

# Statins Inhibit Secretion of Metalloproteinases-1, -2, -3, and -9 From Vascular Smooth Muscle Cells and Macrophages

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**Objective**—Production of several metalloproteinases (MMPs) from smooth muscle cells (SMCs) and macrophages causes matrix destruction and atherosclerotic plaque instability. Statins, which inhibit HMG-CoA reductase and hence cholesterol and isoprenoid synthesis, stabilize plaques. We investigated whether statins inhibit MMP secretion from SMCs and macrophages.

**Methods and Results**—We used human saphenous vein and rabbit aortic SMC and foamy macrophages from cholesterol-fed rabbits. Cerivastatin (50 nmol/L) inhibited inducible MMP-1, -3, and -9 secretion from human SMC by  $52 \pm 19\%$ ,  $71 \pm 18\%$ , and  $73 \pm 17\%$ , respectively ( $P < 0.01$ ,  $n = 3$ ). Similar dose-related effects of cerivastatin (50 to 500 nmol/L), simvastatin (1 to 20  $\mu\text{mol/L}$ ), and lovastatin (5 to 20  $\mu\text{mol/L}$ ) were consistent with their relative potencies against HMG-CoA reductase. Statins also inhibited inducible MMP-1, -3, and -9 and constitutive MMP-2 secretion but not TIMP-1 or -2 secretion from rabbit SMC. Statins also dose-dependently inhibited MMP-1, -3, and -9 secretion from rabbit foam cells; cerivastatin (50 nmol/L) inhibited by  $68 \pm 18\%$ ,  $74 \pm 14\%$ , and  $74 \pm 14\%$ , respectively ( $P < 0.01$ ,  $n = 4$ ). Statins similarly decreased collagenolytic, caseinolytic, and gelatinolytic activity. Mevalonate and geranylgeranylpyrophosphate but not squalene reversed the effects, showing dependence on isoprenoid, not cholesterol depletion. Statins did not affect MMP mRNA levels.

**Conclusions**—Statins inhibit secretion of a several MMPs from both SMCs and macrophages, which could therefore contribute to their plaque-stabilizing effects. (*Arterioscler Thromb Vasc Biol.* 2003;23:769-775.)

**Key Words:** statins ■ metalloproteinases ■ atherosclerosis ■ plaque instability ■ isoprenoids

Matrix metalloproteinases (MMPs) play a major role in atherosclerosis, restenosis after angioplasty, and vein-graft stenosis by remodelling the extracellular matrix.<sup>1,2</sup> Matrix remodelling by MMPs liberates the vascular smooth muscle cells (VSMCs) from their pericellular matrix cage and permits migration during responses to injury.<sup>3-6</sup> Overexpression of MMPs, including MMP-1, MMP-3, and MMP-9, has been demonstrated in human and animal atherosclerotic plaques,<sup>7-16</sup> where it is colocalized with morphological and mechanical determinants of plaque rupture. MMPs together can catalyze the complete destruction of interstitial collagen,<sup>17</sup> which is the main component of fibrous caps responsible for their tensile strength. Loss of collagen leads to structural weakness and less resistance to the mechanical stresses imposed during systole.<sup>18</sup> This results ultimately in plaque rupture, the key event in triggering coronary thrombosis and hence acute coronary syndromes such as unstable angina and myocardial infarction.<sup>19</sup>

Expression of MMPs-1, -3, and -9 is upregulated in cells present in atheromas, including endothelial cells,<sup>20</sup> VSMCs,<sup>21-25</sup> and macrophages.<sup>26-29</sup> Inflammatory mediators, including interleukin-1 (IL-1), CD-40 ligand, and tumor necrosis factor- $\alpha$ , upregulate MMP activity in vascular cells,

especially in combination with platelet-derived growth factor (PDGF) or basic fibroblast growth factor.<sup>23,25</sup> Tissue inhibitors of metalloproteinases (TIMPs) are a family of naturally occurring specific inhibitors of MMPs whose activity in atherosclerotic plaques seems to correlate with decreased MMP activity<sup>30,31</sup> and hence reduced matrix remodelling.

Statins are a structurally related group of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that are widely used to treat hyperlipidemia. Their use is associated with significant reduction of adverse coronary events, including myocardial infarction, and a marginal regression of plaque size.<sup>32,33</sup> Furthermore, recent studies, both in vitro and in vivo, have suggested that the beneficial effects of statins may extend to mechanisms beyond cholesterol reduction.<sup>33-36</sup> These pleiotropic effects of statins are mediated by their ability to block the synthesis of isoprenoid intermediates, which serve as lipid attachments for a variety of intracellular signaling molecules, especially Rho-family small GTP-binding proteins, whose proper membrane localization and function are dependent on isoprenylation.<sup>34,35,37</sup> The pleiotropic effects of statins include improving or restoring endothelial function, inhibiting the proliferation and migration of SMCs, decreasing vascular inflammation, and, importantly,

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enhancing the stability of atherosclerotic plaques.<sup>34,35</sup> Recently, studies demonstrated that statins reduced MMP-9 secretion by macrophages and MMP-1 secretion from vascular endothelial cells.<sup>14,38–40</sup> If these effects were more general to other MMP family members and other plaque resident cells, they might have an important role in plaque stabilization. We therefore investigated whether statins modulate MMP-1, -2, -3, and -9 expression in cultured rabbit and human VSMCs and foam cell macrophages elicited in cholesterol-fed rabbits.

## Methods

### Reagents

Sheep polyclonal anti-rabbit MMP-1 and MMP-3 antibodies were a generous gift from Dr G. Murphy, University of East Anglia, Norwich, UK. Mouse anti-human MMP-1 antibody was purchased from Chemicon International. Sheep anti-human MMP-3 antibody was purchased from the Binding Site. Human recombinant IL-1- $\alpha$  and human recombinant PDGFBB were purchased from R&D System. Cerivastatin was from Bayer, UK, Ltd, and simvastatin was from Merck Research Laboratories. All other reagents were purchased from Sigma Chemical Company unless otherwise stated.

### Tissue Culture

Primary cultures of human saphenous vein and rabbit aortic smooth muscle cells were prepared by modifications of the explant technique, as previously described in detail.<sup>21</sup> Explants were maintained in complete medium composed of DMEM containing penicillin-streptomycin (100 U/mL and 100  $\mu$ g/mL, respectively), 8 mmol/L L-glutamine, and 15% FBS (Advanced Protein Products). After 10 to 14 days, cells were subcultured by trypsin/EDTA treatment. Cells between passages 1 through 3 were plated at a density of  $2 \times 10^5$  cells/well into 6-well culture plates for zymography and Western blotting or  $1 \times 10^6$  cells/75 cm<sup>2</sup> flasks for RNA studies. For all experiments, subconfluent cells were rendered quiescent by incubation in serum-free DMEM supplemented with 0.25% (vol/vol) lactalbumin hydrolysate (Gibco BRL) for 3 days. Cultures were then exposed to fresh serum-free medium containing the appropriate concentration of the agent under investigation for 48 hours.

Rabbit experimental foam cells were isolated from subcutaneous granulomas of cholesterol-fed New Zealand White rabbits, as previously described.<sup>26,29</sup> Briefly, rabbits began a 1% cholesterol diet 2 weeks before implantation of 2 to 6 polyurethane sponges (Baxter Scientific) under the dorsal skin. Sponges remained in place for 4 to 5 weeks to allow macrophage accumulation while the animal remained on a 1% cholesterol diet throughout. The recovered sponges were gently squeezed over sterile test tubes, and the exudates were layered onto a discontinuous metrizamide gradient (bottom cushion 10 mL of 10% metrizamide [wt/vol], top 3 to 4 mL cell suspension) and centrifuged at 1200g for 15 minutes at 10°C. Foam cells were recovered from the floating layer and washed 3 times, and aliquots were prepared for oil red O staining to confirm lipid content and immunocytochemistry by using the rabbit macrophage-specific marker RAM 11. One rabbit yielded  $\approx 2 \times 10^7$  foam cells. Cells were placed at a density of  $5 \times 10^5$  cells/well into 24-well plates, nonadherent cells were discarded after 45 minutes, and the adherent foam cells were then exposed to fresh macrophage serum-free medium (2 g/L bicarbonate-buffered RPMI 1640 media supplemented with 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mmol/L L-glutamine, 0.25% [vol/vol] lactalbumin) containing the appropriate concentration of the agent under investigation for 48 hours.

### Zymography for MMP-9 and Reverse Zymography for TIMP Activity

MMP-9 activity was detected in conditioned media, as previously described.<sup>21</sup> Briefly, 15- $\mu$ L aliquots of conditioned media diluted 1:1

with nonreducing Laemmli sample buffer (2 $\times$ ) were electrophoresed at 4°C in 7.5% SDS-polyacrylamide gels containing 2 mg/mL gelatin derived from calf skin collagen. For reverse zymography, culture supernatants from rabbit SMCs were concentrated 5-fold. Aliquots (40  $\mu$ L) of nonreduced media were electrophoresed at 4°C in 12% SDS-polyacrylamide gels containing 0.5 mg/mL gelatin and 10% baby hamster kidney cell, serum-free, conditioned media as a source of gelatinase. In either case after electrophoresis, SDS was removed and gelatinase activity was revealed by overnight incubation at 37°C and staining with 0.1% Coomassie Brilliant Blue. Zymograms were quantified in the linear range by densitometry with a GS 690 Image Analysis software system (Bio-Rad).

### Western Blotting for MMP-1 and MMP-3

Western blotting was performed on conditioned media samples concentrated 10-fold by ultrafiltration using Amicon 10 centrifugal concentrators (Amicon, Stonehouse). Samples were separated by SDS-PAGE and blotted onto a Hybond-nitrocellulose membrane (Amersham) with the use of a semidry blotting apparatus. Blocking of nonspecific binding and dilutions of the primary (40  $\mu$ g/mL) anti-MMP1 or anti-MMP3 and secondary antibodies (1:2000, DAKO) used 5% skimmed milk powder/Tris-buffered saline/0.2% Tween 20. Protein was visualized using an enhanced chemiluminescence system (Amersham). Bands were quantified by densitometry.

### Collagenolytic, $\beta$ -Caseinolytic, and Gelatinolytic Activity Assays

Freshly isolated culture supernatants were assayed for collagenolytic,  $\beta$ -caseinolytic, and gelatinolytic activity on the basis of the cleavage of fluorescently labeled substrates by using the Type I Collagenase Assay Kit, Stromelysin Activity Assay Kit, and Type IV Collagenase Assay Kit (Yagai Corp), respectively, according to the manufacturer's instructions.

### Semiquantitative Analysis by Reverse Transcriptase-Polymerase Chain Reaction

Total cellular RNA was prepared from  $2 \times 10^6$  rabbit aortic VSMCs using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Procedures for reverse transcription (RT)-polymerase chain reaction (PCR) and the primers used to measure rabbit MMP-1, MMP-3, MMP-9, TIMP-1, TIMP-2, and GAPDH mRNA levels have been described in our previous work.<sup>29</sup>

### Cell Viability and Proliferation Studies

After harvesting conditioned media, viable cell numbers were assessed by the 3-(4,5-dimethylthiazol-2-yl)-2, -5-diphenyltetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's instruction. Apoptosis was assayed by using Cell Death Detection Elisa<sup>plus</sup> (Roche), a photometric enzyme immunoassay for the quantitative determination of cytoplasmic histone-associated DNA fragments.

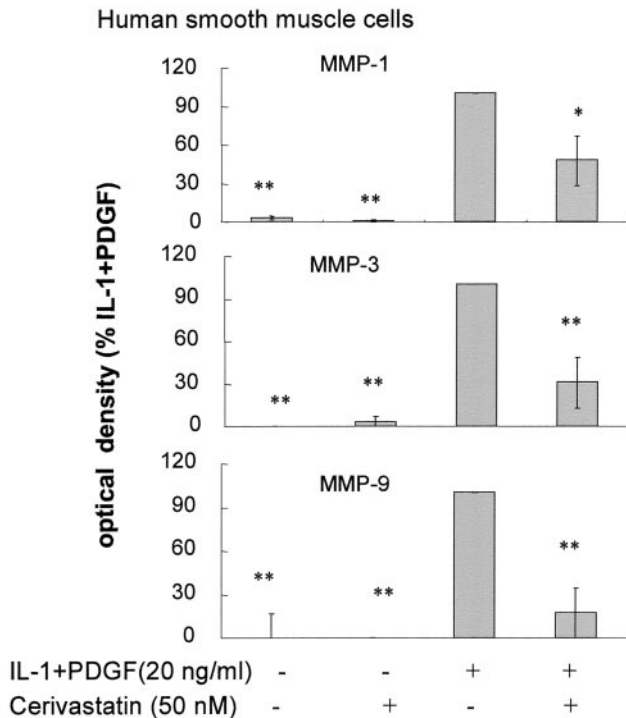
### Statistical Analysis

Each experiment was performed at least 3 times. Data are presented as mean  $\pm$  SEM and analyzed using the Student's *t* test using Bonferroni correction for multiple comparisons.  $P < 0.05$  was considered statistically significant.

## Results

### Statins Inhibit MMP-1, -3, and -9 Production From Human VSMCs

As previously detailed,<sup>25</sup> secretion of MMP-1, -3, and -9 from human VSMC was increased from undetectable levels by a combination of IL- $\alpha$  and PDGFBB (Figure 1A). Secretion of all 3 MMPs was decreased  $52 \pm 19\%$ ,  $71 \pm 18\%$ , and  $73 \pm 17\%$ , respectively ( $P < 0.01$ ,  $n = 3$ ) by 50 nmol/L cerivastatin, a plasma concentration previously shown to be associated with cholesterol-lowering effects in vivo.<sup>41,42</sup> Lov-



**Figure 1.** Cerivastatin inhibited production of MMP-1, -3, and -9 by human VSMCs. Cerivastatin (50 nmol/L) had no effect on MMP-1, -3, and -9 production in unstimulated human VSMCs but inhibited production in response to IL-1 $\alpha$  (20 ng/mL) and PDGF<sub>BB</sub> (20 ng/mL) over 48 hours. Values are mean  $\pm$  SEM of 3 separate observations. \* $P$ <0.05, \*\* $P$ <0.01 compared with cells stimulated with IL-1 $\alpha$  and PDGF<sub>BB</sub> alone.

astatin at a concentration of 5  $\mu$ mol/L also inhibited IL- $\alpha$  and PDGF<sub>BB</sub>-stimulated MMP-1, -3, and -9 secretion by 94 $\pm$ 5%, 78 $\pm$ 24%, and 74 $\pm$ 9.8%, respectively ( $P$ <0.01,  $n$ =3) (Figure I, available online at <http://atvb.ahajournals.org>), consistent with the relative potencies of cerivastatin and lovastatin as HMGCoA reductase inhibitors.<sup>43</sup> Interestingly, cerivastatin (not shown) and lovastatin (Figure I) also decreased constitutive secretion of MMP-2 by 57 $\pm$ 2.3%.

### Lovastatin Decreases MMP-1, -2, -3, and -9 But Not TIMP-1 or -2 Secretion From Rabbit VSMCs

Lovastatin also concentration-dependently decreased MMP-1, -3, and -9 secretion induced by IL- $\alpha$  and PDGF<sub>BB</sub> and constitutive MMP-2 secretion in rabbit SMC (Figure 2A), which demonstrates that the effect is not species-specific. In contrast, it had no effect on constitutive TIMP-1 or -2 secretion (Figure 2B), which implies that statin shifted the MMP/TIMP balance toward inhibition by TIMPs.

Using rabbit VSMCs, we investigated whether decreased MMPs secretion was mediated by decreases in MMP mRNA levels by semiquantitative RT-PCR. Consistent with our previous work,<sup>23,25</sup> mRNA levels of MMP-1, MMP-3, and MMP-9 were upregulated by combination of IL-1 with PDGF<sub>BB</sub> (Figure II, available online at <http://atvb.ahajournals.org>), but MMP-2 was constitutive (not shown). TIMP-1 and TIMP-2 mRNA levels were also constitutive. Lovastatin had no significant effect on mRNA levels of MMPs or TIMPs (Figure II).

### Statins Decrease MMP-1, -3, and -9 Secretion From Rabbit Foam Cell Macrophages

In agreement with previous work,<sup>26,29</sup> rabbit macrophage foam cells expressed MMP-1, MMP-3, and MMP-9 without exogenous stimuli (Figure 3); secretion of MMP-2 was much lower and could not be quantified (results not shown). Cerivastatin concentration-dependently inhibited spontaneous MMP-1, -3, and -9 production from rabbit foam cell macrophages (Figure 3); cerivastatin (50 nmol/L) inhibited by 68 $\pm$ 18%, 74 $\pm$ 14%, and 74 $\pm$ 14%, respectively ( $P$ <0.01,  $n$ =4). Maximal inhibition was also observed with simvastatin at concentrations of 1  $\mu$ mol/L and greater (Figure IIIA, available online at <http://atvb.ahajournals.org>) and concentration-dependently by lovastatin greater than 1  $\mu$ mol/L (Figure IIIB). Lovastatin (10  $\mu$ mol/L) inhibited MMP-1, MMP-3, and MMP-9 secretion by 79 $\pm$ 14%, 80 $\pm$ 10%, and 66 $\pm$ 17%, respectively ( $P$ <0.01,  $n$ =3) but, similarly to VSMC, had no effect on mRNA levels for MMP-1, -3, or -9 levels by semiquantitative PCR (results not shown).

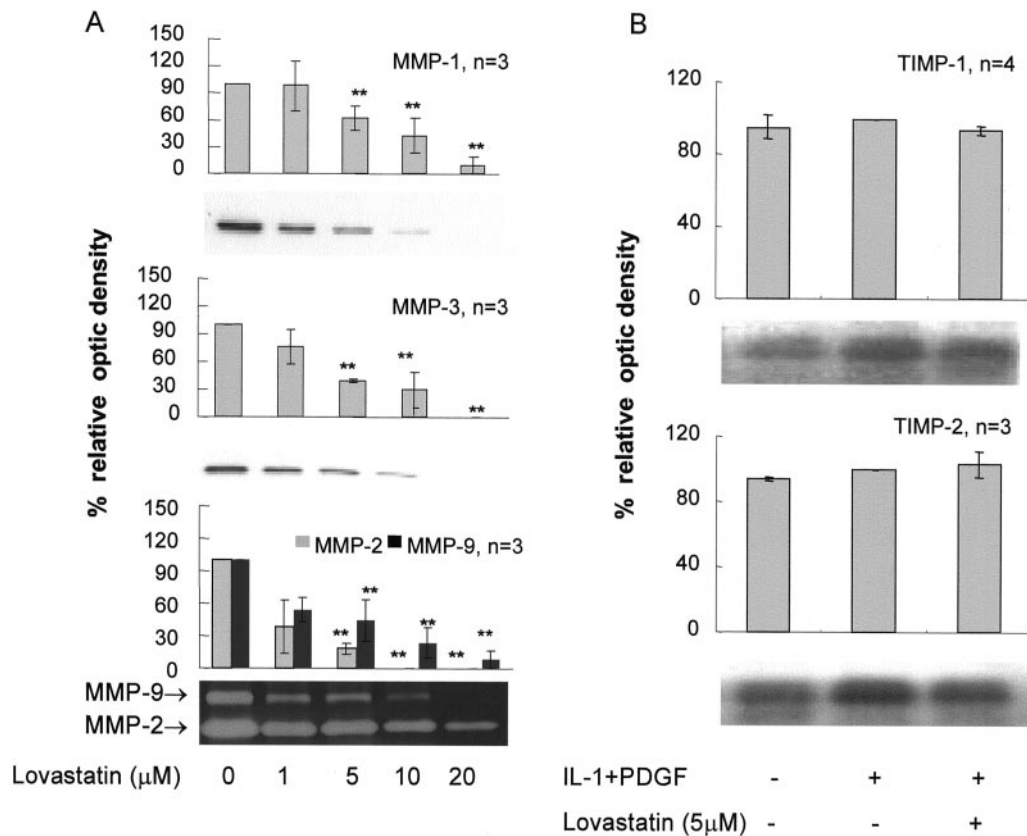
As in rabbit SMCs, viable cell numbers were measured by MTT assay. Neither cerivastatin nor simvastatin had any effect on MTT activity (not shown), but lovastatin inhibited MTT metabolism at a concentration of 1 to 20  $\mu$ mol/L (see Figure IV, available online at <http://atvb.ahajournals.org>). However, this effect on MTT did not seem to be mediated by loss of cell numbers, because total protein levels were not systematically affected (not shown). Furthermore, when we used the Cell Death Detection Elisa<sup>plus</sup> assay as a sensitive method to detect death of foam cells, lovastatin did not lead to cell death even at a concentration of 20  $\mu$ mol/L ( $P$ >0.05,  $n$ =5) (Figure IVB). To correct for any variation in cell numbers, the loading volumes of samples shown in Figure IIIB were normalized according to total protein. When these data were additionally normalized to MTT activity (solid bars in Figure IIIB), 10 and 20  $\mu$ mol/L lovastatin still significantly reduced MMP-1, -3, and -9 secretion, which demonstrates that loss of cell viability could not be the main cause of this inhibition.

### Effects of Mevalonate and Isoprenoids on Action of Lovastatin in Rabbit VSMCs and Foam Cells

Incubation of cells with HMG-CoA reductase inhibitors causes mevalonate starvation. Mevalonate metabolism yields a series of isoprenoids, including the cholesterol precursor, squalene, and geranylgeranyl-pyrophosphate (GGPP), an important lipid attachment for the posttranslational modification of Rho protein.<sup>37</sup> To test by which pathway statins inhibit MMPs, we attempted to rescue MMP secretion from rabbit VSMCs with mevalonate, squalene, or GGPP in the presence of lovastatin. Addition of squalene did not reduce the inhibitory effect of lovastatin on MMP-1, -2, -3, and -9 secretion (Figure 4). In contrast, the addition of mevalonate or GGPP completely reversed the effects of lovastatin on MMP-1 and -3 and partially reversed the effect on MMP-2 and -9 secretion (Figure 4).

Similarly to SMCs, we incubated rabbit foam cells with mevalonate, squalene, or GGPP in the presence of lovastatin. The addition of squalene slightly reversed the effect of





**Figure 2.** Dose-dependent inhibition of MMP-1, -2, -3, and -9 but not TIMP-1 and -2 production from rabbit VSMC by lovastatin. Quiescent RVSMCs were incubated with IL-1 $\alpha$  (20 ng/mL), PDGF<sub>BB</sub> (20 ng/mL), and differing concentrations of lovastatin for 48 hours. A, MMP-1 and -3 were measured in conditioned media by Western blotting, and MMP-2 and -9 by gelatin zymography and related to the production in the absence of lovastatin. B, TIMP-1 and -2 were measured in conditioned media by reverse zymography. Values are mean  $\pm$  SEM of the number of separate observations shown. \*\* $P < 0.01$  vs absence of lovastatin.

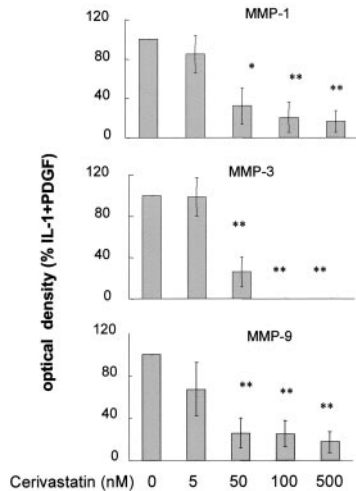
lovastatin on MMP-1 secretion but had no effects on MMP-3 or -9 (Figure 5). The addition of mevalonate (100  $\mu$ mol/L) completely reversed lovastatin effects on MMP-3 and MMP-9 and reversed the effect on MMP-1 by  $92 \pm 2\%$  ( $P < 0.01$ ,  $n = 3$ ). The addition of GGPP completely reversed MMP-1, -3, and -9 secretion (Figure 5).

### Effects of Lovastatin on MMP Activity in Conditioned Media From Rabbit VSMCs and Foam Cells

Using fluorescently labeled substrates, collagenolytic,  $\beta$ -caseinolytic, and gelatinolytic activities could not be detected in rabbit SMC-conditioned media either in the absence or presence of IL-1 and PDGF<sub>BB</sub> (data not shown). Hence, we could not evaluate any effect of lovastatin. However, consistent with our previous work,<sup>29</sup> conditioned media from rabbit granuloma foam cells contained measurable proteolytic activity against fluorescently labeled type I collagen,  $\beta$ -casein, and gelatin substrates, which correspond to activities of MMP-1 through -3 and -9, respectively. Each activity was strongly inhibited by lovastatin, and the inhibition was completely reversed by mevalonate but not squalene (Figure 6). GGPP completely reversed collagenolytic and gelatinolytic activity and partially reversed  $\beta$ -caseinolytic activity by  $81 \pm 10\%$  ( $P < 0.05$ ,  $n = 3$ ).

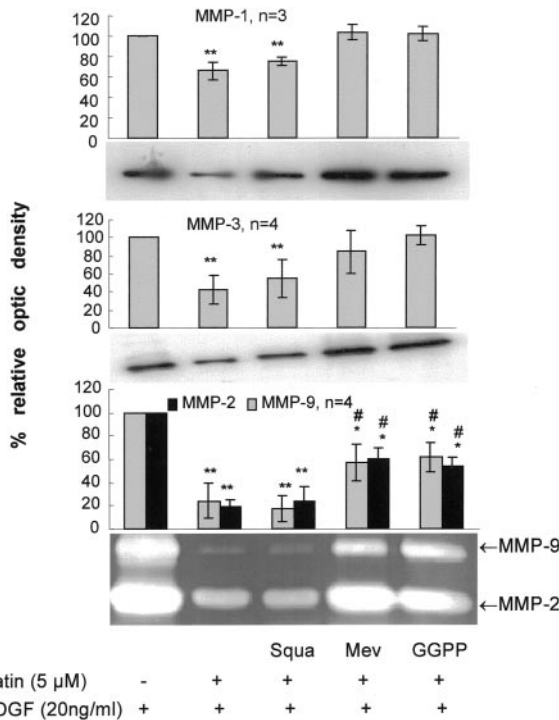
### Discussion

In this study, we demonstrated for the first time that incubation of rabbit and human VSMCs with several statins in vitro reduced the secretion of MMP-1, -2, -3, and -9, but not that of TIMP-1 and -2, by a mechanism that involved isoprenylation and was posttranscriptional. In rabbit foam cells, in which activation of MMP-1, -3, and -9 secretion was activated in vivo, subsequent in vitro incubation with statins also effectively inhibited MMP-1, -3, and -9 secretion and activity by a prenylation-dependent mechanism. The effects of cerivastatin were obtained at 50 nmol/L and above, within the range of concentrations obtained in humans with the highest clinically used dose (0.8 mg/d).<sup>41</sup> They are also in the range of concentrations found to be effective in previous studies in rabbits.<sup>42,44</sup> The dose-related effects of the other statins were obtained at 100-fold higher concentrations, consistent with their known potencies against HMG-CoA reductase and the higher clinical doses 10 to 80 mg/d.<sup>43,45</sup> The concentrations of statins used in our experiments were consistent with those found to cause other non-lipid-lowering effects.<sup>35,46</sup> Furthermore, we established directly that mevalonate starvation is the basis for our observed effects on MMP expression as it is for the cholesterol lowering of statins. Hence, although all of our experiments were conducted in vitro, we believe that they could be of relevance to the action of statins in vivo.

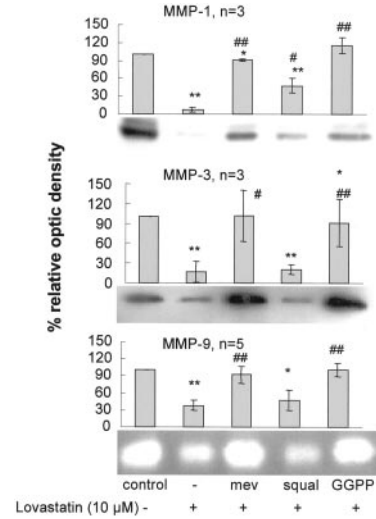


**Figure 3.** Cerivastatin inhibited production of MMP-1, -3, and -9 by rabbit foam cells. Cerivastatin (5 to 500 nmol/L) inhibited production of MMP-1, -3, and -9 by foam cells over 48 hours in a dose-dependent manner. Values are mean±SEM of 3 separate observations. \**P*<0.05, \*\**P*<0.01 compared with control cells.

MMPs are expressed by both SMCs and macrophage foam cells in human atherosclerotic plaques, as demonstrated by immunocytochemistry and in situ hybridization.<sup>7,9–16,47</sup> Our activity measurements showed that part of MMP-1, -3, and -9

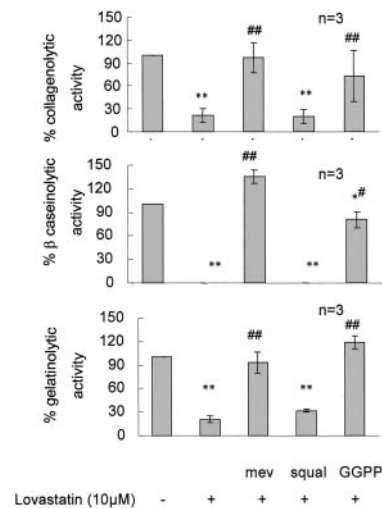


**Figure 4.** Reversal by mevalonate and isoprenoids of the effects of lovastatin on MMP-1, -2, -3, and -9 expression in rabbit VSMCs. Quiescent rabbit VSMCs were incubated with 100 μmol/L mevalonate, 10 μmol/L squalene, or 10 μmol/L GGPP in the presence of IL-1 $\alpha$ , PDGF<sub>BB</sub> (20 ng/mL), and lovastatin (5 μmol/L) for 48 hours. Production of MMP-1, -2, -3, and -9 inhibited by lovastatin was rescued by mevalonate and GGPP but not by squalene. Values are mean±SEM of the number of separate observations shown. \**P*<0.05, \*\**P*<0.01 vs absence of lovastatin. #*P*<0.05 vs inhibited with lovastatin alone.



**Figure 5.** Reversal by mevalonate and isoprenoids of the effect of lovastatin on MMP-1, -3, and -9 production in rabbit foam cells. Rabbit foam cells were incubated with 100 μmol/L mevalonate, 10 μmol/L squalene, or 15 μmol/L GGPP in the presence of 10 μmol/L lovastatin for 48 hours. Inhibition of MMP-1 through -3 and -9 production was reversed by mevalonate (mev) and GGPP but not squalene (squal). Values are mean±SEM of the number of separate observations shown. \**P*<0.05, \*\**P*<0.01 vs absence of lovastatin; #*P*<0.05, ##*P*<0.01 vs lovastatin alone.

secreted by foam cells was in an active form, consistent with in situ zymography data on human and rabbit atherosclerotic plaques.<sup>31,48</sup> In contrast, MMP-1, -2, -3, and -9 secreted from isolated SMC cultures in vitro were either in a latent form or there was an excess of TIMPs so that no proteolytic activity could be detected. Presumably, in atherosclerotic plaques, MMP proenzymes secreted from SMCs can become activated, either through the action of oxidative species<sup>49</sup> or



**Figure 6.** Reversal by mevalonate and isoprenoids of the effect of lovastatin on metalloproteinase activity in rabbit foam cells. Collagenolytic, β-caseinolytic, and gelatinolytic activity, which corresponded to MMP-1, -3, and -9 activity, respectively, were measured using fluorescently labeled substrates. Values are mean±SEM of the number of separate observations shown. \**P*<0.05, \*\**P*<0.01 vs absence of lovastatin, #*P*<0.05, ##*P*<0.01 vs lovastatin alone.

MMPs secreted from macrophages, plasmin, or other serine proteases, for example from mast cells.<sup>50</sup>

Statins decreased secretion of a broad spectrum of MMPs from both SMC and foamy macrophages, which implies a beneficial effect on plaque stability. Indeed, statins have been shown previously to reduce MMP protein expression and activities when administered to hyperlipidemic rabbits,<sup>13,14</sup> and this is accompanied by change in plaque morphology consistent with increased stability.<sup>13,14,51</sup> Moreover, the plaque-stabilizing effects of statins in animal models can apparently be obtained even independently of cholesterol lowering,<sup>36,52</sup> which implies a direct effect on mechanisms leading to plaque instability. However, in previous *in vivo* studies,<sup>13,14,51,52</sup> reduction of macrophage foam cell numbers and MMP activity occurred together, and, except in the case of MMP-9, an effect on MMP secretion per se was not demonstrated. Our experiments conducted *ex vivo* on foam cells produced *in vivo* demonstrate that there is indeed a direct effect of statins on MMP-1 and -3 production from macrophages as well as confirming the reported effect on MMP-9.<sup>14,38</sup> By contrast, statins did not affect the production of TIMP-1 and -2, which potentially inhibit all of the MMP studies here and implies that statins shifted the MMP/TIMP balance toward inactive enzymes. Consistent with this, we showed directly in foam cells that lovastatin decreased the collagenolytic,  $\beta$ -caseinolytic, and gelatinolytic activities, which are predominantly associated with MMP-1, -3, and -9, respectively.

Investigating the mechanism underlying inhibition of MMP secretion, we first showed rescue of MMP-1, -3, and -9 secretion by mevalonate, consistent with bypass of the blockade of HMG-CoA reductase. The potency series cerivastatin >> simvastatin > lovastatin is also consistent with their known potencies against the enzyme.<sup>43,45</sup> Treatment with lovastatin may cause mevalonate starvation inside VSMCs and foam cells. Mevalonate metabolism yields squalene, the precursor of cholesterol and GGPP, which is important in prenylation of proteins. For example, translocation of Rho GTPase family members from the cytoplasm to the plasma membrane is dependent on geranylgeranylation.<sup>35</sup> Rescue of MMP secretion by GGPP implies that inhibition of prenylation is the mechanism for the inhibitory effect of statins on MMP secretion. Our RT-PCR result clearly demonstrated that lovastatin had no significant effects on mRNA level of MMP-1, -3, and -9, even when we titrated the number of cycles of PCR to avoid saturation. Interestingly, the previously reported inhibitory effect of fluvastatin on MMP-9 secretion from human macrophages was accompanied by a doubling of steady-state mRNA levels for MMP-9.<sup>38</sup> Thus, both studies agree that statins inhibit for MMP secretion by a posttranslational mechanism. Such a mechanism helps to explain inhibition of secretion by statins of MMPs with widely differing transcriptional regulation. For example MMP-1, -3, and -9 secretion in VSMC and MMP-1 and -3 secretion in foam cells is regulated and depends on the transcription factor nuclear factor- $\kappa$ B, but MMP-2 secretion in VSMC and MMP-9 secretion in macrophages is constitutive and independent of nuclear factor- $\kappa$ B.<sup>29</sup> Our results showing that secretion of TIMPs is unaffected by statins

implies that the posttranslational mechanism is selective for MMPs, not merely an overall inhibition of protein synthesis.

Additional studies, beyond the scope of this article, will be required to fully understand the mechanisms of how the drug affects posttranscriptional processes for MMPs. Previous studies demonstrated that the inhibition of geranylgeranyl transferase with L-839,867 and the inhibition of Rho by C3 exoenzyme significantly decreased production of MMPs.<sup>39,40</sup> Rho are small GTP-binding proteins that cycle between the inactive GDP-bound state and active GTP-bound state; they play crucial roles in diverse cellular events such as cytoskeleton organization, membrane trafficking, secretion, transcriptional regulation, cell growth control, and development.<sup>35</sup>

In summary, we demonstrate for the first time that statins inhibit the secretion of a broad spectrum of MMPs from both SMC and foam cell macrophages. The effect is mediated by inhibition of prenylation and seems to be mainly posttranslational. Inhibition of MMP secretion could contribute to the plaque-stabilizing potential of statins.

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