD.

Materials and Methods

Human subjects

The study was approved by, and performed in accordance with, the ethical standards of the institutional review board on human experimentation at National Jewish Medical and Research Center. Written informed consent was obtained from each subject.

Experimental animals

Mice were housed and studied under institutional animal care and use committee-approved protocols in the animal facility of National Jewish Medical and Research Center. Experiments were performed on 8- to 12-wk-old, age-matched, female ICR mice (Harlan Sprague Dawley).

Primary cell isolation and culture

Human monocyte-derived macrophages (HMDMs) and neutrophils were isolated and prepared from normal blood, as previously described (44). HMDMs were cultured in X-vivo medium (Cambrex BioScience) with 10% pooled human serum at 37°C in 10% CO2 for 7 days before use.

Human alveolar macrophages were isolated by bronchoalveolar lavage from patients with Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 2 COPD as described (31, 45). The right middle lobe was lavaged with 100 ml of 0.9% saline solution at room temperature. Human alveolar macrophages were resuspended in X-vivo medium (Cambrex BioScience) and were plated on baked glass cover slips in 24-well tissue culture plates (BD Biosciences) at 5.0 × 105 alveolar macrophages/well.

Murine thymocytes were isolated from the thymi of 3- to 4-wk-old, female ICR mice, by passing thymy through a 40-μm strainer (Falcon Scientific) to separate individual cells.

Cell lines and culture

The human Jurkat leukemia T cell line was obtained from the American Medical and Research Center. Experiments were performed on 8- to 12-wk-old, age-matched, female ICR mice (Harlan Sprague Dawley). The human Jurkat leukemia T cell line was obtained from the American Medical and Research Center. Experiments were performed on 8- to 12-wk-old, age-matched, female ICR mice (Harlan Sprague Dawley).

Induction of apoptosis

Apoptosis was induced in human neutrophils, Jurkat T cells, and murine thymocytes by exposure to UV irradiation at 312 nm (Fotodyne) for 10 min, as previously described (31). Neutrophils were cultured in RPMI 1640 with 0.5% endotoxin-free BSA (Sigma-Aldrich) at 37°C in 10% CO2 for 2.5 h before use. Jurkat T cells were cultured in RPMI 1640 with 10% FCS at 37°C in 10% CO2 for 2.5 h before use. Neutrophils and Jurkat T cells treated in this way were ~80% apoptotic by nuclear condensation. Thymocytes were cultured in RPMI 1640 with 10% FCS at 5 × 106 cells/ml at 37°C in 5% CO2 for 3.5 h before use. Neutrophils and Jurkat T cells treated in this way were ~80% apoptotic by nuclear condensation. Thymocytes were cultured in RPMI 1640 with 10% FCS at 5 × 106 cells/ml at 37°C in 5% CO2 for 3.5 h before use. Neutrophils and Jurkat T cells treated in this way were ~80% apoptotic by nuclear condensation.
polyclonal anti-human calreticulin IgY (American Diagnostica) Abs include mouse monoclonal anti-human α-chain CD91 IgG1, Mouse monoclonal anti-human PS recognition structure IgM (217) was prepared in this laboratory as previously described (27). Jackson ImmunoResearch Laboratories Abs include chicken IgY isotype control, Cy-3 goat IgG anti-mouse IgM, and Cy-3 goat IgG anti-mouse IgG, and Cy-3 goat anti-chicken IgY.

Western blotting

HMDM membrane fractions were prepared and separated on SDS-PAGE as described previously (51, 52). Briefly, HMDMs plated on 10-cm tissue-culture dish were harvested and resuspended in PBS. Pelleted cells were lysed by Reporter Lysis Buffer (Promega) and by repeated freeze-thaw cycles. Cell lysates were spun down at 3,000 rpm for 10 min. Supernatants were collected and spun-down at 10,000 × g for 45 min. The pellet was solubilized in lysate buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM DTT, 1% Triton X-100). Samples were run on a 7.5% SDS-PAGE gel, transferred to nitrocellulose, blocked with 3% milk, and sequentially incubated with primary and secondary Abs, Total protein was measured by the BCA Protein Assay kit (Pierce) and equivalent amounts were loaded into gels. Immunoblotting was performed using mAbs against RhoA, RhoB, and Rac-1 (clone 23A8, 1 μg/ml; Upstate Biotechnology) and Rac-1 (clone 23A8, 1 μg/ml; Upstate Biotechnology) and immunodetection was accomplished using a mouse anti-mouse HRP-conjugated secondary Ab (1/100,000). Membranes were developed using Amersham ECL system (Amersham Biosciences).

Statistics

The means were analyzed using ANOVA for multiple comparisons; when ANOVA indicated significance, the Dunnett’s method was used to compare groups with an internal control. For all other experiments in which two conditions were being compared, a Student’s t test assuming equal variance was used. All data were analyzed using JMP (version 3) Statistical Software for Maclntosh (SAS Institute) and are presented ± SEM.

Results

Lovastatin increases uptake of apoptotic cells (effectorcytosis)

We tested the effect of statins on effectorcytosis by incubating HMDMs with lovastatin for 6–48 h. Lovastatin (5 μM) increased effectorcytosis as early as 6 h (Fig. 1B). However, at 24 h, lovastatin increased effectorcytosis in a dose-dependent fashion, exerting a maximum effect at 10 μM. In contrast, lovastatin had no effect on binding of apoptotic cells to HMDMs (Fig. 1C). Lovastatin (1 μM) also increased effectorcytosis in HMDMs after 5 days of treatment compared with untreated cells, suggesting that the effect was prolonged (Fig. 1D).

The effect of lovastatin on effectorcytosis is dependent on HMG-CoA reductase

Most, but not all, statin effects are related to their ability to competitively block HMG-CoA reductase, and thereby decrease production of multiple intermediates in the cholesterol biosynthetic pathway. Mevalonate is the initial product of HMG-CoA reductase (Fig. 1A) and mevalonate levels are decreased by statin therapy. We performed “rescue” experiments with mevalonate to determine whether lovastatin was acting through an HMG-CoA reductase-dependent pathway (Fig. 1E). Mevalonate reversed the ability of lovastatin to potentiate effectorcytosis, confirming HMG-CoA reductase dependency. In contrast, lovastatin suppressed phagocytosis of IgG-opsonized erythrocytes through the FcγR, as has recently been shown (53, 54), and this also appeared to be dependent on HMG-CoA reductase (Fig. 1F).

Lovastatin does not affect expression of effectorcytosis receptors

We considered that lovastatin may exert its positive effect on effectorcytosis by increasing expression of key uptake receptors, especially because statins were recently reported to increase the expression of CD36, a well-known effectorcytosis receptor (55). Contrary to this report, we found that lovastatin had no effect on expression of HMDM effectorcytosis receptors (i.e., α1β2, β3, CD91, calreticulin, CD36, CD44, CD14) or the FcγRIIA (Fig. 2). Lovastatin also had no effect on staining by mAb 217. The protein target of mAb 217 was originally thought to be the phosphatidylserine receptor, but is now not known.

Lovastatin-enhanced effectorcytosis is dependent on prenylation

Statins regulate the posttranslational modification of hundreds of proteins by controlling the production of key substrates of protein prenylation, such as FPP and GGPP. Because mevalonate is an upstream precursor of both FPP and GGPP, statins ultimately decrease their levels as well. We performed dose-response rescue experiments with GGPP and FPP, and found that both reversed lovastatin-enhanced effectorcytosis, albeit with different potencies (Fig. 3, A and B). Even though these experiments suggested a role for both farnesylation and geranylgeranylation, they did not rule out the possibility that the effect of FPP was due to its conversion to GGPP and augmentation of geranylgeranylation. This is an important issue because the known Rho GT-Pase regulators of effectorcytosis are only geranylgeranylated. Incomplete inhibition of HMG-CoA reductase could potentially allow low level synthesis of the 5-carbon isopenoteryl pyrophosphate (IPP), which normally condenses with 15-carbon FPP to produce 20-carbon GGPP (Fig. 1A), thereby creating a pathway for FPP repletion to influence geranylgeranylation.

Specific inhibitors of geranylgeranyltransferase I (GGTI-2133; IC50 = 38 nM) and farnesyltransferase (FTI-276; IC50 = 0.5 nM) were used to further examine the role of these separate prenylation pathways on effectorcytosis. Both inhibitors enhanced effectorcytosis, but the effect of FTI-276 appeared to be more modest than GGTI-2133 (Fig. 3, C and D). It is important to note that farnesyltransferase inhibitors may increase the production of geranylgeranylated proteins, presumably by shunting excess FPP to GGPP (56). This suggests that farnesyltransferase inhibitors may not solely act by decreasing farnesylated proteins, but instead they may also change the balance in favor of geranylgeranylated proteins. Consistent with this possibility, combinations of GGTI-2133 and FTI-276 did not enhance effectorcytosis more thanLovastatin or GGTI-2133 alone (data not shown). Therefore, effectorcytosis appears to be regulated by intracellular signaling molecules that are geranylgeranylated, and possibly by those that are farnesylated.

Lovastatin suppresses membrane-bound RhoA greater than Rac-1

Statins inhibit prenylation and membrane localization of a variety of Rho GTPases, including positive (Rac-1) and negative (RhoA) regulators of effectorcytosis. Yet, in HMDMs, lovastatin consistently enhanced effectorcytosis, implying that lovastatin may exert a prolonged, disproportionate effect on the prenylation and membrane localization of RhoA. To address this hypothesis, HMDMs were treated with and without lovastatin for 24 h, and membrane fractions were assessed for RhoA and Rac-1 staining by Western blot. Lovastatin decreased membrane-bound RhoA greater than Rac-1 (Fig. 4, A and B), suggesting a mechanism for the positive effect ofLovastatin on effectorcytosis.

Lovastatin reverses impaired effectorcytosis by lysophosphatidic acid (LPA), a potent RhoA activator

We next used the potent RhoA activator, LPA, to address whetherLovastatin could reverse impaired effectorcytosis in vitro (57). LPA-suppressed effectorcytosis by HMDMs, and this suppression was prevented byLovastatin (Fig. 4C), suggesting thatLovastatin, or for that matter other Rho pathway inhibitors, could play a therapeutic
role in diseases where suppression of efferocytosis contributes to disease pathogenesis.

**Lovastatin increases efferocytosis in vivo**

We tested whether statins could enhance efferocytosis by treating mice with lovastatin (10 mg/kg) three times over 30 h. Ten million, Cell Tracker Red-labeled apoptotic murine thymocytes were then instilled intratracheally and clearance was assessed. This model has previously been used to evaluate efferocytosis by macrophages and epithelial cells in vivo (45, 50). Defective efferocytosis is suggested by either decreased uptake into alveolar macrophages (i.e., decreased PI; Fig. 5A), or by increased recovery of apoptotic cells in the bronchoalveolar lavage (Fig. 5B). Lovastatin modestly increased efferocytosis in the naive murine lung as measured by an increase in the alveolar macrophage PI (Fig. 5C) and by a decrease in the recovery of apoptotic thymocytes (Fig. 5D).

To examine whether the action ofLovastatin on efferocytosis in vivo was HMG-CoA reductase dependent, mice were treated with...
Lovastatin (10 mg/kg), three times over 30 h, in the presence or absence of rescue mevalonate, and clearance of apoptotic thymocytes by alveolar macrophages was assessed. Lovastatin again increased efferocytosis by alveolar macrophages in vivo, and this effect was prevented by mevalonate (Fig. 5E). Together, these results indicate that lovastatin enhances efferocytosis in the naive murine lung in an HMG-CoA reductase-dependent manner, thus confirming in vitro results.

**Lovastatin enhances efferocytosis by human alveolar macrophages taken from patients with COPD**

Accumulating evidence suggests that efferocytosis is dysregulated in chronic inflammatory lung diseases, such as COPD, and may contribute to disease pathogenesis. For example, several animal models of COPD are associated with increased accumulation (58–61) and impaired removal (45) of apoptotic cells. Likewise, apoptotic cells are increased in COPD lungs (31, 62–65) and efferocytosis is defective in COPD alveolar macrophages ex vivo (32). Therefore, we tested the effect of lovastatin on efferocytosis by alveolar macrophages isolated from GOLD stage 2 (66) COPD patients (Table I). Lovastatin enhanced efferocytosis in these alveolar macrophages in an HMG-CoA reductase-dependent fashion (Fig. 6). Taken together, these data suggest that statins may have therapeutic potential in diseases, such as COPD, where efferocytosis is suppressed and inflammation is dysregulated.

**Discussion**

Apoptotic cell clearance defects are increasingly recognized in diseases of chronic inflammation (31, 32) and autoimmunity (33, 34, 67, 68), suggesting that effective efferocytosis may be necessary for the maintenance of homeostasis. This concept is based on the role efferocytosis plays in suppressing both the innate (69, 70) and adaptive (68) immune response and in removing autoantigens (71). Interestingly, this concept is not new. Over 100 years ago, Metchnikoff (72) recognized this removal process and its importance to homeostasis, which he termed “physiologic inflammation.” Therefore, therapies that enhance efferocytosis may offer a unique therapeutic benefit, especially if impaired efferocytosis is a determinant of disease pathogenesis. Our findings indicate that lovastatin enhances efferocytosis in vitro and in vivo. Lovastatin’s effect on efferocytosis depends on its ability to inhibit HMG-CoA reductase, decrease prenylation substrates, and to alter the balance of Rho GTPases. Lovastatin may have therapeutic potential in chronic inflammatory diseases with impaired efferocytosis because it reverses impaired efferocytosis in vitro and in vivo, and enhances efferocytosis by alveolar macrophages from patients with COPD.

Up to 2% of expressed cellular proteins are prenylated and over 150 prenylated proteins have been identified (73, 74), suggesting that the effect of statins on efferocytosis is likely to be complex. Our findings indicate that lovastatin enhanced efferocytosis in vitro, in part, by altering the membrane balance of RhoA and Rac-1, two key regulators of efferocytosis. Geranylgeranlytransferase I prenylates both RhoA and Rac-1, yet lovastatin suppressed membrane localization of RhoA to a greater extent than Rac-1. The reason(s) for this disproportionate effect is unclear, but it suggests that enzyme kinetics favor prenylation of Rac-1 over RhoA. Because lovastatin increased efferocytosis after 5 days of treatment, the effect appears to be sustained. Possible explanations for this
Lovastatin may enhance efferocytosis by suppressing membrane-bound RhoA (A and B, HMDMs were treated with and without lovastatin for 24 h and assessed for membrane-bound RhoA and Rac-1 by Western blot and densitometry. A, Representative Western blots from five separate experiments are shown. CD71 was used as a membrane marker and control for equal loading. B, Lovastatin significantly decreased membrane-bound RhoA greater than Rac-1 (p < 0.05). C, LPA (10 μM), a potent RhoA activator, decreased ingestion of apoptotic Jurkat T cells by HMDMs in the absence, but not the presence, of lovastatin. Jurkat T cells were used in these experiments instead of neutrophils because they are ingested more avidly by HMDMs. The mean PI as a percentage of control ± SEM is shown for six replicates per group. Control mean PI: 42.2 ± 10.3. *, Significantly different from control (p < 0.05). †, Different from control (p = 0.051).

Lovastatin may enhance efferocytosis by suppressing oxidative stress, because oxidative stress inhibits efferocytosis in an inflammatory environment, lovastatin may enhance efferocytosis by altering the balance of membrane-bound RhoA and Rac-1, as was demonstrated in vitro. Alternatively, in vivo and especially in an inflammatory environment, lovastatin may enhance efferocytosis by suppressing oxidative stress, because oxidative stress inhibits efferocytosis by activating RhoA (K. A. McPhillips, manuscript in preparation). Lovastatin also suppresses matrix metalloproteinase-9 (8), which inhibits efferocytosis in vitro (R. W. Vandivier, unpublished data), and is an important component of lung inflammation. Finally, statins increase PPARγ, which has also been shown to increase efferocytosis (79). Lovastatin did not enhance efferocytosis by altering apoptotic cell binding or by increasing expression of efferocytosis receptors. In contrast, Ruiz-Velasco et al. (55) found that lovastatin treatment (10 μM) increased CD36 surface expression and mRNA in human monocytes at 24 h and THP-1 cells at 48 h. The disparity between our findings may relate to intrinsic differences between human monocytes and HMDMs. Lovastatin suppressed FcγR-mediated phagocytosis in an HMG-CoA reductase-dependent manner, confirming recent reports (53, 54). Interestingly, these authors all concluded that statins suppress FcγR-mediated phagocytosis by inhibiting cholesterol...
biosynthesis, and not by inhibiting prenylation (53, 54). Like effec-
torcytosis, prenylated proteins like Rac-1, Cdc42, and Rab11 are re-
quired for FcγR-mediated phagocytosis (80, 81), and a role for
RhoA has been suggested (82). Therefore, we propose that under
certain conditions statins might also influence FcγR phagocytosis
through modulation of Rho GTPases. The effect of statins on
FcγR-mediated phagocytosis is concerning because of its potential
to impair host defense, but ultimately, its importance remains to be
demonstrated in vivo. In a Klebsiella pneumoniae mouse model of
pneumonia, lovastatin delayed bacterial clearance and enhanced
dissemination (2). In contrast, statins have consistently improved
survival in animal and human bacterial sepsis (16, 22, 83), imply-
ing that the beneficial effects of statins may outweigh their poten-
tial deleterious effects.

The dose and concentration of lovastatin used in the in vitro and
in vivo experiments is certainly higher than is used clinically, sug-
gestig that lovastatin may not augment effectorcytosis when used in
humans. In mice, however, lovastatin induces a 6- to 10-fold in-
crease in hepatic microsomal HMG-CoA reductase after only 24 h,
implying that higher doses may be required to produce clinical
effects in mice compared with humans (48). In humans, prolonged
administration of lovastatin at 80 mg/kg/day results in steady state
concentrations ranging from 0.15 to 0.3 μM (84), which is mar-
ginally less than the lowest effective concentration of lovastatin
used in our study (1 μM). The effectiveness of lovastatin in vivo,
though, may depend on the clinical setting and remains to be de-
termined in COPD. For example, it is possible that prolonged lo-
qvastatin treatment at clinically relevant doses may augment effec-
torcytosis in humans with COPD, especially if RhoA activity is
increased.

The ability of statins to enhance effectorcytosis suggests a new
mechanism by which statins may modulate acute and chronic in-
flammatory diseases, and may help direct statin clinical trials to
specifie diseases. For example, cystic fibrosis, bronchiectasis, and
COPD are attractive targets for statin therapy because they are all
associated with accumulation, and defective clearance, of apo-
totic cells (31, 32). Indeed, our data provides “proof of princie” for
this approach because lovastatin increased effectorcytosis by human
alveolar macrophages taken from patients with COPD. Lee et al.
(19) have also shown that statin treatment inhibits the development
of cigarette smoke-induced emphysema in rats. However, we
would not suggest that potential therapeutic targets be limited to
these chronic inflammatory lung diseases, because accumulation
of apoptotic cells and failed effectorcytosis has also been implicated
in systemic inflammatory diseases, like glomerulonephritis (34),
rheumatoid arthritis (36), and systemic lupus erythematosus (35).

Disclosures

The authors have no financial conflict of interest.

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Table I. Subject demographics*

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<tr>
<td>Age (years)</td>
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<td>FVC (% predicted)</td>
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<td>FEV1/FVC (%)</td>
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* FEV1, Forced expiratory volume in 1 s; FVC, forced vital capacity.


