Statin-Induced Expression of Decay-Accelerating Factor Protects Vascular Endothelium Against Complement-Mediated Injury

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Abstract—Complement-mediated vascular injury is important in the pathophysiology of atherosclerosis and myocardial infarction. Because recent evidence shows that statins have beneficial effects on endothelial cell (EC) function independent of lipid lowering, we explored the hypothesis that statins modulate vascular EC resistance to complement through the upregulation of complement-inhibitory proteins. Human umbilical vein and aortic ECs were treated with atorvastatin or simvastatin, and decay-accelerating factor (DAF), membrane cofactor protein, and CD59 expression was measured by flow cytometry. A dose-dependent increase in DAF expression of up to 4-fold was seen 24 to 48 hours after treatment. Statin-induced upregulation of DAF required increased steady-state mRNA and de novo protein synthesis. L-Mevalonate and geranylgeranyl pyrophosphate reversed the effect, confirming the role of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibition and suggesting that constitutive DAF expression is negatively regulated by geranylgeranylation. Neither farnesyl pyrophosphate nor squalene inhibited statin-induced DAF expression, suggesting that the effect is independent of cholesterol lowering. Statin-induced DAF upregulation was mediated by the activation of protein kinase C and inhibition of RhoA and was independent of phosphatidylinositol-3 kinase and NO activity. The increased DAF expression was functionally effective, resulting in significant reduction of C3 deposition and complement-mediated lysis of antibody-coated ECs. These observations provide evidence for a novel cytoprotective action of statins on vascular endothelium that is independent of the effect on lipids and results in enhanced protection against complement-mediated injury. Modulation of complement regulatory protein expression may contribute to the early beneficial effects of statins in reducing the morbidity and mortality associated with atherosclerosis. (Circ Res. 2002;91:696-703.)

Key Words: atorvastatin ● complement ● cytoprotection ● atherosclerosis ● endothelium

The statins reduce cholesterol synthesis through inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and are widely prescribed to hyperlipidemic patients to reduce their risk of atherosclerotic complications. The efficacy of this group of drugs in reducing coronary morbidity and mortality has been demonstrated in large intervention trials. However, statins have additional benefits on vascular function above and beyond their lipid-lowering effects. Analysis of clinical trial data has shown that the benefits occur early, extend to patients within the normal LDL cholesterol range for Western populations, and exceed those of other lipid-lowering drugs, despite comparable falls in total cholesterol. Through the inhibition of l-mevalonate synthesis, statins also prevent the synthesis of isoprenoid intermediates, including farnesy1 pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Isoprenylation is important in the posttranslational modification of a variety of proteins, including the small GTPases (Rho, Rac, and Ras), and hence plays an integral role in cellular signaling. Moreover, interference with isoprenylation underlies many of the beneficial actions of the statins on vascular endothelium, which include increased endothelial NO synthase (eNOS) expression, proangiogenic effects, increased fibrinolytic activity, reduced secretion of proinflammatory cytokines, and reduced leukocyte adhesion and transmigration.

It is recognized that chronic inflammation is central to the progressive development of atherosclerotic plaques. Complement activation has been proposed as an important component of this response, and this is supported by the presence of C1q through C9 in atherosclerotic lesions, with evidence of complement activation in the form of C3d, C4d, and C5b-9. The generation of sublytic C5b-9 may result in cellular activation, adhesion molecule upregulation, secretion...
of chemokines and growth factors, and proliferation of vascular smooth muscle and endothelial cells (ECs).

Innate mechanisms for the control of complement activation on the cell surface include the membrane-bound regulatory proteins: decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and CD59. DAF prevents the formation and accelerates the decay of C3 and C5 convertases, whereas MCP binds C3b and C4b and facilitates their degradation by factor I, and CD59 acts distally, inhibiting the incorporation of C9 into the C5b-9 membrane attack complex (MAC). In previous work, we have demonstrated that DAF, but not MCP or CD59, is inducible on the EC surface after stimulation with a variety of physiological agonists. We have identified distinct signaling pathways for DAF induction with tumor necrosis factor-physiological agonists. We have identified distinct signaling pathways for DAF induction with tumor necrosis factor-

Northern Blotting

Northern analysis was performed as previously described. Northern blots were quantified by an Appligene Image Analysis System, and densitometry was performed using Image program 1.52 software (National Institutes of Health). Values were corrected with respect to ethidium bromide–stained RNA loading patterns, and an arbitrary value of 1 was assigned to unstimulated ECs.

Western Blotting

Western blot analysis was performed as described previously. The anti-PKCα antibody (C-20) was from Santa Cruz Biotechnology, and anti–phospho-PKCα was from Upstate Biotechnology. Changes in phospho-PKCα were quantified by the Appligene Image Analysis System, and values were corrected with respect to the PKCα bands.

C3 Binding and Cell Lysis Assays

The methods used for detection of cell surface C3 and cell lysis were as described previously. ECs cultured in the presence and absence of atorvastatin for 24 hours were opsonized with mAb RAMC8 and incubated with 5% to 20% normal human serum (NHS) for 3 hours at 37°C before analysis by flow cytometry. In the inhibition studies, blocking mAbs were added at a final concentration of 25 µg/mL. To estimate complement-mediated cell lysis, ECs pretreated with atorvastatin or plain medium alone for 24 hours were loaded with calcein AM (Molecular Probes), opsonized, and incubated with 5% to 20% baby rabbit serum (Serotec) for 30 minutes at 37°C. Percent specific lysis in triplicate wells was calculated as follows: (complement-mediated calcein release–spontaneous release)/(maximal release–spontaneous release) × 100%, where maximal release is complement-mediated release + detergent-mediated release.

Statistical Analysis

Differences between the results of experimental treatments were evaluated by the Mann-Whitney U test. Differences were considered significant at values of P < 0.05.

Results

Statin-Induced Endothelial Cytoprotection

As seen in Figure 1A, 24-hour exposure of HUVECs to atorvastatin led to a significant unimodal increase in DAF expression. In 10 separate experiments, atorvastatin increased DAF expression by 3- to 4-fold above the baseline on unstimulated HUVECs (mean ± SEM RFI was 50.8 ± 11.3 for unstimulated HUVECs and 222.4 ± 38.4 for atorvastatin-stimulated HUVECs, P < 0.01). Dose-response studies showed an increase in DAF expression after treatment with 0.05 to 0.1 µmol/L atorvastatin, with maximal upregulation at 1 to 2.5 µmol/L (Figure 1B). To determine the kinetics of DAF induction, HUVECs were cultured in the presence of atorvastatin for up to 48 hours. An increase in DAF was first...
detectable 12 to 18 hours after treatment and was maximal at 24 to 48 hours (Figure 1C). In contrast, atorvastatin had no effect on the surface expression of MCP, and although an increase in CD59 expression was seen, this did not reach significance (Figure 1D). Significant upregulation of DAF was also seen after treatment with 0.0625 to 0.125 μmol/L simvastatin, with a maximal response at 1.25 μmol/L (Figure 2A). Experiments performed on HAECs (to represent a vascular bed affected by atherosclerosis) and on the human dermal microvascular EC line HMEC-1 confirmed that they behaved in the same way as HUVECs in terms of DAF upregulation in response to atorvastatin and simvastatin (Figure 2B). Therefore, further experiments were performed with HUVECs.

Statin-Induced DAF Expression Requires Increased Steady-State mRNA and De Novo Protein Synthesis

To determine whether the increase in DAF after statin treatment was dependent on gene transcription, HUVECs were pretreated with actinomycin D before the addition of atorvastatin. The presence of actinomycin D completely inhibited atorvastatin-induced DAF expression (Figure 3A). Northern analysis was performed using mRNA extracted from unstimulated HUVECs and cells stimulated with atorvastatin for up to 24 hours. Two DAF mRNA transcripts (2.4 and 1.8 kb) were detected at low levels in unstimulated ECs (lane 1, Figure 3B). In three experiments, atorvastatin treatment led to an increase in DAF mRNA that was first detectable at 6 to 9 hours and was persistent at 24 hours after stimulation. Quantification of mRNA levels using densitometric scanning of the 2.4-kb band demonstrated a 3-fold increase above baseline at 24 hours after stimulation.

To investigate whether the effect of atorvastatin on DAF gene transcription was direct or required synthesis of a transactivating factor, ECs were preincubated for 30 minutes with cycloheximide before the addition of atorvastatin. As previously described, incubation with cycloheximide alone led to a superinduction of steady-state DAF mRNA by up to 60%, as quantified by densitometry (lane 6, Figure 3B). Incubation with atorvastatin and cycloheximide led to a rise in steady-state DAF mRNA similar to that seen with cycloheximide alone (lanes 7 and 8, Figure 3B). This was in contrast to thrombin, which induced an increase in DAF mRNA in the presence of cycloheximide (not shown). The failure of atorvastatin to enhance the cycloheximide-induced rise in steady-state DAF mRNA suggests that the changes in DAF gene transcription observed are indirect and dependent on the synthesis of an intermediary protein.

To determine whether the increase in cell surface DAF was dependent on de novo protein synthesis, HUVECs were
pretreated with cycloheximide before the addition of atorvastatin. This led to a complete abrogation of atorvastatin-induced DAF (Figure 3C). Taken together, these observations suggest that upregulation of DAF expression by statins is associated with gene transcription, an increase in steady-state DAF mRNA, and de novo protein synthesis.

**Effect of Mevalonate and Isoprenoid Intermediates**

Because HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate, we tested the capacity of L-mevalonate to override the effect of statins on DAF expression. L-Mevalonate inhibited DAF upregulation by atorvastatin (Figure 4A), simvastatin, and mevastatin (not shown) in a dose-dependent manner, suggesting that statin-induced DAF expression involves inhibition of HMG-CoA reductase. After this, a series of experiments was performed to further investigate the molecular signal transduction pathway by which the statins modulate EC DAF expression. Pretreatment of ECs with the isoprenoid intermediates GGPP or GGOH led to a dose-dependent inhibition of atorvastatin-induced DAF upregulation (Figure 4B). On the other hand, preincubation of ECs with FPP or squalene, downstream metabolites in the cholesterol synthesis pathway, did not influence statin-induced DAF expression (Figure 4C). These data suggest that geranylgeranylated proteins negatively regulate constitutive endothelial DAF expression.

**Role of Rho-GTPases**

Because geranylgeranylation is known to activate Rho-GTPases, we tested the effect of the geranylgeranyl trans-
ferase inhibitor GGTI-286 and C3 exoenzyme, an inhibitor of Rho-GTPases. Treatment of ECs for 24 hours with GGTI-286 or C3 exoenzyme resulted in a significant increase in DAF expression (Figure 5A). These data indicate that statin-induced DAF expression is mediated, at least in part, by inhibition of the geranylgeranylation of small G proteins.

Effect of PI-3 Kinase, NO, and PKC Pathways on Statin-Induced DAF Expression

Treatment of HUVECs with statins results in the phosphorylation of Akt by a mechanism involving phosphatidylinositol-3 kinase (PI-3 kinase).7,26 To investigate the role of PI-3 kinase in statin-induced DAF upregulation, we preincubated HUVECs with LY290042 before the addition of atorvastatin for 24 hours. As shown in Figure 6A, the presence of LY290042 paradoxically enhanced DAF expression alone and in the presence of atorvastatin.

Statins have also been shown to stabilize eNOS mRNA and increase local synthesis of NO.27 To investigate the role of NO in statin-induced DAF upregulation, we preincubated HUVECs with LY290042 before the addition of atorvastatin for 24 hours. As shown in Figure 6A, the presence of LY290042 paradoxically enhanced DAF expression alone and in the presence of atorvastatin.

We have previously identified distinct PKC-dependent and -independent pathways for the regulation of DAF in ECs.18–20 To examine whether PKC was implicated in statin-induced DAF expression, we initially used a pharmacological inhibitor of the classic and novel PKC isozyme (PKC-β).28 Although G6976 inhibited simvastatin-induced DAF expression, LY379196 had no effect (Figure 7B). This suggested that PKC-β was the principle isoenzyme involved in statin-induced DAF expression, and this was further supported by use of the cell-permeable peptide myr-ιPKC, specific for PKCα and PKCβ,29 which abrogated atorvastatin-induced DAF expression (Figure 7B). Furthermore, Western blotting with an antibody specific for the phosphorylated form of PKCα demonstrated a 3-fold increase in PKCα phosphorylation after 30 minutes of exposure to atorvastatin (Figure 7C), as quantified by densitometry.

Statin-Induced DAF Expression Enhances EC Resistance to Complement-Mediated Injury

To address the functional significance of DAF upregulation by statins, the effects of atorvastatin on complement factor C3 deposition on the EC surface were measured. Unstimulated and atorvastatin-treated HUVECs were opsonized with RMAC8, an IgG2a anti-endoglin mAb. Endoglin is highly expressed on the EC surface and was not influenced by incubation of HUVECs with atorvastatin for 24 hours. Opsonized HUVECs were incubated with 20% NHS for 3 hours, and C3 binding to the cell surface was quantified by flow
cytometry. Compared with no treatment, atorvastatin reduced C3 deposition on the EC surface by 60% (Figure 8A). The inhibitory anti-DAF mAb 1H4 was used to confirm the role of DAF in the reduction of C3 binding observed. In addition, the inhibitory mAb (A35) against CD59 and a control antibody (EN4), which would not be expected to inhibit C3 binding, were studied. As seen in Figure 8B, the addition of 1H4 markedly increased the binding of C3 to unstimulated opsonized ECs exposed to 20% NHS. Moreover, the reduction in C3 binding seen in response to atorvastatin treatment was reversed by 1H4, with levels of C3 deposited on the cell surface becoming equivalent to those observed on unstimulated ECs in the presence of 1H4 (Figure 7B). In contrast, neither A35 nor EN4 had any effect on the level of C3 binding observed (not shown).

To assess the physiological relevance of this reduction in C3 binding, HUVECs were loaded with calcein AM, opsonized with mAb RMAC8, and exposed to baby rabbit serum. Endothelial lysis was subsequently measured by estimation of calcein release. As seen in Figure 8C, pretreatment of ECs with atorvastatin for 24 hours was cytoprotective, significantly reducing cell lysis after exposure to 5% to 10% rabbit serum. However, at serum concentrations of ≥20%, the cytoprotective effect of atorvastatin was overcome. These observations suggest that the increased levels of cell surface DAF seen in response to the treatment of ECs with statins provide additional protection against complement-mediated injury.

Discussion
The effects of statins in the reduction of cardiovascular morbidity and mortality can be detected remarkably early, before significant angiographic changes. Furthermore, statins benefit patients with normal-range LDL cholesterol, raising the possibility that they have beneficial effects beyond cholesterol lowering.1,2 The data presented in the present study demonstrate a novel potentially atheroprotective action of statins, namely, the regulation of complement activation.

Treatment of ECs with three different statins (atorvastatin, simvastatin, and mevastatin) resulted in an upregulation of DAF expression at least equivalent to that previously ob-
served by us with physiological agonists. The concentrations of atorvastatin and simvastatin that we used compare favorably with the concentrations used in other recent investigations. Increases in DAF expression were seen after treatment with 0.05 to 0.25 μmol/L atorvastatin. These equate to the Cmax (maximum amount of drug found in blood after administration) of 15 to 252 ng/mL (0.014 to 0.23 μmol/L) reported in pharmacokinetic studies of atorvastatin using standard oral doses of 20 to 80 mg daily. Furthermore, a Cmax of 34 ng/mL (0.08 μmol/L) after a 40 mg dose of simvastatin is also higher than the minimum concentration (0.0625 μmol/L) capable of inducing DAF expression. However, maximal responses were achieved with supratherapeutic concentrations, and it has yet to be determined how the concentrations of statins used in vitro studies relate to plasma levels achieved in vivo after an oral dosage.

The time course of changes that we observed in EC DAF mRNA and protein expression after culture with statins was similar to that previously reported after stimulation with vascular endothelial growth factor or intestinal trefoil peptide and most probably depend on the synthesis of an intermediate transcriptional activator. According to our in vitro data, statin-induced DAF expression is likely to be not only sustained but also functionally relevant. Thus, treatment of ECs with atorvastatin resulted in a 60% reduction of C3 binding, which was reversed in the presence of the DAF inhibitory mAb 1H4. The relevance of this reduction in C3 deposition was confirmed by the significant fall in complement-mediated cell lysis observed in statin-treated ECs.

Because the response to statins was dependent on gene transcription and was inhibited by cycloheximide, it is likely that de novo protein synthesis is the predominant means by which DAF expression is increased on the cell surface. However, it is possible that statin-induced cholesterol depletion could reduce endocytosis of glycosylphosphatidylinositol-anchored proteins through interference with membrane lipid rafts and hence increase surface expression. Although this may account for the modest increase in CD59 expression observed, it is unlikely to be a major component in statin-induced DAF upregulation because reconstitution of the cholesterol synthesis pathway by the addition of squalene had no effect.

The inclusion of mevalonate prevented DAF upregulation by the statins, confirming the role of HMG-CoA reductase inhibition. Mevalonate is the substrate for cholesterol synthesis and also for isoprenoid intermediates, including FPP and GGPP. The effects of the statins on cell surface DAF were independent of their inhibition of cholesterol synthesis, as squalene, the precursor of cholesterol, had no effect. However, the inclusion of GGPP and GGOH inhibited statin-induced DAF upregulation, suggesting that geranylgeranylated proteins downregulate the expression of DAF. This was supported by the observation that the geranylgeranyltransferase inhibitor GGTI-286 significantly increased EC-surface DAF. In contrast, the addition of FPP had no effect, suggesting that farnesylated proteins do not play a role in DAF regulation. This distinction between the roles of geranylgeranylation and farnesylation in statin-mediated effects on vascular endothelium has been reported previously, with the inhibition of geranylgeranylation seemingly more important.

In vascular ECs, Ras translocation is dependent on farnesylation, whereas geranylgeranylation has been implicated in Rho translocation. Thus, the upregulation of DAF by GGTI-286 and the inhibition of statin-induced DAF by GGPP and GGOH suggest that inhibition of Rho might be important in DAF upregulation. This was confirmed by the observation that C3 exoenzyme, an inhibitor of Rho-GTPase, induced a significant rise in DAF cell surface expression. However, the upregulation of DAF after treatment with C3 exoenzyme or GGTI-286 was always less than that seen with atorvastatin, an observation also made for lovastatin-induced responses, suggesting that statins influence additional downstream signaling pathways in ECs beyond the inhibition of Rho.

Recent studies have revealed a role for the PI-3 kinase/Akt pathway in statin-induced angiogenesis. In addition, statins stabilize eNOS mRNA, resulting in increased local concentrations of NO. Notwithstanding this, statin-induced DAF expression was independent of both PI-3K activation and NO generation. Paradoxically, exposure of ECs to the PI-3K inhibitors LY290042 and wortmannin enhanced both basal DAF and statin-induced DAF expression. This implies that DAF expression is under tight regulation and is downregulated by the activation of PI-3K/Akt and upregulated by a distinct parallel pathway.

We have previously described PKC-dependent pathways of DAF upregulation in ECs. Inhibition of the classic and novel isoforms of PKC with GF109203X abrogated statin-induced DAF upregulation. Furthermore, experiments with Gö6976, LY379196, and particularly the specific inhibitory peptide myristoylated PKC suggest that PKCα is the predominant isozyme involved in this response. This conclusion was supported by the demonstration that atorvastatin induced phosphorylation of PKCα in HUVECs. The mechanism by which the statins activate PKC and confirmation of a link between changes in the activation state of PKCα and Rho in the upregulation of DAF remain to be determined. Evidence to date suggests that protein-protein interactions between PKC isoforms, (including PKCα) and Rho-GTPases do occur. Although the outcome varies between cell types and assay systems, the data suggest that significant cross talk occurs between these two pathways. Indeed, a recent study has demonstrated that phorbol ester–induced PKC activation results in a significant decrease in Rho activity in aortic smooth muscle cells.

Complement activation has an established role in the pathogenesis of inflammatory cardiovascular diseases, including atherosclerosis, myocardial infarction, and the accelerated atherosclerosis of transplantation. Analysis of atherosclerotic plaques has revealed the presence of activation products, including C3d, C4d, and the C5b-9 membrane attack complex. Complement may be activated in the arterial wall by deposition of immune complexes, such as those generated by autoantibodies against oxidized lipoproteins, and by cholesterol crystals, modified LDL, and C-reactive protein. Although DAF and other complement inhibitory proteins have been identified in atherosclerotic lesions, their expression, regulation, and function are poorly understood. However, a recent study demonstrated that although expression of DAF, MCP, and CD59 is evident in normal arteries, there is no increase in their expression in the face of complement activation in atherosclerotic lesions. This led to the proposal that inhibition of complement activation...
may be required to control disease progression. According to our observations, it is possible that the ability of the statins to prevent coronary events in patients with relatively low lipid levels is due, at least in part, to a reduction in complement activation within the atherosclerotic vessel wall through the upregulation of DAF expression on ECs and perhaps also other cells in the vessel wall.

In conclusion, we have demonstrated a novel cytoprotective action of the statins that involves increased expression of the complement regulatory protein DAF. This may represent a means by which endothelium can be therapeutically conditioned for the prevention and treatment of atherosclerosis and other vascular inflammatory diseases involving complement activation.

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References

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