Simvastatin Induces Heme Oxygenase-1
A Novel Mechanism of Vessel Protection

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Background—Evidence from experimental and clinical studies indicates that statins can protect the vessel wall through cholesterol-independent mechanisms. The “pleiotropic” effects include the prevention of inflammation and proliferation of vascular cells. Here, we studied whether heme oxygenase-1 (HO-1), an important cytoprotective molecule, is induced by simvastatin and the role of HO-1 in the pleiotropic effects of simvastatin.

Methods and Results—Human and rat aortic smooth muscle cells treated with simvastatin showed an elevated level of HO-1 for up to 24 hours. The induction of HO-1 by simvastatin was not found in cultured endothelial cells and macrophages. Injecting C57BL/6J mice intraperitoneally with simvastatin increased the level of HO-1 in vascular SMCs (VSMCs) in the tunica media. Treating VSMCs with zinc protoporphyrin, an HO-1 inhibitor, or HO-1 small interfering RNA (siRNA) blocked the antiinflammatory effect of simvastatin, including the inhibition of nuclear factor-κB activation and nitric oxide production. Blockade of HO-1 also abolished the simvastatin-induced p21\(^{WAF1}\), and the associated antiproliferative effect. Simvastatin activated p38 and Akt in VSMCs, and the respective inhibitor of p38 and phosphoinositide 3-kinase (PI3K) greatly reduced the level of simvastatin-induced HO-1, which suggests the involvement of p38 and the PI3K-Akt pathway in HO-1 induction.

Conclusions—Simvastatin activates HO-1 in VSMCs in vitro and in vivo. The antiinflammatory and antiproliferative effects of simvastatin occur largely through the induced HO-1. (Circulation. 2004;110:1296-1302.)

Key Words: vasculature ■ statins ■ muscle, smooth

The beneficial effect of statins, competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase, on reducing mortality rate in patients with coronary heart disease has been evidenced by cohort studies such as the Scandinavian Simvastatin Survival Study and the Heart Protection Study.\(^{1,2}\) The pharmacology of these drugs is explained by their lipid-lowering effect through the upregulation of the hepatic LDL receptor. Recent experimental and clinical evidence demonstrates that the antiatherogenicity of statins also includes cholesterol-independent, or “pleiotropic,” effects.\(^{3,4}\) These pleiotropic effects include immunosuppressive and antiinflammatory properties, which have been suggested to reduce greatly the cardiovascular disease–related morbidity and mortality.\(^{5,6}\) Animal studies have shown that statins can suppress the inflammatory and proliferative responses of vascular smooth muscle cells (VSMCs) after vascular injury.\(^{7,8}\) In vitro experiments showed that statins downregulate several transcription factors involved in inflammation, including nuclear factor (NF)-κB, activator protein-1, and hypoxia-inducible factor-1α, in VSMCs and vascular endothelial cells (ECs).\(^{9}\) In addition, statins exhibit an antiproliferative effect by modulating the expression of p21\(^{WAF1}\).\(^{10}\)

The family of heme oxygenases (HO) degrades heme to biliverdin, carbon monoxide (CO), and free iron in mammalian cells. The HO-1 gene is inducible, whereas those of HO-2 and HO-3 are constitutively expressed.\(^{11,12}\) Because of the antioxidative activity of biliverdin and its metabolite bilirubin and the antiinflammatory effect of CO, HO-1 has been suggested to be cytoprotective. The exogenous administration of HO-1 by the use of agonists or gene transfer reduced the development of atherosclerosis in apolipoprotein E–deficient mice and of restenosis in balloon-injured rat models.\(^{13,14}\) Ample evidence has demonstrated that the HO-1–generated CO modulates guanylyl cyclase/cGMP, p38 mitogen-activated protein kinase (MAPK), p21\(^{WAF1}\), and NF-κB and thus, regulates the expression of genes involved in vasconstriction, proinflammation, and procoagulation.\(^{15–17}\)

Despite extensive studies on the molecular mechanisms of statins, it remains unclear whether HO-1 is functionally related to the cytoprotective effects of statins. In light of this, we explored the effect of simvastatin on the expression of HO-1. Our results demonstrate that simvastatin, at clinical doses, induces HO-1 in VSMCs. Furthermore, the antiinflammatory and antiproliferative effects of simvastatin occur largely through HO-1.
Methods

Materials
Simvastatin was purchased from A.G. Scientific. Hemoglobin (Hb) and bacterial lipopolysaccharide (LPS) were purchased from Sigma. Cobalt protoporphyrin (CoPP) and zinc protoporphyrin (ZnPP) were obtained from Frontier Scientific Inc. SB203580, PD98059, SP600125, and LY294002 were from Calbiochem.

Cell Culture
Rat aortic VSMCs (RASMCs) were isolated from thoracic aortas of Sprague-Dawley rats; human aortic VSMCs (HASMCs) were purchased from CAMBREX. Bovine aortic ECs were cultured in Dulbecco modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Omega), 100 U/mL penicillin, and 100 μg/mL streptomycin. The monocytic cell line THP-1 was cultured in RPMI 1640 medium supplemented with 10% FBS. THP-1 cells were treated with 12-α-tetradecanoyl-phorbol-13-acetate for 7 days to differentiate into macrophages.

Animal Experiments
Male C57BL/6J mice, 8 weeks old, were injected with simvastatin (0.75 to 5 mg/kg) peritoneally. After 3, 6, 12, or 24 hours, mice were euthanized with CO2, and aortas were harvested. Tissue extracts (0.75 to 5 mg/kg) peritoneally. After 3, 6, 12, or 24 hours, mice were euthanized with CO2, and aortas were harvested. Tissue extracts (0.75 to 5 mg/kg) were isolated with the use of a buffer containing 10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L MgCl2, 0.5% Nonidet P-40, 1 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Vessels collected after simvastatin treatment for 12 hours were also fixed with 4% paraformaldehyde.

Plasmids, Small Interfering RNA, and Transient Transfection
RASMCs cultured in 12-well plates were transfected with the NF-κB–Luc plasmid (0.5 μg). Cells were also transfected with CMV-β-gal plasmid (0.5 μg) for transfection control. After various treatments, cells were lysed and the lysates were collected for luciferase activity. The small interfering RNA (siRNA) nucleotide sequences for rodent HO-1 and control pGL3-Basic were as follows: 5’-AAUGGAAGACCACUCCCACUC-3’ and 5’-AAGGCCACAGACAGACGU-AUUGUA-3’; respectively.18 RASMCs were transfected with HO-1 or pGL3 siRNA with the use of Oligofectamine reagent (Invitrogen).

Western Blotting
Cells were lysed in the same buffer as described for aortic tissue extraction. Cytosolic and nuclear extracts were isolated accordingly. Aliquots (25 to 50 μg) of the lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then underwent blotting. After being blocked with 5% skim milk in Tween-20/phosphate-buffered saline, blots were incubated with various primary antibodies (rabbit anti-HO-1 [StressGen], rabbit anti-IκB [Pharmingen], rabbit anti-inducible nitric oxide synthase [iNOS], rabbit anti-p65, rabbit anti-histone H4, mouse anti-p21 [Nek], and mouse anti-α-tubulin [Santa Cruz Biotech]) and then with the horseradish peroxidase–conjugated secondary antibodies. The protein bands in the blots were detected with the use of an enhanced chemiluminescence kit.

Determination of Nitrite Concentration
Accumulated nitrite, a stable breakdown product of NO, in culture medium was determined by use of the Griess reagent. In brief, an aliquot of cell culture medium was mixed with an equal volume of Griess reagent (Sigma) and then incubated at room temperature for 15 minutes. The azo dye production was analyzed by a spectrophotometer with absorbance set at 540 nm. Sodium nitrite was used as a standard.

Immunohistochemistry
The paraffin-embedded mouse aortas were sectioned (6 to 8 μm in thickness). For cultured SMCs, cells were fixed for 4% paraformaldehyde and permeabilized with 70% ethyl alcohol. After being blocked with 2% bovine serum albumin, the deparaffinized sections or fixed SMCs were incubated with anti-p65 antibody or anti-HO-1 antibody. The specimens were then incubated with secondary antibody conjugated with fluorescein isothiocyanate or peroxidase and viewed under a Nikon TE300 fluorescence microscope.

Bromodeoxyuridine Incorporation Assay
VSMCs were cultured in 12-well plates and were transfected with the NF-κB–Luc plasmid (0.5 μg). Cells were also transfected with CMV-β-gal plasmid (0.5 μg) for transfection control. After various treatments, cells were lysed and the lysates were collected for luciferase activity. The small interfering RNA (siRNA) nucleotide sequences for rodent HO-1 and control pGL3 were as follows: 5’-AAUGGAAGACCACUCCCACUC-3’ and 5’-AAGGCCACAGACAGACGU-AUUGUA-3’, respectively.18 RASMCs were transfected with HO-1 or pGL3 siRNA with the use of Oligofectamine reagent (Invitrogen).

Statistical Analyses
Results are expressed as mean±SD from at least 3 independent experiments. For comparison between multiple groups, statistical significance was tested by a single-factor ANOVA. An unpaired t test was used for comparison between two groups.*P<0.05 vs controls.

Figure 1. Simvastatin induces HO-1 in VSMCs. HASMCs and RASMCs were incubated with various concentrations of simvastatin for 24 hours (left). Cells were also treated with 10 μmol/L simvastatin for indicated times (right). Cells under various conditions were then lysed, and cellular proteins were subjected to Western blotting for HO-1 induction assays. Data represent mean±SD from 4 independent repeats. Abbreviations are as defined in text.
test was applied to the comparison of 2 groups. Probability values <0.05 were considered statistically significant.

Results

Simvastatin Induction of HO-1 in VSMCs In Vitro and In Vivo
To test whether a statin could induce HO-1, HASMCs or RASMCs were treated with various concentrations of simvastatin for 24 hours. As shown in Figure 1, the HO-1 protein increased as the simvastatin concentration increased. We also investigated the temporal response of HO-1 in VSMCs treated with simvastatin at 10 μmol/L. The induction of HO-1 by simvastatin was evident as early as 3 hours, and the augmentation lasted for at least 24 hours. At 10 μmol/L, CoPP, an HO-1 inducer, but not simvastatin, was able to induce HO-1 in bovine aortic ECs and THP-1 macrophages (Figure 1 in the online-only Data Supplement). Thus, simvastatin preferentially induced HO-1 in VSMCs in culture.

To explore whether HO-1 was induced by simvastatin in vivo, C57BL/6J mice were injected intraperitoneally with 5 mg/kg simvastatin. Western blotting showed that the HO-1 increase was time dependent (Figure 2A). Doses within the clinical range (0.75 and 1.5 mg/kg) also induced HO-1. Furthermore, immunohistochemical analysis confirmed that the induced HO-1 in the vessel wall was present mainly in VSMCs in the tunica media (Figure 2B). In addition, increased HO-1 was found in hepatocytes, cardiomyocytes, and arterial VSMCs of the collected livers and hearts but not in mesenchymal cells of the lung and kidney (data not shown).

Inhibition of Inflammatory Responses by Simvastatin Is Mediated Through HO-1
A previous study had shown that simvastatin can inhibit the degradation of IkB in VSMCs.9 We tested whether this antiinflammatory effect was mediated through HO-1. Simvastatin prevented the lipopolysaccharide (LPS)–induced IkB degradation in HASMCs (lane 6 versus lane 5 in Figure 3A) and the ensuing nuclear translocation of NF-κB p65 (data not shown). However, treatment with simvastatin plus ZnPP, an HO competitive inhibitor, abolished the preventive effect of simvastatin on NF-κB activation (lane 7 versus lane 6 in Figure 3A). HO-1 can exert its antiinflammatory effect through the derived CO.11,12 Thus, VSMCs were also treated with simvastatin and Hb, a scavenger for CO. Abolition of NF-κB similar to that by ZnPP was observed (lane 8 versus lane 6 in Figure 3A). The role of HO-1 in simvastatin-regulated NF-κB was consistent with transcription mediated by the κB element, because simvastatin significantly repressed the LPS-induced NF-κB–Luc (lane 6 versus lane 5 in Figure 3B). This inhibitory effect was reversed by ZnPP or Hb (lanes 7 and 8 versus lane 6 in Figure 3B).

Increased NO production by upregulated iNOS is a major inflammatory response. We investigated the association of simvastatin and HO-1 in this inflammatory response. As shown in Figure 3C, treating RASMCs with LPS increased the nitrite production, which was inhibited by simvastatin (lane 5 versus lane 4). ZnPP reversed the inhibitory effect (lane 6 versus lane 5). Changes in nitrite level were consistent with those of iNOS (bottom panel of Figure 3C). Hb at concentrations ranging from 5 to 20 μmol/L also effectively reversed the inhibitory effect of simvastatin in a dosedependent manner (Figure 3D). As a control, ZnPP had little effect on simvastatin-inhibited NO production in THP-1 cells (data not shown). These findings suggest that HO-1/CO mediates the inhibitory effect of simvastatin on LPS-induced iNOS gene expression and the ensuing NO production in VSMCs.

We used HO-1 siRNA to confirm further that the simvastatin-inhibited NF-κB is mediated through HO-1. As shown in Figure 4A, attenuation of simvastatin-induced HO-1 by HO-1 siRNA was dose dependent. The interference of the HO-1 transcripts by 600 nmoL/HO-1 siRNA impaired the preventive effect of simvastatin in terms of IkB degradation and NO production caused by LPS (Figure 4B and 4C).
Simvastatin-Inhibited VSMC Proliferation Is Mediated Through HO-1

We also determined whether HO-1 mediates the inhibitory effect of simvastatin on the proliferation of VSMCs. Compared with its effect in untreated controls, simvastatin reduced significantly the incorporation of BrdU into both HASMCs and RASMCs (Figure 5A). This inhibitory effect was partially reversed by inclusion of ZnPP. The antiproliferative effect of statin and HO-1 is mainly due to their upregulation of p21\(^\text{Waf1}\).\(^{10,17}\) We thus investigated whether HO-1 is necessary for the simvastatin-upregulated p21\(^\text{Waf1}\). Treating VSMCs with simvastatin increased the level of p21\(^\text{Waf1}\) (Figure II in the online-only Data Supplement). This induction was abolished in cells cotreated with ZnPP (Figure 5B). These results suggest that, by activating p21\(^\text{Waf1}\), HO-1 is necessary for the antiproliferative effect of simvastatin in VSMCs.

Simvastatin Induction of HO-1 Involves p38 and the Phosphoinositide 3-Kinase–Akt Pathway

In investigating the signaling pathways involved in simvastatin-induced HO-1, we found that simvastatin caused a sustained activation of p38 and Akt and a transient activation of extracellular signal–regulated kinase but had no effect on c-Jun NH\(\text{2}\)-terminal kinase, as indicated by the phosphorylation of these proteins (Figure 6A). In reciprocal experiments, the p38-specific inhibitor SB203580 or the phosphoinositide 3-kinase (PI3K)–specific inhibitor LY294002 attenuated the simvastatin-induced increase in HO-1 (Figure 6B). A combination of SB203580 and LY294002 totally abolished the HO-1 induction. In contrast, PD98059, a specific inhibitor of extracellular signal–regulated kinase, and SP600125, a specific inhibitor of c-Jun NH\(\text{2}\)-terminal kinase, had little effect on HO-1 induction. These results suggest that p38 and PI3K-Akt are involved in the simvastatin-induced increase of HO-1 in VSMCs.

Discussion

Simvastatin, like all other statins, exerts both LDL cholesterol-lowering and pleiotropic effects. In the present study, our data demonstrate that simvastatin increases the expression of HO-1, particularly in VSMCs. The upregulation of HO-1, a critical cytoprotective molecule, provides a novel pleiotropic effect of statins on vessel protection. Interestingly, simvasta-
tin upregulation of HO-1 was found only in cultured VSMCs but not in ECs or macrophages (Figure 1). In the mouse artery in vivo, only the tunica media showed an increased level of HO-1 in response to simvastatin (Figure 2). The expression of HO-1 in the media of our animal models indicates that statins in the circulation could penetrate the arterial endothelium to exert their effect. Furthermore, peritoneal injection of simvastatin was able to maintain a high level of HO-1 expression for up to 24 hours, which may have clinical implications. Indeed, clinical studies indicate that statin therapy before percutaneous coronary interventions is associated with a marked reduction in mortality among patients with elevated levels of C-reactive protein, a marker of inflammation.19,20 Simvastatin reduced the neointimal area and the neointima-to-media ratio in rat carotid arteries after balloon injury or stent deployment.8 Thus, simvastatin, by increasing HO-1, also may protect the injured vessels with impaired or denuded endothelium.

Several clinical trials showed that simvastatin decreased the level of C-reactive protein in human subjects.21–23 In vitro, simvastatin exerts antiinflammatory effects by reducing the expression of proinflammatory cytokines and chemokines in several types of vascular cells.24 Using NF-κB activation and NO production in VSMCs as inflammation markers, we demonstrated that the antiinflammatory effect of simvastatin is largely mediated by HO-1, because ZnPP and HO-1 siRNA abolished these inflammatory responses (Figures 3 and 4). The antiapoptotic and antiinflammatory effects of HO-1 are mainly regulated through its degradation product, CO.16,25 By using Hb to trap CO, we demonstrated that the antiinflammatory effect of simvastatin-induced HO-1 also depended on CO. Statins show a potent antiproliferative effect by increasing cyclin-dependent kinase inhibitors such as p21Waf1 and p27Kip1. The inhibition of HO-1 reduced the simvastatin-induced p21Waf1 and partially inhibited the antiproliferative effect (Figure 5), which indicates that the antiproliferative

Figure 4. HO-1 siRNA inhibits simvastatin-induced HO-1. A, RASMCs were transfected with pGL3 siRNA or HO-1 siRNA for 18 hours and then stimulated with simvastatin (10 μmol/L). After 24 hours, cells were lysed and cell lysates were subjected to Western blotting to determine levels of HO-1 and α-tubulin. B and C, RASMCs transfected with 600 nmol/L pGL3 siRNA or HO-1 siRNA were treated with simvastatin (10 μmol/L) for 24 hours. In B, transfected cells were then stimulated with LPS (1 μg/mL) for 30 minutes before Western blotting to determine levels of IκBα and α-tubulin. In C, transfected cells were stimulated with LPS (1 μg/mL) for 18 hours before assays for NO production. *P<0.05, significantly different between indicated values. Other abbreviations are as defined in text.

Figure 5. HO-1 is necessary for antiproliferative effect of simvastatin. A, HASMCs or RASMCs were serum starved for 24 hours and then stimulated with 10% FBS for 48 hours. Afterward, cells were treated with simvastatin (10 μmol/L) and BrdU in presence or absence of ZnPP (10 μmol/L). Fixed cells were immunostained with anti-BrdU, and nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Proliferated VSMCs were determined by BrdU-positive nuclei. BrdU-positive cells are from 10 randomly selected views at a magnification of ×200x (left). B, RASMCs released from cell cycle by 10% FBS were treated with simvastatin in presence or absence of ZnPP, pGL3 siRNA, or HO-1 siRNA. Cell lysates underwent Western blotting to determine levels of p21Waf1 and α-tubulin. Data represent mean±SD from 3 independent repeats. Abbreviations are as defined in text.
effect of simvastatin was at least in part due to the HO-1–increased p21<sup>kip1</sup>. Results of a previous study suggest that simvastatin attenuates SMC proliferation by preventing the Rho GTPase–downregulated p21<sup>kip1</sup>. Therefore, upregulation of HO-1 and downregulation of Rho may contribute synergistically to simvastatin’s antiproliferative effect.

In addition to simvastatin, many antiinflammatory molecules (eg, interleukin-10) and stress inducers (eg, heavy metals) induce HO-1. These agents have been suggested to activate MAPK family members that in turn induce HO-1 in various cell types. Results presented in Figure 6 suggest that simvastatin activates both p38 and Akt in VSMCs. Furthermore, treatment of VSMCs with the respective inhibitors of p38 and PI3K inhibited the simvastatin-increased HO-1. Simvastatin activation of p38, a stress-induced signaling molecule, and Akt, necessary for cell survival and growth, represents an intricate balance between stress responses and viability cues. Consequently, the cytoprotective HO-1 is induced. Interestingly, neither extracellular signal–regulated kinase nor c-Jun NH<sub>2</sub>-terminal kinase was involved in the simvastatin-activated HO-1, although extracellular signal–regulated kinase was transiently activated by simvastatin. In summary, we have shown that HO-1 mediates the antiinflammatory and antiproliferative effects of simvastatin in VSMCs. Such cytoprotection, in conjunction with other “pleiotropic” effects, would benefit patients with coronary heart disease. Because of the cytoprotective effects of HO-1, gene transfer has been used to deliver HO-1 into hearts and vessels of animal models for study of myocardial protection gene transfer has been used to deliver HO-1 into hearts and vessels of animal models for study of myocardial protection. Therefore, the cytoprotective HO-1 is associated with other “pleiotropic” effects, would benefit patients with coronary heart disease. Because of the cytoprotective effects of HO-1, gene transfer has been used to deliver HO-1 into hearts and vessels of animal models for study of myocardial protection and restenosis prevention. Patients with similar cardiovascular impairments may benefit from statin administration.

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References

Figure 6. p38 and PI3K-Akt pathways are involved in induction of HO-1 by simvastatin. A. RASMCs were treated with 10 μmol/L simvastatin for indicated times. Cell lysates underwent Western blotting with various antibodies against phosphorylated p38, Akt, extracellular signal-regulated kinase, and c-Jun NH<sub>2</sub>-terminal kinase. B, HASMCs or RASMCs were preincubated with 10 μmol/L PD98059, SB203580, SP600125, or LY294002 for 1 hour. Cells were then treated with 10 μmol/L simvastatin or left untreated (controls) for 24 hours. Cell lysates were analyzed by Western blotting to determine levels of HO-1 and α-tubulin. Data represent mean ± SD from 3 independent repeats. Abbreviations are as defined in text.


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