Apelin/APJ Signaling Is a Critical Regulator of Statin Effects in Vascular Endothelial Cells—Brief Report

Danielle L. McLean,* Jongmin Kim,* Yujung Kang, Hong Shi, G. Brandon Atkins, Mukesh K. Jain, Hyung J. Chun

Objective—The endothelial response elicited by the G-protein–coupled receptor pathway involving apelin and APJ predicts an overall vasoprotective effect. As a number of downstream endothelial targets of apelin/APJ signaling are also known to be targeted by statins (3-hydroxy-3-methyl-glutaryl [HMG]-CoA reductase inhibitors) as potential mediators of their known pleiotropic effects, we evaluated for the involvement of apelin/APJ signaling in statin endothelial effects.

Methods and Results—We found that disruption of apelin/APJ signaling in endothelial cells leads to significantly decreased expression of Krüppel-like factor 2, endothelial nitric oxide synthase, and thrombomodulin. We found that statin-mediated induction of Krüppel-like factor 2, endothelial nitric oxide synthase, and thrombomodulin expression, as well as inhibition of monocyte-endothelial adhesion, was abrogated by concurrent apelin knockdown. Moreover, we found that statins can transcriptionally regulate APJ in a Krüppel-like factor 2–dependent manner, demonstrating the presence of a positive-feedback loop.

Conclusion—Our findings provide a novel mechanism by which the apelin/APJ pathway serves as a critical intermediary that links statin to its pleiotropic effects in regulating endothelial gene targets and function. (Arterioscler Thromb Vasc Biol. 2012;32:2640–2643.)

Apelin/APJ (also known as apelin receptor or AGTRL1) signaling is emerging to have a significant role in vascular homeostasis. We previously found that augmentation of apelin/APJ signaling can protect against atherosclerosis in apolipoprotein E–deficient mice. This was found to be mediated, at least in part, via augmentation of nitric oxide production, and our recent studies have demonstrated increased endothelial nitric oxide synthase (eNOS) phosphorylation and expression secondary to apelin/APJ signaling. The considerable atheroprotection offered by statins is believed to be above and beyond that expected from their cholesterol-lowering capabilities. The pleiotropic effect is thought to involve direct targeting of the endothelial cells (ECs) by statins, via upregulation of genes such as Krüppel-like factor 2 (KLF2), eNOS, and thrombomodulin (THBD). Interestingly, 2 recent clinical trials demonstrated that statin treatment in patients with hyperlipidemia results in robust increases in serum apelin levels. Here, we provide a novel mechanism that identifies apelin/APJ signaling as a critical intermediary that mediates the endothelial cellular responses to statin. We show the following: (1) disruption of apelin/APJ signaling leads to abrogation of statin induced KLF2, eNOS, and THBD expression, (2) statins robustly induce the endothelial expression of APJ, and (3) KLF2 is a critical regulator of APJ expression.

Materials and Methods
Please refer to the online-only Data Supplement for a detailed description of the materials and methods used in this study.

Mice
KLF2 flox/flox mice were previously described. These mice were crossed to CAG-CreERT2 mice (Jackson Labs), and Cre-mediated excision was induced in 4-week-old mice by injection with tamoxifen (1mg/injection×10 daily injections) intraperitoneally. All mouse experimentation was done in congruence with the Institutional Animal Care and Use Committee of Yale University and Case Western Reserve University.

Results
Apelin Regulates KLF2 and Downstream Targets in Multiple Endothelial Cells
KLF2 is known to be a critical regulator of endothelial homeostasis. We previously found that KLF2 is downregulated in the lungs of apelin-deficient mice and human pulmonary artery ECs subjected to apelin/APJ knockdown. To determine whether apelin regulation of KLF2 occurs in the ECs from other vascular beds, we evaluated the effect of apelin knockdown in human umbilical vein ECs (HUVECs) and human coronary artery...
We found that in these ECs there was a marked reduction of KLF2 mRNA and protein expression with apelin knockdown (Figure 1A and 1B). We also evaluated the effect of augmented apelin signaling on the KLF2 promoter activity using a luciferase reporter driven by a minimal 221-bp KLF2 promoter. We found that apelin overexpression in HUVECs was able to robustly increase the promoter activity (Figure 1C). We next determined whether apelin-mediated KLF2 expression results in the regulation of the downstream targets of KLF2. We found that both eNOS and THBD, known targets of KLF2, are significantly downregulated with apelin knockdown (Figure 1D and 1G).

**Intact Apelin/APJ Signaling Is Critical for Statin-Mediated Endothelial Gene Regulation and Function**

Previous studies demonstrated that statins can induce a number of genes thought to be critical for endothelial homeostasis, including KLF2, eNOS, and THBD. To determine whether apelin/APJ signaling plays a role in the statin-mediated expression of these target genes, we evaluated the effects of apelin knockdown on statin stimulation in HUVECs. We found a robust induction of KLF2, eNOS, and THBD by stimulation with statin, which was markedly reduced when these cells were concurrently subjected to apelin knockdown (Figure 1E-1G).

We further evaluated whether abrogation of apelin/APJ signaling can impair the physiological effects of statin on ECs. Previous studies demonstrated that statins can reduce monocyte adhesion to ECs, at least in part via induction of THBD and eNOS. To determine whether apelin/APJ signaling plays a role in this process, we determined monocyte adhesion to ECs in response to statin in conjunction with apelin knockdown. Fluorescent-labeled THP-1 monocytes were incubated with HUVECs pretreated with rosvastatin or simvastatin and apelin small interfering RNA. We found a significant reduction of monocyte adhesion to HUVECs that were pretreated with statin as expected (Figure 1H). When statin was administered to cells subjected to apelin knockdown, we found a marked inhibition in the statin-mediated reduction of monocyte adhesion to HUVECs (Figure 1H).
Statins Induce a Known Downstream G-Protein Target of APJ
To further characterize the statin–apelin/APJ link, we determined whether statins have the capacity to augment APJ activation. Apelin–APJ signaling is known to induce activation of the G-protein subunit G\(_{\alpha}\)q, leading to the inhibition of forskolin-induced cAMP production.19,20 We found that apelin stimulation robustly inhibited the forskolin-induced cAMP production in APJ overexpressing COS cells (Figure 1I). Remarkably, we also found a comparable inhibition of cAMP production in APJ overexpressing COS cells when APJ-transfected COS cells were stimulated with statin agents (Figure 1I). This statin effect was dependent on APJ, as control cells not expressing APJ failed to respond to statin or apelin (Figure 1I).

Statins Induce Positive Feedback of Apelin/APJ Signaling via Upregulation of APJ Expression
We next evaluated whether statin can affect the expression of the components of apelin/APJ signaling. We found no significant change in apelin expression in HUVECs with statin treatment, suggesting that the increased apelin levels observed in recent clinical trials8,9 likely originate from another cell type (Figure I in the online-only Data Supplement). Interestingly, we found that all statin agents tested robustly induced APJ expression in HUVECs (Figure 2A and 2B). A previous study demonstrated that KLF2 may be involved in transcriptional regulation of APJ in Xenopus.21 Indeed, we found putative KLF2 binding sites in the human APJ promoter (data not shown), and found that APJ upregulation temporally followed the simvastatin-mediated induction of KLF2 expression (Figure 2C). To determine whether KLF2 may be involved in the statin-mediated induction of APJ, we evaluated the effects of small interfering RNA-mediated KLF2 knockdown on APJ expression. We found a significant reduction of APJ expression in HUVECs treated with KLF2 small interfering RNA, as well as abrogation of statin-mediated induction of APJ in the context of KLF2 knockdown (Figure 2D and 2E). We also assessed APJ mRNA and protein expression using lung tissue from floxed control (KLF2:fl/fl+tamoxifen) and KLF2 knockout (KLF2:fl/fl:CAGCreERT2) mouse lungs (Figure 2F and 2G). KLF2 knockdown also abrogates statin-induced APJ induction (\(P<0.01\)).

**Figure 2.** APJ is transcriptionally regulated by statin and Krüppel-like factor 2 (KLF2). A, Stimulation of human umbilical vein endothelial cells (HUVECs) with 3 statin agents leads to a robust induction of APJ mRNA. \(^*P<0.01\). B, Statin stimulation leads to increased APJ protein expression in HUVECs. \(^*P<0.01\) and \(^*P<0.001\). C, Simvastatin stimulation leads to induction of APJ, which is preceded by KLF2 induction in HUVECs. CAGCreERT2, Cre dependent for the generation of mice lacking KLF2. D and E, Knockdown of KLF2 in HUVECs leads to significant reduction of APJ mRNA (graph) and protein levels. KLF2 knockdown also abrogates statin-induced APJ induction. \(^*P<0.01\). F and G, Significantly decreased APJ mRNA and protein levels in the lungs of KLF2-deficient mice. \(^*P<0.05\). H, KLF2 overexpression via adenovirus induces luciferase reporter driven by the putative APJ promoter containing consensus KLF2 binding sites (−1392 bp) but not shorter promoter constructs lacking KLF2 binding sites. \(^*P<0.01\). I, Putative model of statin and apelin/APJ-mediated endothelial transcriptional regulation. Statin-mediated augmentation of apelin–APJ signaling results in induction of downstream targets, including KLF2, endothelial nitric oxide synthase (eNOS), and thrombomodulin (THBD). KLF2 in turn induces transcription of APJ. siRNA indicates small interfering RNA.
(KLF2:fl/fl:CAGCreERT2+tamoxifen) mice, given the embryonic lethality of KLF2-null mice. In accordance with the cell culture data, APJ levels were significantly reduced in the KLF2 knockout mice (Figure 2F and 2G). Moreover, we generated luciferase reporter constructs that contain the upstream putative promoter regions of the human APJ gene. Three different lengths of APJ promoter (−201 bp, −450 bp, or −1392 bp) reporter constructs were generated, with only the longest reporter constructs containing the putative KLF2 binding sites. There was no effect of KLF2 overexpression on the promoter activity of the 2 shorter (−201 bp and −450 bp) constructs that lack KLF2 binding sites. However, cells transfected with the −1392 bp construct displayed significantly increased promoter activity when treated with the KLF2 virus (Figure 2H).

Discussion

Our findings are the first to demonstrate that an intact apelin/APJ signaling pathway is critical for the previously described pleiotropic effects of statins on ECs. Pleiotropic effects of statins have been well documented and are associated with the improvement of cardiovascular outcomes above and beyond the lipid-lowering effects. Here, we demonstrate that abrogation of apelin/APJ signaling leads to a significant inhibition of statin-mediated induction of KLF2, eNOS, and THBD. Moreover, we found that statin stimulation leads to activation of Gαi in an APJ-dependent manner, as well as transcriptional induction of APJ, the only known receptor for apelin. Further studies are warranted to determine whether statins directly activate APJ or potentiate apelin-mediated activation of APJ. These findings suggest the presence of a positive-feedback loop, where statin-mediated target gene induction is dependent on apelin/APJ signaling in the endothelium, and the resulting increase in KLF2 expression leads to further augmentation of apelin/APJ signaling via upregulation of APJ (Figure 2I). Our findings provide a novel mechanism of the effects of statin on the endothelium and significantly extend our understanding of the importance of apelin/APJ signaling in atherosclerosis and vascular homeostasis.

Sources of Funding

This work was supported by grants from the National Institutes of Health (HL095654 and HL101284 [to H.J. Chun]; HL088740 [to G.B. Atkins]; and HL086548, HL097593, and HL076754 [to M.K. Jain]) and the Howard Hughes Medical Institute (Physician Scientist Early Career Award [to H.J. Chun]).

Disclosures

None.

References

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Arterioscler Thromb Vasc Biol. 2012;32:2640-2643; originally published online September 20, 2012;
doi: 10.1161/ATVBAHA.112.300317

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary Figure 1. Statin does not alter apelin expression in HUVECs. A) HUVECs stimulated with 10 µM of rosvustatin for 24 hours showed no change in apelin mRNA levels. B) Apelin ELISA using supernatants from HUVECs stimulated with statins show no change in apelin protein levels.
Supplementary Table 1: Oligonucleotide sequences for generation of APLNR promoter

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1392bp 5’ oligo</td>
<td>TTTCTCTATCGATAAGTACCCTGCAGGGATCATCTGAGGTCA</td>
</tr>
<tr>
<td>450bp 5’ oligo</td>
<td>TTTCTCTATCGATAAGTACCCTGCAGGGATCATCTGAGGTCA</td>
</tr>
<tr>
<td>201bp 5’ oligo</td>
<td>TTTCTCTATCGATAAGTACCTGCAGGGAGACAGGCTTC</td>
</tr>
<tr>
<td>Common 3’ oligo</td>
<td>CCGGAATGCCAAGCTTCCTGCCCCATAGTAGTTGTC</td>
</tr>
</tbody>
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**Red**: restriction site  
**Black**: genomic sequence  
**Green**: recombination site
Supplementary Materials and Methods

Cell Culture and reagents. HUVECs (Yale VBT Core) were cultured in EBM-2 (Lonza) and THP-1 monocytes (ATCC) were grown in RPMI. COS cells (ATCC) were grown in DMEM (Invitrogen). Rosuvastatin (AstraZeneca) and fluvastatin (Novartis) were solubilized in DMSO and used at 10 µM concentration. Simvastatin (Sigma) was first activated to its active form as previously described. In brief, 4 mg of simvastatin was dissolved in 100 µl of ethanol. Then, 150 µL of 0.1 N NaOH was added to the solution that was subsequently incubated at 50°C for 2 h. The pH was brought to 7.0 by HCl, and the final concentration of the stock solution was adjusted to 4 mg/ml. Cells were stimulated with the statin agents for 24 hours. KLF2 and control adenoviruses were purchased from Welgen.

Real-time Quantitative PCR. Total RNA was harvested using miRNeasy kit (Qiagen), and cDNA was generated with iScript kit (BioRad). Real-time PCR reactions were carried out using TaqMan probes (Invitrogen) with Ssofast Probes Supermix (BioRad).

Apelin ELISA. Analysis of apelin in cell culture medium was performed using a quantitative ELISA kit (Phoenix Pharmaceuticals).

Assay for inhibition of cAMP. Either COS cells (ATCC) or COS cells transfected with APJ (www.cdna.org) were plated in 96-well plates and preincubated with serum-free medium containing 500µM isobutyl-1-methylxanthine (IBMX, Sigma) and 100µM 4-(3-butoxy-4-methoxybenzyl) imidazolidone (Ro 20-1724, Sigma) at 37 °C for 20 min. Cells were then treated with stimulation buffer containing forskolin (4 µM) in the absence or presence of apelin-13 (1uM), rosvastatin (10uM) or simvastatin (10uM) at 37 °C for 15 min. The cAMP level was determined using a cAMP-Glo™ Assay (Promega) according to the manufacturer's instructions.
**APJ Promoter Luciferase Reporter.** The 201bp, 450bp, and 1392bp proximal human APJ promoter was generated using the respective primers (Supp. Table 1) by PCR from genomic DNA and cloned into the pGL3 vector using the In-Fusion cloning kit (Clontech).

**Luciferase Reporter Assays** HUVECs were transfected with KLF2-luciferase, APJ-luciferase, renilla-luciferase (Promega), and apelin (Origene) constructs and luciferase assay was performed using the Dual-Luciferase Reporter Assay kit (Promega).

**SiRNAs.** SiRNAs against apelin and KLF2 (Invitrogen) were transfected using RNAiMax (Invitrogen).

**Monocyte Adhesion Assay.** HUVECs were transfected with control or apelin siRNA for 24h before statin treatment for an additional 24h. Fluorescently labeled THP-1 moncytic cells (BCECF-AM, Invitrogen) were incubated with the HUVECs for 1h at 37°C. Following removal of non-adherent cells by washing, monocyte adhesion to HUVECs was quantified by measuring the fluorescent cell areas (Adobe Photoshop CS4) in five independent images per condition. The graph depicts averages of three independent experiments.

**Western Blot.** Western blots were carried out using antibodies against eNOS (BD Transduction Laboratories), KLF2 (mouse samples: Santa Cruz, human samples: Aviva Systems), THBD (Santa Cruz), APJ (MBL International) and GAPDH (Cell Signaling) purchased from the respective vendors and as previously described.

**Statistical Analysis.** All experiments (including qPCR analyses, western blots, and monocyte binding assays) were repeated a minimum of three times in triplicates and data expressed as means ± S.E.M. Data were analyzed by the unpaired Student’s t test or by one-way analysis of variance with Bonferroni post-hoc test using GraphPad Prism software. Differences were considered significant at \( p < 0.05 \).
References


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A) HUVECs stimulated with 10 µM of rosuvastatin for 24 hours showed no change in apelin mRNA levels.  B) Apelin ELISA using supernatants from HUVECs stimulated with statins show no change in apelin protein levels.