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BMPER Is Upregulated by Statins and Modulates Endothelial Inflammation by Intercellular Adhesion Molecule–1

Thomas Helbing, René Rothweiler, Jennifer Heinke, Lena Goetz, Philipp Diehl, Andreas Zirlik, Cam Patterson, Christoph Bode, Martin Moser

Objective—In addition to lowering cholesterol, statins exert pleiotropic effects on endothelial cells. Bone morphogenetic proteins (BMPs) have recently been implicated in vascular inflammation and disease. We set out to investigate the effect of statins on BMP endothelial cell precursor–derived regulator (BMPER), a novel member of the BMP pathway.

Methods and Results—Mevastatin enhanced BMPER expression in cultured endothelial cells in a time- and concentration-dependent manner as determined by immunocytochemistry, RT-PCR, and Western blotting. Similar effects were observed in vitro and in vivo using simvastatin. Actinomycin D chase analysis and BMPER promoter reporter assays revealed that this is mostly a posttranscriptional event resulting in prolonged BMPER RNA half-life. We confirmed that the RhoA/Rho-associated coiled-coil containing protein kinase Rho kinase (Rock)/actin pathway is involved using the specific pathway activator cytotoxic necrotizing factor of *Yersinia pseudotuberculosis,* which prevented upregulation of BMPER expression by mevastatin and pathway inhibitors (C3-toxin, RhoAN19 mutant, fasudil, and cytochalasin D) that enhanced BMPER expression. Increasing concentrations of BMPER exert antiinflammatory features in endothelial cells as reflected by intercellular adhesion molecule–1 downregulation. Accordingly, silencing of BMPER enhances intercellular adhesion molecule–1 expression. Furthermore, mevastatin reduced the expression of proinflammatory BMP4, a well-known direct interaction partner of BMPER.

Conclusion—Mevastatin modulates the BMP pathway by enhancing BMPER via the RhoA/Rock/actin pathway, as well as by reducing BMP4 expression. BMP4 downregulation and BMPER upregulation contribute to the antiinflammatory pleiotropic effects of statins. **(*Arterioscler Thromb Vasc Biol*. 2010;30:554-560.)**

Key Words: statins ■ ICAM-1 ■ bone morphogenetic proteins ■ vascular biology

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily. BMPs are important regulators in blood vessel formation and vascular disease.[1] BMP2 and BMP4 are upregulated in athero-prone regions in blood vessels, induce[2,3] a proinflammatory endothelial phenotype, and may contribute to the development of atherosclerotic plaques and vascular calcification.[4,5] Infusion of BMP4 in vivo leads to endothelial dysfunction and arterial hypertension.[6,7] Important insights have also come from the discovery of mutations of the BMP receptors in patients with familial pulmonary artery hypertension or teleangiectasia.[8]

BMP endothelial cell precursor–derived regulator (BMPER) is a secreted glycoprotein that binds directly to BMPs and modulates their function in a dose-dependent fashion. In gain-of-function assays, BMPER behaves as a BMP antagonist,[9,10] whereas in loss-of-function models, BMPER may also exert pro-BMP functions.[11–14] BMPER was originally identified in a screen for differentially expressed proteins in embryonic endothelial precursor cells.[9] In mouse and zebrafish, it is expressed at sites and at the time of vasculogenesis, consistent with a regulatory role for BMPER in vascular events. When BMPER is inactivated in zebrafish embryos, intersomitic angiogenesis is severely perturbed.[11] Consistent with this vascular phenotype, BMPER may confer proangiogenic activity in endothelial cells in a dose-dependent fashion.[15] Taken together, these data indicate that BMPER acts as a context-dependent BMP modulator and is essential for BMP4 function in endothelial cells.[15]

It has been shown that BMP4 exerts its proinflammatory effects by increased nuclear factor-κB activation and induction of intercellular adhesion molecule–1 (ICAM-1).[16,17] ICAM-1 is an adhesion molecule that is expressed on the endothelium and leukocytes and is upregulated in inflammation by proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, and interferon-γ.[18] Increased expression of ICAM-1 was identified in all subtypes of atheroscle-

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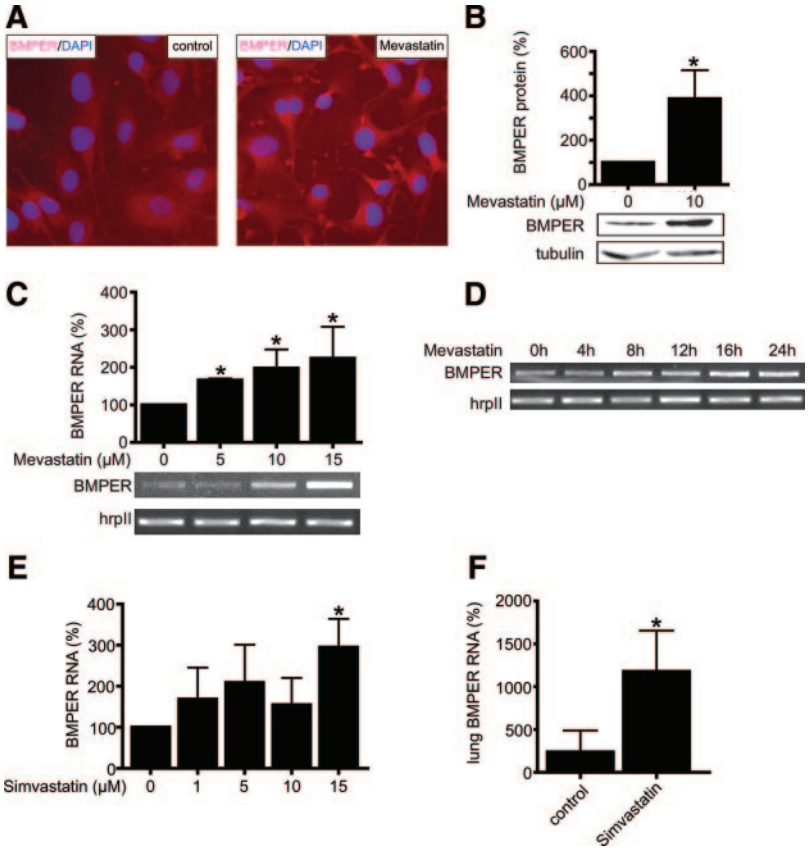

Figure 1. Inhibition of endothelial HMG-CoA reductase upregulates BMPER expression in endothelial cells. A, After 24 hours of mevastatin (10 μmol/L) treatment, BMPER expression was increased in HUVECs, as shown by immunocytochemistry (right panel) compared with a negative control with the corresponding serum (left panel). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). B, After 24 hours of mevastatin (10 μmol/L), HUVECs were lysed and used for Western blot with the indicated antibody. β-Tubulin was used as a loading control. Expression was quantified by densitometric analysis of 3 independent experiments. C and D, BMPER RNA expression depends on concentration (C) and duration (D) of mevastatin treatment, shown by RT-PCR (gel) and by reverse transcription–quantitative polymerase chain reaction (bar graph) of 3 independent experiments. RNA expression was analyzed by specific primers for human BMPER and human RNA polymerase II (hrpII). E, Simvastatin increased the BMPER RNA level in HUVECs in vitro. F, C57/BL6 mice were treated with activated simvastatin (n=5) SC or PBS (n=5) as control for 14 days. Lungs were isolated, and RNA was prepared and used for quantitative polymerase chain reaction. *$P<0.05$ versus control.

rotic lesions and is involved in the recruitment of monocytes to the lesion, as suggested by its role in the entry of leukocytes into foci of inflammation. Along the same lines, ICAM-1-enhanced monocyte recruitment is a potential mechanism for the growth of an atherosclerotic plaque.[19] Therefore, it is important to understand the regulation of ICAM-1 on the endothelial surface and to identify regulators of ICAM-1 expression because of their potential in the treatment of vascular inflammation.

In addition to their ability to lower plasma cholesterol levels, statins have been shown to decrease ICAM-1 expression in endothelial cells.[20,21] They demonstrate antiatherogenic properties by improving endothelial function, stabilizing atherosclerotic plaques, and reducing oxidative stress, as well as endothelial inflammation and thrombogeneity.[22] Therefore, statins are used in the primary and secondary prevention of cardiovascular disease. By inhibition of the 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, statins block the conversion of HMG-CoA to mevalonate and cause a depletion of isoprenoids, such as mevalonate, farnesylpyrophosphate (FPP), and geranylgeranylpyrophosphate (GGPP). These isoprenoids serve as important lipid anchors for the posttranscriptional modification of small GTPases, such as Ras, Rho, Rac, and Rap, by isoprenylation. Small GTPases are involved in cell signaling, and perturbed isoprenylation of small GTPases by statins mediates antiinflammatory effects partially by downregulation of proinflammatory BMP2.[6]

In this study, we identified the extracellular BMP modulator BMPER as a new mediator of antiinflammatory effects of statins in endothelial cells.

Methods

Reagents, antibodies, cell culture, immunocytochemistry, transfection of promoter constructs, luciferase assays, RT-PCR, quantitative real-time polymerase chain reaction, small interfering RNA (siRNA) transfection, Western blotting, and animal procedures are described in the online Data Supplement, available at http://atvb.ahajournals.org.

Statistical Analysis and Quantification

Statistical analyses were performed using GraphPad Prism 4.0. Data are presented as mean±SD, and comparisons were calculated by Student's t test (2-sided, unpaired). Results were considered statistically significant when $P<0.05$. Densitometric analysis of Western blots was performed using Quantity One 1-D Analysis Software (version 4.4, Bio-Rad) and levels of significance were calculated by 1-sample t test.

Results

Mevastatin Upregulates BMPER Expression in Endothelial Cells

To test the hypothesis that statins may exert pleiotropic effects by regulating BMPER, we treated human umbilical vein endothelial cells (HUVECs) with mevastatin. Indeed, as visualized by immunocytochemistry and quantified by Western blotting, mevastatin increased BMPER protein expression (Figure 1A and 1B). Treatment with 10 μmol/L mevastatin for 24 hours resulted in a 4-fold upregulation of BMPER protein. Similar results were obtained for BMPER RNA levels in a concentration- and time-dependent manner (Figure 1C and 1D). To investigate whether BMPER regulation was confined to mevastatin or whether other statins have similar properties, we tested simvastatin and pravastatin. Indeed, these compounds also increased BMPER RNA levels in vitro,

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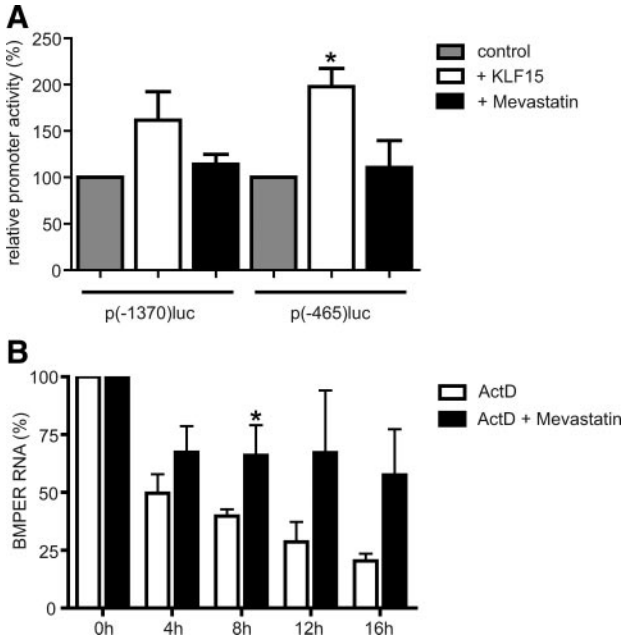

Figure 2. Mevastatin increases BMPER levels by RNA half-life prolongation. A, Mevastatin does not induce BMPER promoter activity in endothelial cells. Cells were transfected with the respective promoter construct, and luciferase activity was quantified after 24 hours of mevastatin (10 μmol/L) treatment. Krüpple-like factor 15 (KLF15) was used as a positive control for BMPER promoter activation. Values represent the mean±SD of 3 independent experiments normalized to β-galactosidase. B, After 24 hours of preincubation with or without mevastatin, HUVECs were treated with de novo transcription inhibitor actinomycin D (ActD) for the indicated times. RNA levels were normalized to the RNA level at 0 hours of the same group. Each experiment was performed at least 3 times, with similar results. *$P<0.05$ versus ActD control.

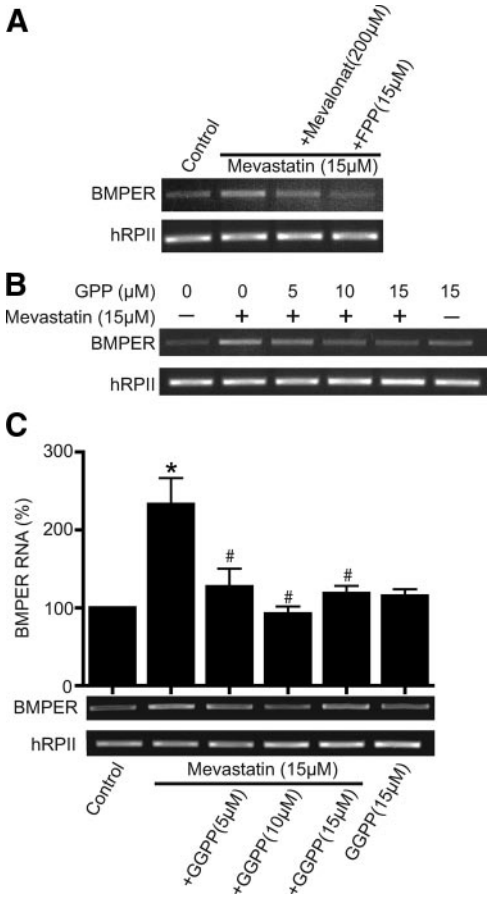

Figure 3. Mevastatin-induced BMPER expression is reversed by isoprenoids in endothelial cells. HUVECs were treated with mevastatin alone or in combination with mevalonate (A), FPP (A), geranylpyrophosphate (GPP) (B), and GGPP (C) for 24 hours. Total RNA was harvested and assessed for BMPER expression by RT-PCR. Human RNA polymerase II (hRPII) was used as a loading control. One representative gel out of 3 independent experiments with similar results is shown. In addition, corresponding quantitative polymerase chain reactions were performed (C), and results are shown as a bar graph. *$P<0.05$ versus control; #$P<0.05$ versus mevastatin.

suggesting a class effect of statins on BMPER regulation (Figure 1E). These data were confirmed in vivo by treating C57/BL6 mice with subcutaneous injection of simvastatin for 14 days. In simvastatin-treated animals, BMPER RNA levels were upregulated in the lungs compared with control. These data clearly demonstrate that statins increase BMPER expression in vitro and in vivo.

Mevastatin Increases BMPER Expression by Posttranscriptional Modification

To analyze the mechanism by which statins regulate BMPER expression, we pursued 2 separate approaches. First, we tested the effect of mevastatin on 2 BMPER promoter constructs of different sizes that contain luciferase as a reporter of BMPER promoter activity. Krüpple-like factor 15, a known activator of the BMPER promoter, was used as positive control.[23] As demonstrated in Figure 2A, both BMPER promoter constructs did not respond to mevastatin, suggesting a regulatory mechanism that is independent of the promoter. In the second set of experiments, we pretreated HUVECs with actinomycin D, an inhibitor of de novo transcription (Figure 2B). When these cells, in which RNA content depends completely on RNA degradation, were exposed to mevastatin, we found that BMPER RNA half-life was prolonged compared with cells that were not exposed to mevastatin. This indicates that mevastatin stabilized BMPER RNA. Taken together, these data cannot completely exclude transcriptional regulation but strongly suggest a posttranscriptional mechanism of regulation.

Mevastatin-Mediated Induction of BMPER Expression Is Dependent on Isoprenoid Intermediates

Statins inhibit the HMG-CoA reductase and cause a depletion of downstream isoprenoids, such as mevalonate, geranylpyrophosphate, FPP, or GGPP, in the cells.[24] To determine which downstream isoprenoid in the cholesterol biosynthetic pathway regulates BMPER expression, HUVECs were exposed to mevastatin alone or in combination with individual downstream isoprenoids. As shown in Figure 3A, supplementation of mevastatin-treated cells with mevalonate completely reversed the mevastatin-dependent induction of BMPER, confirming the specificity of the effect of mevastatin. Similarly, the effect of mevastatin could be reversed using geranylpyrophosphate, FPP, and GGPP, whereas the respective isoprenoid alone had no effect (Figure 3B and 3C). These

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data underline that the statin-mediated BMPER upregulation is dependent on the cholesterol synthesis pathway and demonstrate that all tested isoprenoids are able to reverse the effect of mevastatin on BMPER.

RhoA Is Involved in BMPER Regulation in Endothelial Cells

The isoprenoids FPP and GGPP have regulatory roles in a number of signaling cascades, such as the Ras and Rho pathways. Because Rho is a major target of geranylgeranylation, inhibition of Rho and its downstream target, Rho kinase, mediates some of the pleiotropic effects of statins on the vascular wall.[24,25] As a consequence, we hypothesized that activation of RhoA reverses mevastatin-induced BMPER expression. Therefore, we tested whether activation of RhoA using a direct and highly specific RhoA activator (cytotoxic necrotizing factor of *Yersinia pseudotuberculosis* [CNFY]) would reverse the statin effect.[26] As expected, mevastatin increased the BMPER RNA level to 241% compared with basal level, whereas CNFY alone reduced the BMPER RNA level to 71%. Cotreatment of cells with mevastatin and CNFY completely reversed the upregulation of BMPER RNA by mevastatin (Figure 4A). These findings indicate that the statin-mediated inhibition of RhoA contributes to the increased BMPER expression.

To confirm these results, we tested the effect of RhoA inhibition on BMPER. Therefore, HUVECs were coincubated with clostridium botulinum C3 transferase, an exotoxin that inactivates Rho by ADP-ribosylation.[27] Indeed, as shown in Figure 4B, treatment of HUVECs with the C3 toxin for 24 hours augmented BMPER RNA level to 140%. To increase the specificity, we overexpressed a dominant-negative RhoA mutant (RhoA19N) in HUVECs (Figure 4C). Specific inhibition of RhoA resulted in upregulation of BMPER RNA. These findings demonstrate that RhoA inhibition increases BMPER expression to an extent similar to inhibition of Rho GTPases by mevastatin. Taken together, these findings indicate that RhoA is an important regulator of BMPER RNA levels in endothelial cells and that the statin-mediated inhibition of geranylgeranylation of RhoA is responsible for the increased BMPER expression.

Inhibition of Rock Increases BMPER RNA in Endothelial Cells

Rho-associated coiled-coil containing protein kinase Rho kinase (Rock) is an important downstream target of RhoA activity.[24,28] To determine the involvement of Rock in BMPER expression, endothelial cells were incubated with the specific Rock inhibitor fasudil for 24 hours. Confirming our hypothesis, fasudil upregulated BMPER RNA levels (246%) and protein levels (134%) compared with control (Figure 4D and 4E). These findings support the notion that the RhoA/Rock pathway is involved in the regulation of BMPER RNA levels.

Disruption of the Endothelial Actin Cytoskeleton Increases BMPER Expression

Rock phosphorylates various targets and mediates a range of cellular responses that involve the assembly of the actin cytoskeleton. The number of actin stress fibers and the reorganization of the cytoskeleton are mediated in part by

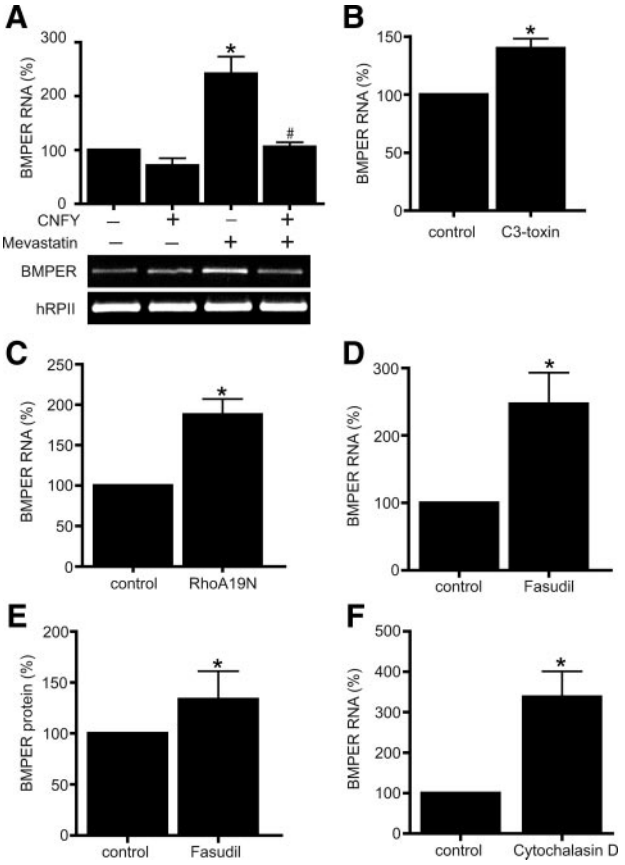

Figure 4. Mevastatin-mediated upregulation of BMPER depends on RhoA and Rho kinase activity. A, Specific activation of the Rho pathway by CNFY prevents mevastatin-induced BMPER upregulation. HUVECs were treated with CNFY (400 ng/mL) and mevastatin (15 μmol/L) alone or in combination. Reverse transcription–quantitative polymerase chain reaction was performed to quantify BMPER RNA levels. Human RNA polymerase II (hRPII) was used as a loading control. *$P<0.05$ versus control; #$P<0.05$ versus mevastatin. B–E, Inhibition of the Rho pathway enhances BMPER expression. B, The Rho inhibitor C3 toxin augments BMPER RNA levels. HUVECs were treated with C3 toxin (250 ng/mL) for 24 hours. C, Overexpression of the dominant-negative RhoA (RhoN19) mutant in HUVECs increases BMPER RNA levels compared with control (empty vector). D and E, The Rock inhibitor fasudil upregulates BMPER RNA and protein expression in endothelial cells. HUVECs were treated with fasudil (50 μmol/L) for 24 hours before cells were harvested for RNA or protein analysis. F, Disruption of actin cytoskeleton increases BMPER RNA level in endothelial cells. HUVECs were incubated with the actin cytoskeleton disruptor cytochalasin D (5 μmol/L) for 8 hours. *$P<0.05$ versus control.

Rocks. To address the question of whether this downstream step of RhoA/Rock signaling is also involved in BMPER regulation, we treated endothelial cells with cytochalasin D, a well-characterized disruptor of the actin cytoskeleton, for 8 hours (Figure 4F). Cytochalasin D causes a strong increase in BMPER RNA levels to 338% compared with control, suggesting that the actin cytoskeleton is indeed involved in the regulation of BMPER expression.

Mevastatin Downregulates Proinflammatory BMP4 in Endothelial Cells

Having demonstrated that mevastatin upregulates BMPER, we asked whether other BMP pathway members are also

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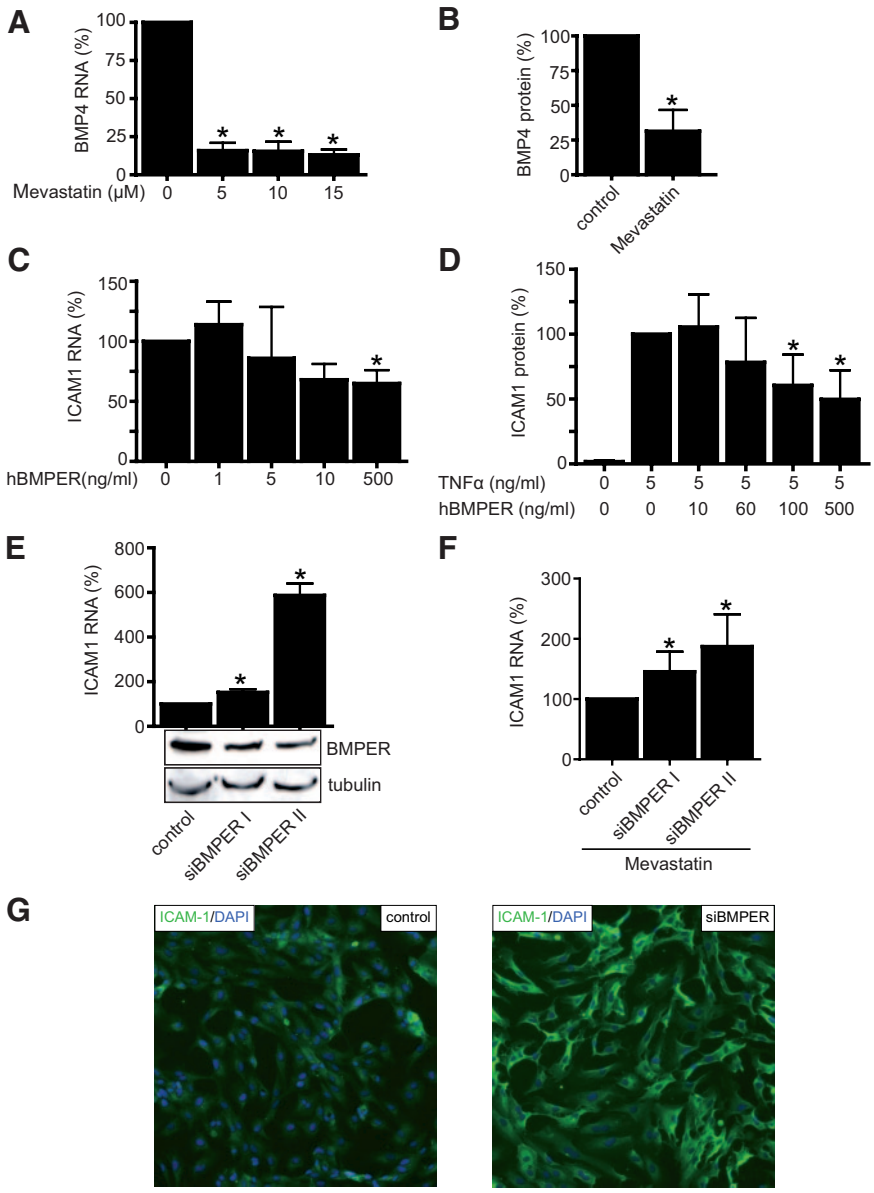

Figure 5. Mevastatin downregulates BMP4. A and B, HUVECs were incubated with mevastatin. BMP4 RNA (A) and BMP4 protein (B) were quantified at 24 and 48 hours, respectively. These experiments were performed 3 times, with comparable results. *$P<0.05$ versus control. BMPER regulates ICAM-1 expression. C, Serum-starved HUVECs were treated with or without BMPER at the indicated concentrations for 24 hours before RNA was harvested, reverse transcribed, and used for reverse transcription–quantitative polymerase chain reaction to quantify ICAM-1. hBMPER indicates human recombinant BMPER. D, HUVECs were stimulated with tumor necrosis factor-α (TNFα) (2 ng/mL) and increasing concentrations of BMPER. After 8 hours, cells were harvested and used for Western blotting. *$P<0.05$ versus control. E and F, Specific silencing of BMPER in endothelial cells by 2 different siRNAs results in increased ICAM-1 expression. siBMPER indicates BMPER-specific small interfering RNA. E, Two different siRNAs for BMPER or control siRNA were transfected in HUVECs. After 48 hours, protein and RNA were prepared. Sufficient BMPER knockdown at the protein level is shown by Western blotting compared with tubulin as the loading control (top panels). ICAM-1 expression was quantified by reverse transcription–quantitative polymerase chain reaction, as shown in the bar graph. Three independent experiments were quantified. *$P<0.05$ versus control. F, After silencing of BMPER expression, HUVECs were treated with mevastatin (10 μmol/L) for 24 hours, and RNA was used for quantitative real-time polymerase chain reaction to analyze ICAM-1 RNA expression. *$P<0.05$ versus control. G, After silencing of BMPER in HUVECs, ICAM-1 protein expression (right panel) is increased compared with control (left panel), as shown by immunocytochemistry. 4,6-Diamidino-2-phenylindole (DAPI) was used for staining of nuclei.

regulated by statins. We decided to focus on BMP4 because we had shown earlier that BMPER interacts directly with BMP4 and because BMP4 is a known inducer of vascular inflammation.[17] In contrast to BMPER, BMP4 was markedly downregulated by mevastatin (Figure 5A and 5B). This finding is consistent with the notion that downregulation of BMP4 contributes to the antiinflammatory effect of statins.

BMPER Modulates ICAM-1 Expression

Next, we asked whether regulation of BMPER by statins is also involved in vascular inflammation. To quantify vascular inflammation we chose ICAM-1 expression as a surrogate marker. When we added BMPER to HUVECs, ICAM-1 RNA was downregulated (Figure 5C). To investigate whether BMPER was capable of antagonizing proinflammatory stimuli, we coincubated HUVECs with tumor necrosis factor-α, a strong inducer of endothelial inflammation, and with increasing concentrations of BMPER. Indeed, BMPER inhibited tumor necrosis factor-α-induced ICAM-1 expression in a concentration-dependent manner (Figure 5D). To confirm these findings, we silenced BMPER in HUVECs. Two different siRNAs designed to target BMPER were used in these experiments. Consistent with an inhibitory role of BMPER on ICAM-1 expression, we found that ICAM-1 RNA and protein are increased in BMPER silenced endothelial cells (Figure 5E and 5G). In our hands, BMPER-specific small interfering RNA (siBMPER) II consistently reached higher knockdown efficiencies than siBMPERI, and consequently, more pronounced effects on ICAM1 were observed using siBMPERII. To analyze whether BMPER is a mediator of ICAM-1 regulation in the presence of statins, HUVECs were transfected with siBMPER and treated with mevastatin for 24 hours. As expected, silencing of BMPER increased ICAM-1 expression in mevastatin-treated cells (Figure 5F).

Taken together, these data suggest that statins reduce vascular inflammation by interfering with the BMP pathway at 2 ends. First, they downregulate proinflammatory BMP4, and second, they increase BMPER, for which we suggest an

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antiinflammatory role by its suppressing activity on ICAM-1 expression.

Discussion

In this study, we characterize the BMP modulator BMPER as a novel pleiotropic target of statins in endothelial cells and present novel findings with regard to the regulation of BMPER and the BMP pathway. First, HMG-CoA reductase inhibitors upregulate BMPER expression in vitro and in vivo. Second, upregulation of BMPER expression by mevastatin is a posttranscriptional event. Third, the effect of mevastatin on BMPER involves inhibition of the RhoA/Rock/actin pathway. Fourth, mevastatin differentially regulates BMPER and BMP4 and thereby inhibits vascular inflammation as reflected by ICAM-1.

BMPER is an extracellular BMP modulator that is expressed by endothelial cells. Data from *Drosophila*, *Xenopus*, zebrafish, chicken, and mouse reveal that BMPER is necessary to sharpen BMP gradients and that its activity is sensitive to dose changes.[9,12–14,29–32] At low doses, BMPER is needed to enhance BMP4 activity, but at higher doses, BMPER increasingly inhibits BMP4 activity.[9,15] Therefore, a detailed understanding of BMPER regulation is crucial to control BMP effects.

Here we present data demonstrating that mevastatin increases BMPER expression in endothelial cells (Figure 1). Until now, this is the first drug that has been shown to increase BMPER expression. Pleiotropic effects of statins are frequently controlled by posttranscriptional events rather than by control of gene promoter activity.[25,33] This is also the case for BMPER. Although our data cannot completely rule out transcriptional modification of BMPER expression by statins, they strongly suggest a posttranscriptional effect, resulting in prolongation of BMPER RNA half-life (Figure 2). The isoprenoids downstream of HMG-CoA reductase are important modulators of small GTPases. For example, inhibition of Rho geranylgeranylation and membrane translocation of Rho by mevastatin lead to a greater accumulation of inactive Rho in the cytoplasm.[25] Because supplementation of GGPP could reverse the statin effect on BMPER, we hypothesized that the Rho/Rock/actin pathway was involved in BMPER regulation (Figure 3). Indeed, specific activation of this pathway by CNFY reduced BMPER expression, whereas pathway inhibition by either a specific RhoA inhibitor (C3-transferase toxin), a dominant-negative RhoA mutant (RhoA19N), or a Rock inhibitor (fasudil) results in upregulation of BMPER (Figure 4). Similarly, inhibition of downstream actin cytoskeleton assembly increases BMPER RNA. Taken together, this is compelling evidence that the RhoA/Rock/actin pathway plays a pivotal role in mevastatin-mediated BMPER expression. Remarkably, this is a very similar mechanism to the regulation of endothelial nitric oxide synthase by statins.[25,34,35] The mechanistic data dissecting the RhoA/Rock/actin pathway of BMPER activation are of great value, as recently specific inhibitors of Rock have emerged as novel therapeutic strategies to treat vascular dysfunction and its long-term consequences such as atherosclerosis or pulmonary artery hypertension.[36,37] Our data demonstrate that Rock inhibition, in addition to the well-described consequences for endothelial nitric oxide synthase, also modifies the BMP pathway.

Modification of the BMP pathway has a major impact on vascular inflammation. BMP4 exerts prooxidant, proinflammatory, and prohypertensive effects on endothelial cells and is involved in vascular calcification. By downregulation of BMP4 expression, statins decrease BMP activity, which mediates antiinflammatory, antiatherogenic, and vasculoprotective effects.[17,38] Another new target of statins within the BMP pathway is BMPER. Here we show that high levels of BMPER modulate expression of adhesion molecules on endothelial cells. This is demonstrated by downregulation of ICAM-1, a marker of endothelial cell activation and inflammation (Figure 5). Thus, statins modulate the BMP pathway at different levels: they downregulate BMP4, and at the same time they upregulate the dose-dependent BMP4 modulator BMPER, together resulting in a strong antiinflammatory activity.

In conclusion, we demonstrate that BMPER is upregulated by mevastatin via posttranscriptional modification involving the RhoA/Rock/actin pathway. At the same time, BMP4 is downregulated by mevastatin. This dual modification of the BMP pathway results in decreased vascular inflammation and thus represents a hitherto unknown antiinflammatory effect of statins.

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Disclosures

None.

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