Original Paper

Atorvastatin and Simvastatin, but not Pravastatin, Up-regulate LPS-Induced MMP-9 Expression in Macrophages by Regulating Phosphorylation of ERK and CREB

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Key Words

CREB • ERK • Macrophage • LPS • MMP-9 • Statin

Abstract

Statins suppress expression of pro-inflammatory cytokines in endothelial cells, whereas they enhance it in immune cells. Pro-inflammatory cytokines and lipopolysaccharide (LPS) induce matrix metalloproteinase (MMP)-9 gene expression in macrophages, which has been linked to progress of various inflammatory diseases. The aim of this study was to identify effects of various statins on LPS-induced MMP-9 gene expression in macrophages and microglia. MMP-9 expression was analyzed by real-time PCR or zymography. Effect of statins on activation of signaling pathways was analyzed by time-dependent phosphorylation of signaling molecules. Atorvastatin and simvastatin, but not pravastatin, up-regulated LPS-induced MMP-9 expression in murine RAW 264.7 macrophages and BV2 microglia. The phosphorylation duration of extracellular signal regulated kinases was extended by simvastatin, but not by atorvastatin or pravastatin. The up-regulation of LPS-induced MMP-9 gene expression by the statins was dependent on extracellular calcium ions and mediated by enhancing phosphorylation of cAMP-responsive element binding protein. Geranylgeranyl pyrophosphate, a precursor for cholesterol synthesis, could suppress up-regulation of LPS-mediated MMP-9 gene expression by atorvastatin and simvastatin. Atorvastatin and simvastatin-mediated up-regulation of LPS-induced MMP-9 gene expression in macrophages and microglia *in vitro* raises an important concern about use of the widely-prescribed statins in certain inflammatory conditions that are mediated by LPS.

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Introduction

Matrix metalloproteinase (MMP)-9 may mediate beneficial tissue remodeling during stroke recovery [1]. However, dysregulated expression of MMP-9 in macrophages has been linked to various inflammatory diseases such as atherosclerosis and chronic obstructive pulmonary disease as well as stroke and post-infarct hemorrhagic transformation [2-6]. The expression level and activity of MMP-9 in macrophages can be up-regulated by lipopolysaccharide (LPS) and proinflammatory cytokines [7].

LPS interacts with Toll-like receptor 4 (TLR4) to function as an endotoxin, and phosphorylates I κ B kinases and mitogen activating protein (MAP) kinases, which induce expression of pro-inflammatory cytokines by activating nuclear factor kappa B (NF κ B) and activator protein-1 (AP-1) [8, 9]. Sequence analysis of the MMP-9 gene promoter region identified binding sites for AP-1 and NF κ B [10]. In addition, LPS has been reported to induce influx of extracellular calcium, which is linked to the activation of calcium ion-dependent transcription factors including nuclear factor of activated T cells or cAMP-responsive element binding protein (CREB) [11, 12].

Statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, decrease the incidence of cardiovascular diseases by lowering low-density lipoprotein and triglyceride levels [13]. In addition, statins exert vascular protective effects by inhibiting synthesis of isoprenoids including geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate, intermediate metabolites of cholesterol synthesis pathway [14]. Inhibition of GGPP-mediated post-translational prenylation of small GTPases has been reported to attenuate NADPH-mediated free radical production [15, 16], which modulates MMP-9 gene expression [17].

Although statins have been reported to show pleiotropic beneficial effects with anti-oxidant and immunomodulation capability [14, 15], all statins are associated with myopathy [18]; a disproportionately high incidence of myopathy occurs with high-dose simvastatin [19]. In addition, there is evidence that various statins display mixed metabolic effects ranging from beneficial to adverse effects on insulin resistance [20]. While statins have been reported to decrease expression of proinflammatory cytokines [21, 22] and MMP-9 [23] in endothelial cells, studies using immune cells, such as dendritic cells and macrophages, reported that statins increase expression of pro-inflammatory cytokines [24, 25].

We hypothesized that distinct statins, probably also depending on concentrations, might display differential effects on LPS-induced MMP-9 gene expression in macrophages. Our results demonstrate that various statins show distinct effects on LPS-induced MMP-9 gene expression in macrophages by modulating phosphorylation degrees of MAP kinases and CREB, which are involved in the LPS-mediated signaling pathways. We also investigated if GGPP could affect up-regulation of LPS-induced MMP-9 gene expression in macrophages by the statins.

Materials and Methods

Reagents

LPS from *E.coli* 0111:B4, atorvastatin, pravastatin, simvastatin, TSA, and actinomycin D were purchased from Sigma-Aldrich (St Louis, MO). Primary antibodies against p38, phosphorylated p38, ERK 1/2, phosphorylated ERK 1/2, JNK 1/2, phosphorylated JNK 1/2 which is also known as stress-activated protein kinases, phosphorylated CREB, and beta-tubulin were purchased from Cell Signaling Technology (Danvers, MA). FastStart PCR Master Mixes and phosphatase inhibitor cocktail were purchased from Roche Applied Sciences (Mannheim, Germany).

Cell culture and treatment

RAW 264.7 murine macrophage cells (purchased from ATCC) and BV2 murine microglia (a generous gift from HS Kim [26]) were maintained in phenol red free DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified incubator containing 5% CO₂. In all experiments, cells of less than 27 cell passage number were used. Lipophilic statins, atorvastatin and simvastatin, and TSA were dissolved in dimethylsulfoxide (DMSO) and diluted with cell culture medium just before the treatment. Final concentrations of DMSO in all cell culture experiments were adjusted to 0.05%. Hydrophilic pravastatin was dissolved in PBS.

Synthesis of cDNA and quantitative real-time polymerase chain reaction (PCR)

When cells in 12 well plates reached 70% confluency, cells were washed with PBS, incubated in serum-free DMEM, and treated with 0.1 µg/ml LPS from *E.coli* 0111:B4 and various concentrations of atorvastatin, pravastatin, or simvastatin for 8 h or 16 h, as indicated. Medium was collected for zymography assay, and total RNA was extracted with TRIsure (Bioline, London, GB) according to the manufacturer's instruction. Synthesis of cDNA was performed using Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Analysis of mRNA expression was determined with quantitative real-time PCR using FastStart PCR Master Mixes, and 10 pmole primers according to the manufacturer's instruction. The sequences of β -actin as a reference gene, MMP-9, and MMP-2 are as follows: β -actin (forward: 5'-CTAAGGCCAACCGTGAAAAG, reverse: 5'-ACCAGAGGCATACAGGGACA); MMP-9 (forward: 5'-AAACCAGACCCAGACTCCTC, reverse: 5'-GAGGACACAGTCTGACCTGAA); and MMP-2 (forward: 5'-TCTGGTGCTCCACCACATACAAC, reverse: 5'-CTGCATTGCCACCCATGGTAAACA). Abundance of MMP-9 mRNA in each sample was determined by the ΔC_t (cycle threshold), the difference between the C_t values for MMP-9 and β -actin. Relative ratios of MMP-9 mRNA expression levels were defined as $2^{-\Delta\Delta C_t}$ which reflects changes of MMP-9 expression levels from cells with treatment compared to those from unstimulated cells. All experiments were performed at least 3 times with duplicate samples.

Gelatin zymography

Aliquots of medium after 16 – 20 h culture were mixed with zymography sample buffer (Bio-Rad, CA, USA), incubated for 1 h at room temperature, and analyzed by SDS-PAGE with 10% gels containing 1mg/ml gelatin at 4 °C. Proteins in the gels were renatured by incubating for 30 min at 37 °C in 50 mM Tris-HCl/pH 7.4, 2.5% Triton X-100, 5 mM CaCl₂, and 1 µM ZnCl₂. The gels were incubated in 50 mM Tris-HCl/pH 7.4, 5 mM CaCl₂, and 1 µM ZnCl₂ overnight at 37 °C. The gels were then stained with 0.25% (w/v) Coomassie Brilliant Blue R250 solution in 20% methanol and 5% acetic acid, and destained in 20% methanol and 5% acetic acid.

Preparation of cell lysates and Western blotting analysis

Cells in serum-free DMEM were treated with statins and/or LPS for 40 min or otherwise indicated, lysed by adding high salt cell lysis buffer (20 mM Tris-HCl/pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml leupeptin, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 0.3 M NaCl, 0.5 mM phenylmethanesulfonyl fluoride) plus phosphatase inhibitor cocktail, and centrifuged at 12,000 x g for 5 min at 4°C. Total cell lysates separated by SDS-PAGE was processed for Western blot analyses using enhanced chemiluminescence detection kit.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical comparison of data was determined using one-way analysis of variance, followed by the Dunnett post-hoc adjustment. A value of $P < 0.05$ was considered significant.

Results

Atorvastatin and simvastatin up-regulate LPS-induced MMP-9 gene expression

Quantitative real-time PCR and zymography assays showed that LPS (0.1 µg/ml) induced gene expression of MMP-9 in RAW 264.7 macrophages. Treatment of cells with

Fig. 1. Atorvastatin and simvastatin up-regulate LPS-induced MMP-9 gene expression in a dose dependent manner. RAW 264.7 cells were treated with LPS (0.1 μg/ml) and/or various concentrations of atorvastatin, pravastatin, and simvastatin for 16 hrs. Final concentrations of DMSO in all cell culture treatments were adjusted to 0.05%. Zymography (A) of media was carried out as described in the Method section. Real-time RT-PCR of MMP-9 (B) was carried out with β-actin as an internal control. Ratios of MMP-9 mRNA to β-actin mRNA in the presence of LPS without statins were calculated as 1 in each set of experiments for statistical analysis. Results are presented as means ± SD; n (numbers of experiments performed) = 4. * $p < 0.01$ vs LPS only. ** $p < 0.001$ vs LPS only. Statistical comparison of data was determined using one-way ANOVA followed by the Dunnett posttest adjustment. Real-time RT-PCR of MMP-2 (C) was carried out with β-actin as an internal control as described above.

atorvastatin or simvastatin, unlike pravastatin treatment, significantly enhanced LPS-induced MMP-9 gene expression (Fig. 1A and B). Effects of vehicle (DMSO) on LPS-induced MMP-9 gene expression could be excluded, since final concentrations of DMSO in all cell culture experiments were adjusted to 0.05%. While simvastatin up-regulated LPS-induced MMP-9 gene expression approximately two-fold at 0.5 μM, atorvastatin did it at 5 μM. Zymography showed an enhanced expression of MMP-9 by atorvastatin or simvastatin at the protein level as well. Unlike MMP-9 gene expression, atorvastatin and simvastatin did not enhance MMP-2 mRNA expression (Fig. 1C). Up-regulation of LPS-induced MMP-9 gene expression by atorvastatin and simvastatin was also observed in microglia BV2 cells (Fig. 2). Effects of the statins on LPS-induced MMP-9 gene expression were less pronounced in BV2 microglia than in RAW 264.7 macrophages. Statins at the concentrations used in this study neither changed the cell viability nor proliferation rate of the cells (data not shown).

Fig. 2. Up-regulation of LPS-induced MMP-9 gene expression is also observed in BV 2 microglia. RAW 264.7 or BV 2 cells were treated with LPS and 5 μ M of atorvastatin, pravastatin, or simvastatin for 8 hrs. Ratios of MMP-9 mRNA to β -actin mRNA in the presence of LPS without statins were calculated as 1 in each set of experiments for statistical analysis. Results are presented as means $\pm$ SD; n (numbers of experiments performed) = 3. * $p < 0.05$ vs LPS only. ** $p < 0.01$ vs LPS only.

Fig. 3. Atorvastatin and simvastatin do not increase stability of MMP-9 mRNA. A, RAW 264.7 cells were incubated in media containing LPS and/or atorvastatin (A), pravastatin (P) or simvastatin (S) for given hours and subjected for RNA extraction. An amount of β -actin mRNA in each sample was used as a standard to determine relative abundance of MMP-9 mRNA. A ratio of MMP-9 expression to β -actin expression in the presence of LPS without statins for 3 hr incubation was calculated as 1 in each set of experiments for statistical analysis. Results are presented as means $\pm$ SD; n = 3 independent experiments. * $p < 0.01$ vs LPS only. B, Cells were incubated in media containing LPS with or without atorvastatin (AS) or simvastatin (SS) for 8 hrs before addition of actinomycin D (2 μ g/ml) and further incubated as indicated. Real-time RT-PCR of MMP-9 was carried out with β -actin as an internal control to determine relative abundance of MMP-9 mRNA. Ratios of MMP-9 mRNA to β -actin mRNA at time 0 h of actinomycin D were calculated as 1 in each set of experiments for statistical analysis. Results are presented as means $\pm$ SD; n = 3 independent experiments.

Atorvastatin and simvastatin do not increase stability of MMP-9 mRNA

An optimal time interval required for atorvastatin and simvastatin to initiate up-regulation of MMP-9 gene expression was determined to explore mechanisms by which atorvastatin and simvastatin enhanced LPS-mediated MMP-9 gene expression. Up-regulation of LPS-induced MMP-9 mRNA expression by atorvastatin and simvastatin became statistically significant at 5 h after the treatment, and increasingly pronounced with the lapse of time (Fig. 3A).

Fig. 4. Simvastatin extends duration of ERK phosphorylation. A, Cells in serum-free medium were stimulated with LPS (0.1 $\mu\text{g}/\text{ml}$) with or without 5 μM statins for 0.5, 1.5, or 3 hrs. Whole cell lysates separated on SDS-polyacrylamide gels were analyzed with P-p38, p38, P-ERK 1/2, ERK 1/2, P-JNK, or JNK. Representative results out of 3 independent experiments are shown. B, Quantitation of protein levels of P-p38, P-ERK, and P-JNK was calculated with those of non-phosphorylated p38, ERK, and JNK, respectively. Ratios of protein levels of interest to those of non-phosphorylated forms obtained from cells incubated in the presence of LPS for 0.5 h were calculated as 1 in each set of experiments for statistical analysis. Results are shown as means $\pm$ SD; n = 3 independent experiments. * $p < 0.05$ vs LPS only.

We determined if atorvastatin or simvastatin changed stability of MMP-9 mRNA based on requirement of incubation of cells with statins for several hours for the up-regulation of LPS-induced MMP-9 gene expression by statins. Decay rates of the existing MMP-9 mRNA, after the LPS-induced expression had been modulated by statins, were analyzed by incubating the cells for 0 - 12 h with actinomycin D (2 $\mu\text{g}/\text{ml}$) to prevent the synthesis of a new RNA. Decay rates of MMP-9 mRNA in the presence of simvastatin or atorvastatin (slopes of the top and middle lines, respectively, in Fig. 3B) were not lower but slightly higher than in the absence of the drugs (bottom line). These results indicate that atorvastatin or simvastatin

Fig. 5. Up-regulation of LPS-induced MMP-9 gene expression is dependent on calcium ion. **A**, Cells were treated with LPS and 5 μM of atorvastatin (AS), pravastatin (PS), or simvastatin (SS) in regular medium (+ calcium) or calcium free medium (- calcium) and subjected to RNA extraction. A ratio of MMP-9 to β-actin mRNA in the presence of LPS without statins in regular medium was calculated as 1. * $p < 0.01$ vs LPS only. ** $p < 0.001$ vs LPS only. **B**, Total cell extracts prepared from cells incubated with LPS and statins (5 μM) for 4 hrs were analyzed by Western blot assay. **(C)** Quantitation of protein levels of P-CREB was calculated with beta-tubulin on the same gels as an internal control. Ratios of protein levels of P-CREB to beta-tubulin obtained from cells incubated in the presence of LPS for 4 h were calculated as 1 in each set of experiments for statistical analysis. Results are shown as means ± SD; $n = 3$ independent experiments. * $p < 0.01$ vs LPS only.

did not decrease, but rather slightly increased the decay rate of MMP-9 mRNA. In summary, atorvastatin and simvastatin do not up-regulate LPS-mediated MMP-9 gene expression by stabilizing MMP-9 mRNA.

Simvastatin extends phosphorylation duration of extracellular signal kinases (ERK)

Treatment of RAW 264.7 cells with LPS resulted in phosphorylation and activation of the MAP kinases p38, ERK1/2, and c-Jun N-terminal kinases (JNK), which play a key role in activation of AP-1 family transcription factors to induce MMP-9 gene expression [9]. Co-treatment with atorvastatin or simvastatin at various concentrations did not enhance the initial phosphorylation degree of p38, ERK1/2, or JNK at 30 min (data not shown). We, therefore, analyzed whether statins could regulate activities of the kinases by changing duration of phosphorylation. Effects of the statins on phosphorylation degree of MAP kinases were analyzed from 0.5 to 3 h after treatment with LPS and statins. The amounts of phosphorylated ERK from cells treated with LPS gradually decreased with the lapse of time from 0.5 to 3 h (Fig. 4). Atorvastatin and pravastatin showed little effect on phosphorylation

Fig. 6. Addition of GGPP or TSA suppresses the up-regulation of LPS-induced MMP-9 gene expression by atorvastatin or simvastatin. **A**, Cells were incubated in media containing 0.1 μ g/ml LPS and with or without 5 μ M atorvastatin (AS) or simvastatin (SS). GGPP (final concentration 15 μ M) was added to cell culture wells as indicated. A ratio of MMP-9 to β -actin mRNA in the presence of LPS without statins and GGPP was calculated as 1 and used as a standard to compare statin effects with or without GGPP. Results are presented as means $\pm$ SD; $n = 3$ independent experiments. * $p < 0.01$ vs LPS only. ** $p < 0.001$ vs LPS only. **B**, Cells were treated with LPS and with or without 5 μ M atorvastatin (AS) or simvastatin (SS). TSA was added to cell culture wells as indicated. A ratio of MMP-9 to β -actin mRNA in the presence of LPS without statins and TSA was calculated as 1 and used as a standard to compare statin treatment groups in experiments with or without TSA. Results are presented as means $\pm$ SD; $n = 3$ independent experiments. * $p < 0.01$.

degree of ERK induced by LPS. However, the amount of phosphorylated ERK did not significantly decrease at 1.5 h and slightly decreased at 3 h after the treatment with LPS and simvastatin (Fig. 4).

In contrast to the phosphorylation degree of ERK, phosphorylation degrees of JNK as well as p38 were not changed by atorvastatin or simvastatin (Fig. 4B). In addition, treatment with the statins did not change the amounts of I κ B (Fig. 4B), indicating that atorvastatin and simvastatin do not enhance LPS-induced MMP-9 gene expression by modulating NF κ B activity.

Up-regulation of LPS-induced MMP-9 gene expression by atorvastatin or simvastatin is dependent on extracellular calcium ion, and mediated by phosphorylation of CREB

Based on the correlation between induction of influx of extracellular calcium ion by LPS and gene expression of proinflammatory cytokines [12, 27], we analyzed if up-regulation of LPS-induced MMP-9 gene expression by atorvastatin or simvastatin was dependent on calcium ions in the media. LPS could induce MMP-9 gene expression regardless of the presence of calcium ions in the media, although the expression level was much lower in the media without calcium ions (Fig. 5A). However, up-regulation of LPS-induced MMP-9 expression by atorvastatin or simvastatin was not observed in the calcium ion-free media (inlet figure of Fig. 5A).

Given the reports that phosphorylation of CREB at residue 133, which is triggered by calcium ion or cAMP [11], induces expression of MMP-9 [28], we determined whether atorvastatin or simvastatin modulates phosphorylation degree of CREB. For accurate quantitation, intensities of phosphorylated CREB (MW 43 kD) were compared with those of β -tubulin (MW 55 kD) on the same gels. In line with the report [29], LPS induced

phosphorylation of CREB (Fig. 5B), which was further enhanced by 5 μ M atorvastatin or simvastatin, but not by 5 μ M pravastatin (Fig. 5B and C).

GGPP suppresses the up-regulation of LPS-mediated MMP-9 gene expression by atorvastatin and simvastatin

Statins inhibit the conversion of HMG-Co A to mevalonate that acts not only as a precursor for cholesterol synthesis but also as an intermediate metabolite for protein prenylation. The addition of 15 μ M GGPP, an intermediate metabolite of the cholesterol synthesis pathway, enhanced LPS-induced MMP-9 mRNA expression approximately two-fold (Fig. 6A). Contrary to the control group, where GGPP was not added, atorvastatin and simvastatin did not further up-regulate LPS-induced MMP-9 gene expression when 15 μ M GGPP was added. Up-regulation of LPS-induced MMP-9 gene expression by 20 μ M atorvastatin, which was similar to the induction fold by 5 μ M simvastatin, was also suppressed with the addition of 15 μ M GGPP (data not shown).

HDAC inhibitor down-regulates LPS-induced MMP-9 gene expression

Recent studies have reported that histone deacetylase (HDAC) inhibitor, trichostatin A (TSA), suppresses LPS-induced gene expression of interleukin-1, tumor necrosis factor α , and nitric oxide synthase in macrophages [30, 31]. Thus, we investigated if TSA could also suppress the statin-mediated up-regulation of LPS-induced MMP-9 gene expression. Treatment of cells with TSA suppressed LPS-induced MMP-9 gene expression in the absence or presence of the statins (Fig. 6B). However, the ratios of LPS-induced MMP-9 mRNA expression in the presence of atorvastatin or simvastatin to those in the absence of statins did not change with TSA treatment.

Discussion

To our knowledge, this is the first study to report that atorvastatin and simvastatin, but not pravastatin, up-regulated LPS-induced MMP-9 gene expression in RAW 264.7 macrophages and BV2 microglia.

The half maximal inhibitory concentration (IC_{50}) of atorvastatin, pravastatin, and simvastatin to inhibit HMG-CoA reductase are 5, 23, and 95 nM, respectively [32, 33]. Differences in IC_{50} values of the statins to inhibit HMG-CoA reductase may not explain distinct effects of the statins on LPS-induced MMP-9 gene expression. First, less than 5 fold difference of IC_{50} values between pravastatin (23 nM) and simvastatin (95 nM) cannot explain their different effects on LPS-induced MMP-9 gene expression observed in this study. Second, while the concentration of atorvastatin required to up-regulate LPS-induced MMP-9 gene expression (5 μ M) is approximately thousand-fold higher than its IC_{50} (5 nM), that of simvastatin (0.5 μ M) is approximately five-fold higher than its IC_{50} (95 nM).

Thus, we considered other potential molecular mechanisms to explain the up-regulation of LPS-induced MMP-9 gene expression by atorvastatin or simvastatin: increasing MMP-9 mRNA stability, and enhancing activity of transcription factors involved in MMP-9 mRNA expression by changing phosphorylation degree of CREB and/or the kinases involved in activation of the AP-1 family transcription factors.

MMP-9 mRNA stability

It has been reported that nitric oxide suppressed the expression of HuR, an mRNA stabilizing factor interacting with the AU-rich elements in the 3' untranslated region, and increased the decay rate of MMP-9 mRNA in glomerular mesangial cells [34]. In the present study, atorvastatin and simvastatin did not decrease but slightly increased the decay rate

of MMP-9 mRNA, indicating that these drugs did not up-regulate LPS-induced MMP-9 gene expression by increasing MMP-9 mRNA stability.

ERK phosphorylation

A number of plant extracts, such as epigallocatechin gallate from green tea and curcumin from curry, have been reported to suppress NF κ B and/or AP-1 by changing phosphorylation efficiency of I κ B and/or kinases in the MAP kinase signaling pathway [35, 36]. Although the statins did not alter initial phosphorylation efficiency of the MAP kinases involved in activation of AP-1 family transcription factors, simvastatin increased duration of phosphorylation of ERK. The effect of atorvastatin on the extended duration of ERK phosphorylation was less pronounced. Pravastatin changed neither the degree of the initial phosphorylation nor the duration of phosphorylation of the kinase. This suggests that up-regulation of LPS-induced MMP-9 gene expression by simvastatin may be in part due to the extended time of ERK phosphorylation.

Extracellular calcium and CREB phosphorylation

Calcium ion overload due to enhanced influx of extracellular calcium ion has been reported to activate inflammatory signaling pathways [37] and cause ischemic brain damage that could be attenuated by inhibiting calcium influx [38, 39]. We showed that extracellular calcium ions were required for maximum induction of MMP-9 gene expression by LPS, which is in line with the study using neutrophils demonstrating that cAMP/Ca²⁺ pathway is involved in LPS-induced MMP-9 secretion [40]. We also showed that extracellular calcium ions were necessary for up-regulation of LPS-induced MMP-9 gene expression by atorvastatin and simvastatin. In the following experiments, which were performed after consideration of the reports that phosphorylation and activation of CREB were mediated by calcium ions, we demonstrated that atorvastatin and simvastatin changed phosphorylation efficiency of CREB, linking the CREB-related pathways to the statin-mediated up-regulation of LPS-induced MMP-9 gene expression. In conclusion, simvastatin enhanced phosphorylation degree of CREB and ERK, whereas atorvastatin enhanced that of CREB, which would explain the more pronounced effect on LPS-induced MMP-9 gene expression by simvastatin.

Lipophilic atorvastatin and simvastatin intercalate into the upper hydrocarbon core of the lipid bilayer, while hydrophilic pravastatin interacts with the polar surface of the membrane [41]. Further studies are required to see if a lipophilicity-related differential modulation of membrane calcium channels by atorvastatin and simvastatin vs. pravastatin could affect LPS-induced influx of extracellular calcium ion and consequently CREB phosphorylation, followed by differential expression of MMP-9 gene.

Suppression of statin-mediated up-regulation of LPS-induced MMP-9 expression

Trying to reverse the effect of atorvastatin and simvastatin, we investigated the effect of GGPP, which is a precursor for cholesterol synthesis and an intermediate metabolite for protein prenylation. Atorvastatin and simvastatin failed to up-regulate LPS-induced MMP-9 gene expression after the addition of GGPP. Isoprenoids produced from mevalonate, such as GGPP or farnesyl pyrophosphate, initiate post-translational prenylation of Rac and Ras homologue (RHO) which enables small GTPase proteins to associate with membrane for biological function [15]. Inhibition of translocation of Rac-1 GTPase results in attenuation of NADPH-mediated free radical production. Although inhibition of prenylation of proteins, especially small GTPases, has been reported to play a role in most pleiotropic effects of statins [16], it appears to result in up-regulation of LPS-induced MMP-9 gene expression by simvastatin.

Biochemical and molecular analyses of MMP-9 gene expression in HeLa cells using chromatin immunoprecipitation and transient transfection assays demonstrated that

Fig. 7. A schematic diagram explains mechanisms by which atorvastatin and simvastatin up-regulate LPS-induced MMP-9 gene expression. An italic letter *p* represents phosphorylation. Interaction of LPS with the CD14/toll-like receptor 4 (TLR4) complexes on the cell surface does not only stimulate phosphorylation of the MAP kinases but also induce influx of calcium ions into cells. Atorvastatin enhances the phosphorylation degree of CREB, whereas simvastatin enhances the phosphorylation degree of both ERK and CREB.

HDAC-1 and HDAC-3, but not HDAC-2 and HDAC-4, participate in transcriptional regulation of MMP-9 [42]. TSA belongs to a class I HDAC inhibitor that blocks the activity of HDAC-1, HDAC-2, HDAC-3, and HDAC-8 [43]. Our result showing that treatment of cells with TSA reduced LPS-induced MMP-9 gene expression is in line with the report that TSA suppresses gene expression of inflammatory cytokines [30, 31, 44]. However, atorvastatin and simvastatin could up-regulate LPS-induced MMP-9 gene expression in the presence of TSA, demonstrating that the effects by atorvastatin and simvastatin do not result from regulating activity of HDAC-1 or -3.

Conclusion

We report on atorvastatin- and simvastatin-mediated up-regulation of LPS-induced MMP-9 expression in macrophages and microglia, reveal their mechanisms linked to the LPS – phosphorylation of ERK/CREB – MMP-9 signaling pathway, and introduce GGPP and TSA as inhibitors of the off-target effects of the statins (Fig. 6 and 7). Considering the clinical implications of the widely-prescribed statins as well as the importance of proper regulation of MMP-9 expression in the pathologic vs. physiologic remodeling [45], our study merits further investigation in clinical settings as well as in animal models, particularly in certain inflammatory conditions that are mediated by LPS.

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References

- 1 Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, Wang X, Lo EH: Role of matrix metalloproteinases in delayed cortical responses after stroke. *Nat Med* 2006;12:441-445.
- 2 Lim CS, Shalhoub J, Gohel MS, Shepherd AC, Davies AH: Matrix metalloproteinases in vascular disease--a potential therapeutic target? *Curr Vasc Pharmacol* 2010;8:75-85.

- 3 Kim DE, Kim JY, Schellingerhout D, Kim EJ, Kim HK, Lee S, Kim K, Kwon IC, Shon SM, Jeong SW, Im SH, Lee DK, Lee MM, Kim GE: Protease imaging of human atheromata captures molecular information of atherosclerosis, complementing anatomic imaging. *Arterioscler Thromb Vasc Biol* 2010;30:449-456.
- 4 Shon SM, Park JH, Nahrendorf M, Schellingerhout D, Kim JY, Kang BT, Jeong SW, Kim EJ, Ryu JH, Kim K, Kwon IC, Lee DK, Lee MM, Kim DE: Exercise attenuates matrix metalloproteinase activity in preexisting atherosclerotic plaque. *Atherosclerosis* 2011;216:67-73.
- 5 Foronjy R, Nkyimbeng T, Wallace A, Thankachen J, Okada Y, Lemaitre V, D'Armiento J: Transgenic expression of matrix metalloproteinase-9 causes adult-onset emphysema in mice associated with the loss of alveolar elastin. *Am J Physiol Lung Cell Mol Physiol* 2008;294:L1149-1157.
- 6 Del Zoppo GJ, Frankowski H, Gu YH, Osada T, Kanazawa M, Milner R, Wang X, Hosomi N, Mabuchi T, Koziol JA: Microglial cell activation is a source of metalloproteinase generation during hemorrhagic transformation. *J Cereb Blood Flow Metab* 2012.
- 7 Rouis M: Matrix metalloproteinases: A potential therapeutic target in atherosclerosis. *Curr Drug Targets Cardiovasc Haematol Disord* 2005;5:541-548.
- 8 Mackman N, Brand K, Edgington TS: Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in thp-1 monocytic cells requires both activator protein 1 and nuclear factor kappa b binding sites. *J Exp Med* 1991;174:1517-1526.
- 9 Lin CC, Tseng HW, Hsieh HL, Lee CW, Wu CY, Cheng CY, Yang CM: Tumor necrosis factor-alpha induces mmp-9 expression via p42/p44 mapk, jnk, and nuclear factor-kappa b in a549 cells. *Toxicol Appl Pharmacol* 2008;229:386-398.
- 10 Masure S, Nys G, Fiten P, Van Damme J, Opdenakker G: Mouse gelatinase b. Cdna cloning, regulation of expression and glycosylation in wehi-3 macrophages and gene organisation. *Eur J Biochem* 1993;218:129-141.
- 11 Illario M, Giardino-Torchia ML, Sankar U, Ribar TJ, Galgani M, Vitiello L, Masci AM, Bertani FR, Ciaglia E, Astone D, Maulucci G, Cavallo A, Vitale M, Cimini V, Pastore L, Means AR, Rossi G, Racioppi L: Calmodulin-dependent kinase iv links toll-like receptor 4 signaling with survival pathway of activated dendritic cells. *Blood* 2008;111:723-731.
- 12 Zanoni I, Ostuni R, Capuano G, Collini M, Caccia M, Ronchi AE, Rocchetti M, Mingozzi F, Foti M, Chirico G, Costa B, Zaza A, Ricciardi-Castagnoli P, Granucci F: Cd14 regulates the dendritic cell life cycle after lps exposure through nfat activation. *Nature* 2009;460:264-268.
- 13 LaRosa JC, He J, Vupputuri S: Effect of statins on risk of coronary disease: A meta-analysis of randomized controlled trials. *JAMA* 1999;282:2340-2346.
- 14 Bu DX, Griffin G, Lichtman AH: Mechanisms for the anti-inflammatory effects of statins. *Curr Opin Lipidol* 2011;22:165-170.
- 15 Greenwood J, Steinman L, Zamvil SS: Statin therapy and autoimmune disease: From protein prenylation to immunomodulation. *Nat Rev Immunol* 2006;6:358-370.
- 16 Biasucci LM, Biasillo G, Stefanelli A: Inflammatory markers, cholesterol and statins: Pathophysiological role and clinical importance. *Clin Chem Lab Med* 2010;48:1685-1691.
- 17 Gargiulo S, Sottero B, Gamba P, Chiarpotto E, Poli G, Leonarduzzi G: Plaque oxysterols induce unbalanced up-regulation of matrix metalloproteinase-9 in macrophagic cells through redox-sensitive signaling pathways: Implications regarding the vulnerability of atherosclerotic lesions. *Free Radic Biol Med* 2011;51:844-855.
- 18 Thompson PD, Clarkson P, Karas RH: Statin-associated myopathy. *JAMA* 2003;289:1681-1690.
- 19 Egan A, Colman E: Weighing the benefits of high-dose simvastatin against the risk of myopathy. *N Engl J Med* 2011;365:285-287.
- 20 Koh KK, Sakuma I, Quon MJ: Differential metabolic effects of distinct statins. *Atherosclerosis* 2011;215:1-8.
- 21 Anderson TJ, Meredith IT, Yeung AC, Frei B, Selwyn AP, Ganz P: The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion. *N Engl J Med* 1995;332:488-493.
- 22 Zeuke S, Ulmer AJ, Kusumoto S, Katus HA, Heine H: Tlr4-mediated inflammatory activation of human coronary artery endothelial cells by lps. *Cardiovasc Res* 2002;56:126-134.
- 23 Massaro M, Zampolli A, Scoditti E, Carluccio MA, Storelli C, Distanto A, De Caterina R: Statins inhibit cyclooxygenase-2 and matrix metalloproteinase-9 in human endothelial cells: Anti-angiogenic actions possibly contributing to plaque stability. *Cardiovasc Res* 2010;86:311-320.

- 24 Monick MM, Powers LS, Butler NS, Hunninghake GW: Inhibition of rho family gtpases results in increased tnf-alpha production after lipopolysaccharide exposure. *J Immunol* 2003;171:2625-2630.
- 25 Konat GW, Krasowska-Zoladek A, Kraszpuski M: Statins enhance toll-like receptor 4-mediated cytokine gene expression in astrocytes: Implication of rho proteins in negative feedback regulation. *J Neurosci Res* 2008;86:603-609.
- 26 Lee EJ, Kim HS: Inhibitory mechanism of mmp-9 gene expression by ethyl pyruvate in lipopolysaccharide-stimulated bv2 microglial cells. *Neurosci Lett* 2011;493:38-43.
- 27 Zhou X, Yang W, Li J: Ca²⁺- and protein kinase c-dependent signaling pathway for nuclear factor-kappab activation, inducible nitric-oxide synthase expression, and tumor necrosis factor-alpha production in lipopolysaccharide-stimulated rat peritoneal macrophages. *J Biol Chem* 2006;281:31337-31347.
- 28 Park JK, Park SH, So K, Bae IH, Yoo YD, Um HD: Icam-3 enhances the migratory and invasive potential of human non-small cell lung cancer cells by inducing mmp-2 and mmp-9 via akt and creb. *Int J Oncol* 2010;36:181-192.
- 29 Caivano M, Cohen P: Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and il-1 beta in raw264 macrophages. *J Immunol* 2000;164:3018-3025.
- 30 Adcock IM: Hdac inhibitors as anti-inflammatory agents. *Br J Pharmacol* 2007;150:829-831.
- 31 Yu Z, Zhang W, Kone BC: Histone deacetylases augment cytokine induction of the inos gene. *J Am Soc Nephrol* 2002;13:2009-2017.
- 32 Cohen LH, van Vliet A, Roodenburg L, Jansen LM, Griffioen M: Pravastatin inhibited the cholesterol synthesis in human hepatoma cell line hep g2 less than simvastatin and lovastatin, which is reflected in the upregulation of 3-hydroxy-3-methylglutaryl coenzyme a reductase and squalene synthase. *Biochem Pharmacol* 1993;45:2203-2208.
- 33 Gerber R, Ryan JD, Clark DS: Cell-based screen of hmg-coa reductase inhibitors and expression regulators using lc-ms. *Anal Biochem* 2004;329:28-34.
- 34 Akool el S, Kleinert H, Hamada FM, Abdelwahab MH, Forstermann U, Pfeilschifter J, Eberhardt W: Nitric oxide increases the decay of matrix metalloproteinase 9 mrna by inhibiting the expression of mrna-stabilizing factor hur. *Mol Cell Biol* 2003;23:4901-4916.
- 35 Hosokawa Y, Hosokawa I, Ozaki K, Nakanishi T, Nakae H, Matsuo T: Catechins inhibit cxcl10 production from oncostatin m-stimulated human gingival fibroblasts. *J Nutr Biochem* 2009;21:659-664.
- 36 Bachmeier B, Nerlich AG, Iancu CM, Cilli M, Schleicher E, Vene R, Dell'Eva R, Jochum M, Albini A, Pfeffer U: The chemopreventive polyphenol curcumin prevents hematogenous breast cancer metastases in immunodeficient mice. *Cell Physiol Biochem* 2007;19:137-152.
- 37 Coultrap SJ, Vest RS, Ashpole NM, Hudmon A, Bayer KU: Camkii in cerebral ischemia. *Acta Pharmacol Sin* 2011;32:861-872.
- 38 Domercq M, Perez-Samartin A, Aparicio D, Alberdi E, Pampliega O, Matute C: P2x7 receptors mediate ischemic damage to oligodendrocytes. *Glia* 2010;58:730-740.
- 39 Davidson JO, Green CR, Nicholson LE, O'Carroll SJ, Fraser M, Bennet L, Gunn AJ: Connexin hemichannel blockade improves outcomes in a model of fetal ischemia. *Ann Neurol* 2012;71:121-132.
- 40 Ernens I, Rouy D, Velot E, Devaux Y, Wagner DR: Adenosine inhibits matrix metalloproteinase-9 secretion by neutrophils: Implication of a2a receptor and camp/pka/ca²⁺ pathway. *Circ Res* 2006;99:590-597.
- 41 Mason RP, Walter ME, Day CA, Jacob RF: Intermolecular differences of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors contribute to distinct pharmacologic and pleiotropic actions. *Am J Cardiol* 2005;96:11F-23F.
- 42 Ma Z, Shah RC, Chang MJ, Benveniste EN: Coordination of cell signaling, chromatin remodeling, histone modifications, and regulator recruitment in human matrix metalloproteinase 9 gene transcription. *Mol Cell Biol* 2004;24:5496-5509.
- 43 Dokmanovic M, Clarke C, Marks PA: Histone deacetylase inhibitors: Overview and perspectives. *Mol Cancer Res* 2007;5:981-989.
- 44 Leoni F, Fossati G, Lewis EC, Lee JK, Porro G, Pagani P, Modena D, Moras ML, Pozzi P, Reznikov LL, Siegmund B, Fantuzzi G, Dinarello CA, Mascagni P: The histone deacetylase inhibitor itf2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. *Mol Med* 2005;11:1-15.
- 45 Rosell A, Lo EH: Multiphasic roles for matrix metalloproteinases after stroke. *Curr Opin Pharmacol* 2008;8:82-89.