Statins Exert Endothelial Atheroprotective Effects via the KLF2 Transcription Factor*

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3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, have been shown to positively impact vascular function independent of their plasma lipid-lowering action. Several of these beneficial effects involve modulation of gene expression. Here we explored whether the transcription factor Kruppel-like factor 2 (KLF2), a biomechanically activated gene we recently identified as part of the endothelial “atheroprotective phenotype,” is regulated by statins and whether this mechanism is important for the non-lipid lowering beneficial effects mediated by these drugs in endothelium. The mRNA levels of KLF2 in human umbilical vein endothelial cells increased in the presence of various statins. KLF2 induction was observed within 8 h after drug treatment and remained elevated for at least 24 h. This statin effect on KLF2 expression was reversed by addition of mevalonate and its downstream metabolite geranylgeranyl pyrophosphate. Furthermore, inhibition of protein geranylgeranylation with GGTI-298 significantly induced KLF2 levels, whereas inhibition of farnesylation did not. Statin-mediated KLF2 expression was followed by the up-regulation of several of its downstream transcriptional targets. Using small interfering RNA to block KLF2 expression, we demonstrated that this transcription factor is necessary for the statin-mediated regulation of several pathophysiologically relevant genes. These results strongly implicate KLF2 as a transcriptional regulator of the statin-mediated effects in vascular endothelium and provide a novel mechanism for the well established non-lowering beneficial cardiovascular effects of statins.

Statins act to reduce plasma cholesterol levels by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and currently constitute the most widely prescribed class of drugs for the reduction of morbidity and mortality associated with cardiovascular disease. Interestingly, many of the clinical benefits afforded by these drugs appear to be mechanistically unrelated to their lipid-lowering effects (1, 2). These non-lipid-lowering beneficial effects include improvement in the production of endothelial derived relaxing factor as well as other aspects of endothelial dysfunction (2, 3). Indeed, short term statin treatment improves NO-mediated vasodilation and myocardial perfusion before changes in LDL-cholesterol can be detected (4, 5). Because the clinical benefits in patients on statins occur early in the course of therapy, are seen in patients with LDL-cholesterol levels that are within the normal range, and often appear not to be correlated directly with the degree of LDL-cholesterol reduction, it has been suggested that these benefits may be a result of yet-to-be-defined direct vascular actions, in particular in the endothelium (1, 6). At the cellular level, statins have been shown to transcriptionally up-regulate eNOS gene expression, stabilize eNOS mRNA, as well as directly influence the activity of the enzyme in endothelium (7). In addition, statins also suppress the expression of the vasoconstrictor endothelin-1 (EDN-1) in endothelial cells (EC) (8). Several studies have demonstrated anti-inflammatory effects of these drugs on various cell types implicated in atherosclerosis, including endothelial cells (1). These effects include endothelial suppression of adhesion molecule (VCAM-1, E-selectin) and cytokine/chemokine (MCP-1, IL-8, RANTES (regulated on activation normal T cell expressed and secreted)) expression. Both in vitro and in vivo; additionally, these effects are correlated with reduced macrophage infiltration in early atheroma (9, 10). Statins also have been shown to reduce endothelial thrombogenicity, in part via induction of thrombomodulin expression. Thrombomodulin is an endothelial-specific cell surface molecule that generates activated protein C, a potent inhibitor of the coagulation cascade as well as an anti-inflammatory substance (11). The net beneficial pathophysiological influence of these various non-lipid lowering transcriptional effects of statins on endothelial cells strongly suggests these changes may be a major component of their clinical efficacy.

KLF2 is a zinc finger-containing transcription factor implicated in blood vessel development and T lymphocyte activation (12–14). Expression of KLF2 in cultured EC is increased by steady laminar shear stress, a biomechanical stimulus (15, 16). Moreover, we have demonstrated induction of KLF2 in cultured human EC in response to the complex hemodynamic environment found in regions of the carotid bifurcation that are resistant to the development of atherosclerosis, thus suggesting that this transcription factor may exert an “atheroprotective” function (15, 17). Interestingly, using in situ hybridization, Dekker et al. (15) have shown a relative lack of KLF2 expression in the endothelial lining at bifurcations in the human aorta, a region where early lesions of atherosclerosis are prone to develop. Recent work in our laboratory indicates this...
transcription factor regulates multiple endothelial functions by acting as an integrator of hemodynamic and humoral stimuli. KLF2 overexpression inhibits the proinflammatory and prothrombotic endothelial gene expression observed in the face of proinflammatory stimuli (16, 18). Because the KLF2-dependent transcriptional and functional effects observed in endothelial cells exposed to atheroprotective flow were reminiscent of the previously reported statin-mediated changes in endothelial gene expression, we directly examined whether KLF2 and its transcriptional targets are regulated directly by statins.

**MATERIALS AND METHODS**

**Cell Culture—**Human umbilical vein endothelial cells (HUVEC) were plated at densities of 60,000 cells/cm² in 6-well plates (Costar) in medium 199 (BioWhittaker) supplemented with 50 μg/ml endothelial cell growth supplement (Collaborative Research), 100 μg/ml heparin (Sigma), 100 units/ml penicillin-G + 100 μg/ml streptomycin (BioWhittaker), 2 mM L-Gln (Invitrogen), and 20% fetal bovine serum (BioWhittaker). Cells were allowed to grow to confluence for 24 h while incubated under 37 °C and 5% CO₂ conditions.

**RNA Isolation and Quantitative Real-time Taqman PCR—**RNA isolation, cDNA preparation, and real-time Taqman PCR were performed essentially as described previously (17). Briefly, RNA was ethanol-precipitated from the TRIzol lysates, and integrity was assessed using the RNA 6000 Nano-Assay using a microfluidics system, 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Quantitative reverse transcription-PCR was performed on a 7900HT sequence detection system (Applied Biosystems). Expression of β-2-microglobulin was used as an endogenous control in all experiments.

**Statin Dose and Time Response—**After initial culturing as described above, 2 ml of fresh media were added with different statins at concentrations of either 100 nM or 1 μM. Cerivastatin was a kind gift from K. Shimizu and R. Mitchell. Simvastatin, lovastatin, and pravastatin were obtained from Calbiochem, Inc. and solubilized in ethanol. Control medium (0 nM statin) was also assessed, as was medium treated solely with 20 μl of ethanol to serve as a vehicle control for the lovastatin and simvastatin solutions. Cells were again incubated for 24 h under 37 °C and 5% CO₂ conditions, rinsed with DPBS, and harvested in 500 μl of TRIzol (Invitrogen). For the cerivastatin dose response, cells were cultured in fresh media with either 0 (control), 10 nM, 100 nM, 1 μM, or 10 μM cerivastatin, and cells were incubated for 24 h. Cells were then rinsed with DPBS and harvested in 500 μl of TRIzol. For the cerivastatin time course response, HUVECs were plated as usual before addition of either 0 (control) or 100 nM concentration of cerivastatin in fresh medium. Cells were then incubated for 2, 4, 8, 12, or 24 h. At the corresponding time intervals, cells were rinsed with DPBS and harvested in 500 μl of TRIzol.

**Mevalonate, Isoprenoid, and Inhibitor Experiments—**HUVEC were cultured in the presence of 100 nM cerivastatin with or without varying concentrations of mevalonate, geranylgeranyl pyrophosphate (GGPP), or farnesyl pyrophosphate (FPP) before RNA isolation and quantitation. Purity of GGPP or FPP was reported by the manufacturer to be 100% by TLC for the batches used in these experiments. Methanol was used as carrier control also used the same dilution of Me²SO in media. Cells were cultured with the indicated concentrations of inhibitors for 8 h and total RNA was isolated 24 h later.

**RESULTS**

To investigate whether KLF2 may play a role in controlling the transcriptional expression of multiple endothelial genes modulated by statins, we asked whether statins regulate the expression levels of KLF2. Incubation of cultured HUVEC with two doses (100 nM and 1 μM) of four different statins for 12 h revealed a significant up-regulation of KLF2 for three of them, with cerivastatin mediating the strongest increase of about 8-fold (Fig. 1A). Interestingly, pravastatin had no effect on KLF2 expression at either of the concentrations tested, which is consistent with other studies examining transcriptional changes in response to this relatively hydrophilic statin (11). Thus, the up-regulation of KLF2 in HUVEC appears to be a general class effect of statins.

The time course of KLF2 induction by cerivastatin was assessed by measuring KLF2 levels in HUVEC at different time points following incubation with the drug at a dose of 100 nM (Fig. 1B). The first detectable increase of KLF2 was observed at 4 h, and it peaked at 8 h. While KLF2 levels remained elevated past 8 h compared with control, the cerivastatin-mediated induction was slightly less at the later time points. These data indicate that KLF2 levels peak at 8 h in response to cerivastatin treatment and remain elevated up to at least 24 h, suggesting the change in KLF2 expression is not a rapid transient response.

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FIG. 1. **Statins induce endothelial KLF2 expression.** A, KLF2 mRNA levels were measured by reverse transcription-PCR in HUVEC cultured for 24 h in normal medium, medium with ethanol vehicle, or 100 nM and 1 μM concentrations of simvastatin, lovastatin, cerivastatin, or pravastatin. B, KLF2 expression in HUVEC exposed to 100 nM cerivastatin for varying amounts of time. All data are expressed as mean ± S.E. (n = 5; *, p < 0.05; **, p < 0.01).
By inhibiting the activity of HMG-CoA reductase, statins deplete cells of this immediate metabolite of the enzyme, mevalonate, and its downstream products (Fig. 2A). To test whether the cerivastatin-mediated KLF2 up-regulation is dependent on inhibition of HMG-CoA reductase, we tested whether adding back mevalonate to the cells would reverse the effect of cerivastatin. Cells were incubated with or without cerivastatin, and with a range of concentrations of mevalonate, for 24 h. Mevalonate, or the ethanol vehicle alone, had no effect on KLF2 expression if cerivastatin was not present (Fig. 2B). Cerivastatin increased KLF2 levels as shown previously, and with a range of concentrations of mevalonate in the media. Mevalonate thus reverses the effects of cerivastatin on KLF2 expression in a dose-related manner, indicating these changes are dependent on inhibition of HMG-CoA reductase.

Mevalonate yields the downstream products FPP and GGPP. These isoprenoids serve to tether important signaling proteins (e.g. Ras, Rho, Rac) to cell membranes and thereby regulate their activity (Fig. 2A). To determine whether one of these arms of the mevalonate pathway was particularly important for the observed statin-mediated effects on KLF2 expression, FPP or GGPP was added to the medium of HUVEC treated with cerivastatin for 8 h. Incubation of HUVEC with FPP or GGPP alone had no effect on basal KLF2 expression (Fig. 2C). Addition of FPP (10 μM) modestly yet significantly reduced KLF2 expression and functional phenotypes (17). For example, EC exposed to the wall shear stress waveforms present in atherosclerosis-resistant arterial geometries expressed genes characteristic of anti-inflammatory and anti-thrombotic endothelial phenotypes. We have also recently demonstrated that the expression of the transcription factor KLF2 and several of its transcriptional targets are uniquely induced by the atheroprotective waveform and that the flow-mediated KLF2 expression confers an atheroprotective endothelial phenotype.2 If statins also modulate their major transcriptional targets by up-regulating KLF2 expression, we would predict that KLF2 induction by statins would precede the statin-mediated regulation of its downstream transcriptional targets. Because we found KLF2 up-regulation to first appear at 4 h and peak by 8 h, we tested the expression of important established and novel KLF2 targets after 8 and 24 h of incubation with 100 nM cerivastatin (Fig. 2D). Three of the most highly regulated transcriptional targets of KLF2 are argininosuccinate synthetase (ASS), C-type natriuretic peptide (CNP), and prostaglandin D2 synthase (PTGDS). ASS is a limiting factor for eNOS substrate bioavailability, while CNP is an endothelial secreted peptide that also functions in vasodilation. PTGDS produces as one of its products the most potent known endogenous ligand of peroxisome proliferator-activated receptor-γ, 15d-PGJ2, which has well characterized anti-inflammatory activity (19). Our analysis revealed, for the targets tested, a significant up-regulation of CNP by the cerivastatin treatment at the 8 and 24 h time points and only at the 24 h time point for the rest of the targets.
(Fig. 3A). Because KLF2 induction is detectable at 4 h, it is clear that these targets are regulated at a later time point, consistent with a primary regulatory role of KLF2 in their transcripitional response to cerivastatin.

To test the role of KLF2 up-regulation in statin-mediated effects in endothelial cells, we prevented the cerivastatin-mediated induction of KLF2 using siRNA. Cerivastatin treatment of endothelial cells preincubated with KLF2 siRNA resulted in KLF2 levels similar to control conditions (Fig. 3B). To assess the functional consequences under these conditions, we analyzed the expression of various transcriptional targets previously identified as being responsive to statins (20). Of the genes known to be regulated by statins, we found that the up-regulation of thrombomodulin, eNOS, and integrin β4 by cerivastatin (100 nM) was completely lost when up-regulation of KLF2 by cerivastatin was inhibited (Fig. 3B). Down-regulation of MCP-1, which has been proposed as an important anti-inflammatory effect of the statins, also was lost if KLF2 up-regulation was blocked. We also asked whether the novel transcriptional targets we have identified as KLF2-regulated were responsive to cerivastatin. All three of these targets (ASS, CNP, PTGDS) were potently regulated by cerivastatin, but abrogation of KLF2 up-regulation abolished their induction by the drug. Up-regulation of KLF2 thus appears to be required for multiple pathophysiologically relevant changes in endothelial gene expression induced by cerivastatin.

**DISCUSSION**

There is growing evidence that the clinical benefits from the use of statins may derive from their effects on processes and cell types beyond those involved in systemic reduction of plasma cholesterol levels (1). One aspect of great interest is the direct vascular effects of statins, particularly on the endothelium (2). While some biologically important direct effects of these drugs on the vascular endothelium are clearly rapid, and therefore probably not a result of transcriptional regulation (e.g. phosphorylation of eNOS and Akt) (21, 22), many studies indicate that important transcriptional changes do occur in endothelial cells exposed to statins, changes that would act to confer a “vasoprotective” or atheroprotective effect (11, 23, 24). Here we identify KLF2 as a transcriptional target of statins and demonstrate its requirement in statin-dependent endothelial transcriptional changes. Of four statins tested, three (simvastatin, cerivastatin, lovastatin) were shown to induce endothelial KLF2 expression at pharmacological concentrations achieved in patients (25, 26). Pravastatin had no effect at the concentrations tested, possibly due to its relative hydrophilicity, which would predictably limit its entry into endothelial cells by passive diffusion (2). Our finding that pravastatin had the least efficacy in inducing KLF2 also parallels findings regarding this drug in other studies using several cultured cell types, including endothelial cells (11). The time course of KLF2 up-regulation is rapid and precedes the regulation of all the other statin targets ass, consistent with an upstream role of KLF2 on these transcriptional targets. Importantly, these statin-mediated effects are reversed by addition of mevalonate, indicating that the effect is dependent on inhibition of HMG-CoA reductase.

The downstream products of mevalonate include isoprenoids that are critical for post-translational modification of major signaling molecules such as Ras, Rho, and Rac, allowing for membrane localization and signaling (27). We found that exogenous addition of GGPP, but not FPP, could completely reverse the effects of statins on KLF2 expression, implying that the observed changes are mediated by depleting cells of GGPP-dependent biochemical processes. Because FPP is metabolized to form squalene and ultimately cholesterol, this result also suggests that deprivation of these molecules cannot explain our observed induction of KLF2. Nevertheless, the modest yet significant contribution we observed for FPP in the statin-dependent up-regulation of KLF2 may suggest a minor contribution of the sterol pathway. Importantly, we observed that the cerivastatin-dependent induction of KLF2 was reproduced with a geranylgeranyl transferase inhibitor in a dose-related fashion but not with a farnesyl transferase inhibitor. Taken together, our data thus point to statins up-regulating endothelial KLF2 via depletion of GGPP-dependent signaling pathways in these
cells. GGPP, but not FPP, is critical for the activity of Rho superfamily proteins (e.g. Rho, Rac) among many others. Determining which of the geranylgeranylated signaling molecules responsible for the change in KLF2 expression could possibly reveal a pathway that mediates tonic suppression of KLF2 under control (no drug) conditions, possibly at the transcriptional level or via epigenetic mechanisms (e.g. chromatin modifications). Addition of statins or GGTIs in this model would limit the activity of this suppressive pathway, thus allowing KLF2 to be expressed highly. It will thus be of great interest to test the in vivo effects on atherosclerosis by GGTIs, which would have no predicted effect on the lipid profile while potently inducing KLF2 in endothelial cells and possibly other cell types. When statins are administered to patients, these drugs most likely affect other cell types involved in inflammation in addition to endothelial cells, including lymphocytes. Importantly, KLF2 expression has been demonstrated in lymphocytes, and its absence leads to a constitutively activated phenotype in T cells (13). Given the importance of KLF2 for endothelial gene expression in response to statins, and the demonstrated inhibitory effects of statins on lymphocyte function, it will be critical to assess whether there exists an analogous role for KLF2 in lymphocytes to further explain the currently growing immunomodulatory view of statins in the context of atherogenesis (28).

We have identified CNP, an endothelial secreted vasodilatory and anti-inflammatory peptide that acts on adjacent smooth muscle cells (29, 30), as a potently induced target of KLF2 and statins in endothelial cells. To date, biomarkers for pathobiological changes in the cardiovascular system largely detect later stage processes in which various cell types (e.g. platelets, inflammatory cells) and molecules (e.g. PA, PAI-1) are involved (31). Most recently, attention has been focused on serum markers that serve as an overall index of "systemic inflammation" (e.g. C-reactive protein, CRP) and show a strong correlation with the morbidity and mortality associated with coronary artery disease (32–34). However, it would be very desirable to identify circulating biomarkers that can act as more direct indicators of endothelial dysfunction, because current clinical evidence suggests that the earliest pathophysiologic predictors of atherosclerosis and its comorbidities (e.g. metabolic syndrome X) may be subtle deficiencies in proper endothelial functions including but not limited to blood vessel formation, control of vascular tone, inflammation, and coagulation. Moreover, RNAi induced "silencing" of KLF2 expression in endothelial cells exposed to atheroprotective shear stress stimulation leads to the marked suppression of atheroprotective gene expression and the flow-mediated atheroprotective phenotype. In addition, overexpression experiments have demonstrated a role for KLF2 in the maintenance of an anti-inflammatory and anti-thrombotic endothelial phenotype (16, 18).

The identification of KLF2 as a major target of statins, one of the most widely used class of drugs for cardiovascular disease, raises the possibility that statins could promote a global atheroprotective endothelial phenotype that is largely dependent on KLF2 up-regulation. Indeed, experiments recently performed in our laboratory have shown that statins are able to shift the flow-mediated atheroprotective phenotype observed in endothelial cells exposed to a waveform characteristic of atherosclerosis-susceptible regions of the human carotid toward the flow-mediated atheroprotective phenotype, as assessed by genome-wide analysis of transcriptional regulation. Importantly, here we demonstrated that KLF2 is both necessary and sufficient to modulate many of the known endothelial atheroprotective transcriptional responses characterized to date for statins. Given the pleiotropy of KLF2 in endothelial function, and its intimate relationship to the beneficial effects of statins on the vascular endothelium, designing drugs specifically to modulate endothelial KLF2 expression, or its downstream effectors, could result in novel therapies promoting vascular health in various diseases in which endothelial dysfunction plays a pathogenic role.

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