Kruppel-Like Factor 2 as a Novel Mediator of Statin Effects in Endothelial Cells

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Background—Although 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are known to modulate endothelial function, the transcriptional mechanisms underlying these effects are incompletely understood. We hypothesized that Lung-Kruppel–like factor (LKLF/KLF2), a novel and potent regulator of endothelial gene expression, may mediate the downstream effects of statins. Here we report that statin-induced expression of endothelial NO synthase (eNOS) and thrombomodulin is KLF2 dependent.

Methods and Results—KLF2 mRNA was induced by treatment with multiple statins in a concentration-dependent manner. Multiple lines of evidence suggest that this induction is dependent on inhibition of the Rho pathway and requires de novo transcription. Furthermore, promoter deletion and mutational analyses suggest that mevastatin induced KLF2 promoter activity through a single myocyte enhancer factor binding site. Finally, small-interfering RNA–mediated knockdown of KLF2 strongly attenuated the ability of mevastatin to increase eNOS and thrombomodulin accumulation in endothelial cells.

Conclusions—Taken together, these observations indicate that statin-dependent induction of eNOS and thrombomodulin requires KLF2 and thereby provides a novel molecular target for modulating endothelial function in vascular disease. (Circulation. 2005;112:720-726.)

Key Words: endothelium ■ nitric oxide synthase ■ vessels ■ transcription
novel transcription involving the myocyte enhancer factor 2 (MEF2) proteins. Finally, small-interfering-RNA (siRNA)–mediated knockdown of KLF2 attenuates the ability of statins to induce eNOS and thrombomodulin. Our results identify a novel pathway by which statins regulate endothelial gene expression.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVECs) were acquired from Cambrex Bioscience (Walkersville, Md) and cultured in EBM-2 media according to manufacturer’s instructions. All statins were purchased from Calbiochem and prepared according to manufacturer’s recommendations. The thrombomodulin and MEF2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); the eNOS antibody was from BD Biosciences (Palo Alto, Calif); the α-tubulin antibody was from Sigma (St Louis, Mo). The adenoviral constructs were generated by the Harvard Gene Therapy Initiative (Boston, Mass). The MEF2A and MEF2C plasmids were kindly provided by E.N. Olson (Dallas, Tex). The −1.7-kB KLF2-Luc promoter was a kind gift from J. Leiden (Abbott Laboratories). All deletion constructs of the KLF2 promoter were generated by polymerase chain reaction (PCR) and cloned into the PGL2 basic vector. Mutation of the MEF site was accomplished by using the QuikChange mutagenesis following the manufacturer’s instruction (Stratagene). The RNA polymerase inhibitor 5,6-dichlorobenzimidazole riboside (DRB) was purchased from Calbiochem and prepared according to manufacturer’s instructions (Stratagene). The RNA polymerase inhibitor 5,6-dichlorobenzimidazole riboside (DRB) was purchased from Calbiochem and prepared according to manufacturer’s instructions (Stratagene). The RNA polymerase inhibitor 5,6-dichlorobenzimidazole riboside (DRB) was purchased from Calbiochem and prepared according to manufacturer’s instructions (Stratagene).

Northern and Western Blot Analysis

Cellular protein was extracted in RIPA buffer, and Western blot analyses were performed with the use of the indicated antibodies as previously described. HUVECs were infected with Ad-GFP and Ad-GFP-KLF2 for 24 hours and then harvested for total protein analysis. Total RNA was obtained by using Trizol following the manufacturer’s instructions, and Northern blot studies were performed as previously described. The KLF2 and KLF6 cDNA fragments were generated by reverse transcriptase (RT)–PCR.

mRNA Half-Life Studies

Determination of KLF2 mRNA stability was performed as follows. HUVECs were treated with the RNA polymerase inhibitor DRB under basal conditions or after 24 hours of statin treatment (n = 3). Total RNA was harvested at 0, 20, 40, 60, 80, and 100 minutes after addition of DRB. After hybridization with KLF2 cDNA, band intensities were analyzed by densitometry, normalized to 18S ribosomal RNA, and plotted semilogarithmically as a function of time.

Transient Transfection Assays

HUVEC cells were plated at a density of 5 × 10^4 per well in 12-well plates 1 day before transfection. A total of 0.5 μg of the indicated plasmid DNA were transiently transfected with the use of FuGene 6 reagent (Roche Molecular Biochemicals) according to instructions by the manufacturer. Cells were treated with mevastatin 24 hours after transfection, harvested 48 hours after transfection, and assayed for luciferase activity normalized with β-galactosidase activity in each sample. For all transfections, n = 6 to 9.

Gel-Shift Studies

The MEF site within the KLF2 promoter was used for gel-shift studies. The wild-type sequence was 5'-CCAGCGCTTATATAACGGCGAGAGAGCGCAATGCTATCGGT-

Results

Effect of Mevastatin and KLF2 on Endothelial Gene Expression

Previous studies indicate that statins can induce factors such as eNOS and thrombomodulin. Consistent with these observations, we found that treatment of human HUVECs with mevastatin (10 μmol/L) induced eNOS and thrombomodulin protein levels (Figure 1A).

We recently reported the observation that adenoviral overexpression of KLF2 in HUVECs alters the expression of endothelial products such as eNOS and thrombomodulin. As shown in Figure 1B, by comparison with uninfected (mock) and control infected cells (Ad-GFP), adenoviral...

Figure 1. Induction of eNOS and thrombomodulin (TM) by mevastatin and KLF2. A, Mevastatin induces eNOS and thrombomodulin. HUVECs were treated with mevastatin (10 μmol/L) for 24 hours, and expression of eNOS and thrombomodulin was assessed by Western blot analysis. Graph represents quantitative results from 3 independent experiments. *P<0.008 (statin vs vehicle). B, KLF2 induces eNOS and thrombomodulin. HUVECs were infected with control (Ad-GFP) and KLF2 adenovirus for 24 hours, and expression of eNOS and thrombomodulin was assessed by Western blot analysis. Graph represents quantitative results from 3 independent experiments. *P<0.004 (KLF2-infected cells vs control virus).
overexpression of KLF2 (Ad-GFP-K2) robustly induced both eNOS and thrombomodulin protein levels.

**Statin-Mediated Induction of KLF2 Expression Is Dependent on Cholesterol Synthesis**

The similar effect of statins and KLF2 on the expression of these target factors raised the possibility that KLF2 may mediate some of the statin effects. As a first step, we assessed the effect of several statins on KLF2 mRNA expression. As shown in Figure 2A, mevastatin, simvastatin, and lovastatin all induced KLF2 mRNA expression in HUVECs ≈3.5 to 4-fold (Figure 2A, graph). In contrast, no significant effect was seen with pravastatin. The specificity of this inductive effect was verified by the fact that expression of KLF6, another member of this family known to be expressed in the endothelium, was not significantly altered by statin treatment (Figure 2A). Furthermore, the induction of KLF2 by mevastatin, lovastatin, and simvastatin occurred in a concentration-dependent manner (Figure 2B).

As a consequence of their ability to inhibit HMG-CoA reductase, statins cause cells to be depleted in mevalonate. To test whether the statin-mediated induction of KLF2 expression was specific and dependent on mevalonate depletion, HUVECs were incubated with mevastatin in the presence or absence of GGPP, FPP, or mevalonate.
absence of mevalonate. As shown in Figure 2C (lane 2 versus lane 5), supplementation with mevalonate completely blocked statin-dependent induction of KLF2.

Mevalonate is a precursor for cholesterol as well as isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). The isoprenoids are important posttranslational lipid modifications of a variety of proteins such as Ras and Rho. Ras proteins are predominantly farnesylated, whereas the Rho proteins are mainly geranylgeranylated. To test whether Ras or Rho has a role in the statin-dependent induction of KLF2, HUVECs were incubated with mevastatin in the presence of the isoprenoid intermediates FPP and GGPP. As shown in Figure 2C, GGPP (lane 3) partially reversed the mevastatin-mediated induction of KLF2 mRNA. In contrast, no effect was seen with FPP (lane 4). Furthermore, treatment of HUVECs with the Rho inhibitor exotoxin C3 augmented KLF2 expression (Figure 2D). These data suggested that inhibition of Rho maybe important for KLF2 induction.

To address this more directly, HUVECs were coinfected with an adenovirus expressing RhoA under the control of tetracycline transactivator and an adenovirus constitutively expressing the transactivator. Rho expression was seen only on coinfection with both adenoviruses. As a consequence, KLF2 expression was strongly reduced (Figure 2E). Taken together, these data strongly suggest that activation of RhoA reduces KLF2 expression.

Statins Induce the KLF2 Promoter via MEF Binding Site

To gain greater insight about the molecular basis for statin-mediated induction of KLF2, we first performed experiments in the presence or absence of the RNA polymerase inhibitor DRB. As shown in Figure 3A, the induction of KLF2 by mevastatin was completely abrogated by pretreatment with DRB (50 μmol/L). Furthermore, half-life studies revealed that KLF2 mRNA stability was not significantly affected by statin treatment. In the absence of mevastatin, KLF2 mRNA half-life was 56 minutes, whereas in the presence of mevas- tatin the half-life was 58 minutes (data not shown). These data suggest that the statin-mediated induction of KLF2 requires de novo transcription.

To understand the transcriptional basis for statin-mediated induction, promoter studies were undertaken. As shown in Figure 3B, mevastatin treatment induced the KLF2 promoter activity (221-bp Luc promoter fragment) but lost with further deletion to the 114-bp Luc construct.
we mutated this region in the context of the
events mediated via MEF factors as being operative in the
supershift studies. These data implicate downstream nuclear
specificity of this binding was verified by competition and
able to strongly bind this region of the KLF2 promoter. The
Finally, as shown in Figure 3E, both MEF2A and MEF2C are
inductive effect is largely abrogated when this site is mutated.
MEF2A can transactivate the KLF2 promoter
through a critical 107-bp region.

Thus, treatment with statins induces the KLF2 promoter
cylooxygenase.

Figure 4. KLF2 knockdown attenuates mevastatin-induced accumulation of eNOS and thrombomodulin (TM) protein. A, siRNA-mediated knockdown of KLF2. Specific siRNA (KLF2
siRNA) and nonspecific siRNA (Ctrl siRNA) were transfected into HUVECs for 48 hours, and KLF2/KLF6 mRNA levels were assessed by Northern blot analysis; B, KLF2 knockdown reduces mevastatin induction of eNOS and thrombomodulin. KLF2 and control siRNA were transfected into HUVECs and then treated with mevastatin 24 hours after transfection. Total protein was harvested and assessed for the indicated factors.

Graph represents the results of 3 independent experiments. \(^{*}p<0.01\) (treatment vs vehicle). COX indicates cyclooxygenase.

Through close inspection of this region of the KLF2
promoter, we identified a single consensus MEF binding site. This site is typically bound by members of the MADS-box family of transcriptional regulators that are key regulators of muscle development. Interestingly, 2 members of this family, MEF2A and MEF2C, have also been implicated as regulators of endothelial cell biology.28–30 To assess the importance of this site in statin-mediated induction of the KLF2 promoter, we mutated this region in the context of the −221-bp Luc construct. As shown in Figure 3C, mutation of the MEF site completely prevented the mevastatin-mediated induction of the KLF2 promoter.

To determine whether MEF factors were able to bind and transactivate via this site, we performed cotransfection and gel mobility shift assays. Indeed, as shown in Figure 3D, MEF2A can transactivate the KLF2 promoter \(\approx 7\)-fold. This inductive effect is largely abrogated when this site is mutated. Finally, as shown in Figure 3E, both MEF2A and MEF2C are able to strongly bind this region of the KLF2 promoter. The specificity of this binding was verified by competition and supershift studies. These data implicate downstream nuclear events mediated via MEF factors as being operative in the statin-mediated induction of the KLF2 promoter.

Effect of KLF2 “Knockdown” on Endothelial Gene Expression

To determine whether KLF2 is required for statin-mediated
effects, we undertook siRNA-mediated knockdown studies.31 As shown in Figure 4A, a strong reduction in KLF2 mRNA expression can be achieved with specific siRNA. This effect was maximal by 48 hours and sustained for 72 to 96 hours (data not shown). Furthermore, the knockdown was specific as no reduction in KLF6 levels was observed by KLF2 siRNA (Figure 4A).

Next, HUVECs were transfected with nonspecific or specific siRNA for 48 hours followed by treatment with mevastatin for 24 hours. As shown in Figure 4B and consistent with recent observations,23 the basal level of eNOS protein was reduced by \(\approx 40\%\). Furthermore, the \(\approx 2.7\)-fold induction of eNOS protein by mevastatin was completely abrogated after knockdown of KLF2. Similarly, an \(\approx 72\%\) reduction in thrombomodulin levels was noted under basal conditions after knockdown of KLF2.23 In addition, although treatment with mevastatin induced thrombomodulin levels \(\approx 3.1\)-fold in the nonspecific siRNA-treated cells, only a 1.4-fold induction was noted after KLF2 knockdown. Taken together, these data support a critical role for KLF2 in mediating the ability of mevastatin to induce endothelial gene expression.

Discussion

The central findings of this study are that statins induce KLF2 expression and that a reduction in KLF2 expression attenuates statin-mediated accumulation of eNOS and thrombomodulin levels (Figure 4B). To our knowledge, KLF2 is the first transcription factor to be implicated as critical for the ability of statins to induce these key endothelial factors. Our data also implicate another family of transcription factors, the MEFs, as being involved in the induction of KLF2. Taken together, these observations provide a novel pathway by which statins may exact some of their favorable effects in endothelial cells.

Conditions that predispose to atherosclerosis such as dia-
betes, hypercholesterolemia, and hypertension are associated
endothelial dysfunction. As a consequence, the endothelial
phenotype is altered to one that is proadhesive and prothrombotic. Basic and clinical observations strongly sup-
port the ability of statins to improve endothelial health
through differential effects on the expression of certain factors.5,10,32 In this regard, one of the most important effects of statins is to increase eNOS and thrombomodulin levels in endothelial cells. This is thought to occur through both transcriptional and posttranscriptional means.6,7,26 Studies from our laboratory indicate that KLF2 can induce eNOS and thrombomodulin mRNA and promoter activity, suggesting that transcription events underlie, at least in part, the ability of this factor to induce these targets.22,23 However, this does not exclude the possibility that the KLF2 induction of eNOS and thrombomodulin accumulation in endothelial cells may also be, in part, through posttranscriptional effects as well as effects on protein synthesis and stability. Indeed, the require-
ment for KLF2 in the ability of mevastatin to induce eNOS and thrombomodulin strongly suggests that additional mech-
nisms are likely involved.

Another mechanism by which statins confer favorable
effects is by preventing the cytokine-mediated induction of proadhesive molecules and procoagulant substances such as vascular cell adhesion molecule-1 (VCAM-1) and tissue factor.1,2 This is thought to occur by inhibiting the function of proinflammatory mediators such as NF-κB and AP-1.21 In light of our previous study demonstrating that KLF2 inhibits...
NF-κB–mediated induction of VCAM-1/E-selectin, it is possible that some of the inhibitory effects on NF-κB function may be secondary to an increase in KLF2 levels.

Our results also demonstrate that multiple statins, namely, mevastatin, simvastatin, and lovastatin, can all induce KLF2 in a concentration-dependent manner (Figure 2A and 2B). In contrast, no effect was seen with pravastatin, probably because the uptake of this compound is poor in endothelial cells. Furthermore, the induction by mevastatin was dependent on inhibition of cholesterol synthesis (Figure 2C). Our data also support a potential relationship between Rho proteins and KLF2 expression. The Rho proteins are mainly geranylgeranylated, whereas Ras proteins are predominantly farnesylated. The fact that GGPP but not FFP was able to attenuate the ability of mevastatin to induce KLF2 suggests that activation of the Rho pathway negatively regulates KLF2 expression. Consistent with this possibility, treatment with a Rho inhibitor, C3 exotoxin, induced KLF2 expression (Figure 2D). Finally, adenoviral overexpression of Rho strongly reduced KLF2 expression. Interestingly, activation of Rho is thought to be an upstream event that mediates many of the deleterious effects of proinflammatory substances (e.g., interleukin-1β, tumor necrosis factor-α, and thrombin) in endothelial cells. As we have shown previously, these stimuli also reduce KLF2 expression, raising the possibility that one of the mechanisms by which Rho activates the endothelium is through the inhibition of KLF2 expression.

An intriguing observation made in this study is that the inhibition of de novo transcriptional activity prevents the ability of mevastatin to induce KLF2 promoter activity (Figure 3A). These data support a link between the statin-mediated inhibition of cholesterol/Rho activation and a transcriptional event that culminates in the induction of KLF2 expression. Although the details of this link remain incompletely understood, our studies provide an important initial clue. Our promoter deletion studies suggest that the transcriptional mediator(s) inducing the KLF2 promoter likely bind within a specific 107-bp region (from −114→−221; Figure 3B). Our laboratory has recently assessed this critical region and identified a MEF site within the proximal promoter region as critical for activity (A. Kumar, PhD, unpublished data, 2004). The importance of this site, in the context of statin-mediated induction of the KLF2 promoter, was verified by mutational analyses (Figure 3C). These observations are particularly timely in light of a recent report that mutations in MEF2A are associated with severity of coronary atherosclerotic disease. Indeed, it will be interesting to determine whether MEF2A mutations affect KLF2 levels as well as patient response to statin therapy.

On the basis of its ability to differentially regulate endothelial genes, our previous observations implicate KLF2 as a key “molecular switch” governing endothelial function in health and disease. The observations presented here identify KLF2 as a downstream mediator of statin effects in the endothelium. The identification of this regulatory pathway may provide the foundation for novel strategies in the treatment of vascular disease.

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References

Clinical studies over the past decade demonstrate that 3-hydroxy-3-methylglutaryl coenzyme A inhibitors (statins) can substantially reduce cardiovascular mortality. Although originally designed to treat elevated cholesterol levels, accumulating evidence suggests that the beneficial effects of statins exceed what may be anticipated by simply lipid lowering alone. Recent studies suggest that statins can impart favorable properties to vascular cells by alterations in gene expression. For example, treatment of endothelial cells with statins has been shown to result in the accumulation of key factors such as endothelial NO synthase (eNOS) and thrombomodulin that are known to confer antithrombotic, antiadhesive, and antiinflammatory properties to the vessel wall. However, the nuclear mechanism underlying the ability of statins to induce these factors has remained poorly understood. This study provides evidence that the transcription factor termed Kruppel-like factor 2 (KLF2) is a key mediator of statin effects. KLF2 is expressed in endothelial cells, and its levels are potently induced by multiple statins. Furthermore, it is shown that the statin-induced accumulation of eNOS and thrombomodulin in endothelial cells is KLF2 dependent. These observations identify a novel mechanism by which statins regulate endothelial gene expression and provide the foundation for the development of novel strategies in the treatment of vascular disease.
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