Critical Promoter Region for Statin-Induced Human Endothelial Nitric Oxide Synthase (eNOS) Transcription in EA.hy926 Cells

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Aim: Statins have many anti-atherogenic effects apart from reducing the serum level of low density lipoprotein cholesterol (LDL-C). For instance, statins can enhance the expression of endothelial nitric oxide synthase (eNOS), at least partly by upregulating its transcription. Although it has been reported that -786 T/C polymorphism of the promoter region has an important influence on statin-induced transcription of the human eNOS gene, much remains unclear about statin-induced eNOS transcription. We tried to identify other statin-responsive promoter regions.

Methods: A human endothelial cell line (EA.hy926 cells) was treated with pitavastatin, atorvastatin, or fluvastatin, after which eNOS mRNA levels were assessed by quantitative real-time RT-PCR. EA.hy926 cells were also transiently transfected with luciferase reporter genes driven by various lengths of the human eNOS promoter and were treated with statins before luciferase activity was measured.

Results: Statin treatment increased eNOS mRNA levels in EA.hy926 cells. In addition, cells transfected with the reporter gene driven by the eNOS promoter fragment starting from position -740 exhibited a pitavastatin-induced increase of luciferase activity, which was not observed in cells transfected with the reporter gene driven by the fragment starting from -727. Similar results were also obtained with atorvastatin and fluvastatin.

Conclusions: Statins enhanced eNOS expression in EA.hy926 cells, at least partly by inducing its transcription. Although a statin-responsive sequence that could function even in a heterologous promoter was not precisely identified, the region of the human eNOS promoter around position -730 seems to be critical for statin-induced transcriptional activation.


Key words: Statin, Endothelial cells, eNOS, Gene regulation

Aim

It is recognized that deterioration of endothelial cell function can trigger the development of atherosclerosis. Endothelial cells not only act as a physical barrier that prevents the infiltration of monocytes or macrophages into the intima, but also produce various factors, including nitric oxide (NO), that regulate vascular tone and blood flow. NO is produced by endothelial NO synthase (eNOS), which is expressed in endothelial cells, and NO production is decreased at the sites of atherosclerotic lesions as a result of endothelial dysfunction. On the other hand, NO deficiency itself can be atherogenic, as has been demonstrated by a study performed in eNOS-null mice; therefore, preservation of normal eNOS expression is essential for the prevention of atherosclerosis.

Hydroxymethyl glutamyl coenzyme A (HMG-CoA) reductase inhibitors, so-called statins, have been extensively used for the treatment of dyslipidemia associated with elevation of low-density lipoprotein (LDL) cholesterol levels. These drugs inhibit hepatic cholesterol synthesis in the liver and increase LDL receptor expression, thereby enhancing hepatic uptake of LDL from the blood. Thus, statins have an anti-atherogenic effect by lowering serum LDL-cholesterol
RT-PCR

EA.hy926 cells were purchased from the ATCC (Manassas, VA, USA). After incubation in DMEM with 50 mg/mL LPDS for 24 h, the cells were treated with either 9.0 μM pitavastatin calcium (Wako, Osaka, Japan), 50 μM atorvastatin calcium (Sigma-Aldrich, St. Louis, MO, USA), 50 μM fluvastatin sodium (Wako), or 0.1% (v/v) dimethyl sulfoxide (DMSO, the vehicle) for a further 24 h. Three dishes of cells received each treatment. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and was treated with RNase-free DNase (Promega, Madison, WI, USA). Reverse transcription was performed using the extracted total RNA, random hexamer primers (Amersham, Uppsala, Sweden), dNTPs, RNAsin (Promega), and Moloney murine leukemia virus reverse transcriptase (Promega). The cDNA thus obtained was subjected to quantitative real-time PCR, which was performed using a LightCycler TaqMan Master kit (Roche Applied Science, Penzberg, Germany) and a LightCycler Nano (Roche Applied Science). The primers for eNOS (5’-CTCACCCGCTACACATCCTGCTG-3’ and 5’-TTTCCACAGGGACGAGGT-3’) and the probe used for detection (probe #85; Roche Applied Science) were determined using the Roche Applied Science Universal Probe Library Assay Design Center (http://www.roche-applied-science.com). For each sample, real-time PCR amplifica-

<table>
<thead>
<tr>
<th>Table 1. Primers used for PCR amplification of the human eNOS promoter sequence</th>
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<tr>
<td>Forward Primers:</td>
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<tr>
<td>F-1695 5’-GACTGTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<tr>
<td>F-1668 5’-GACTGTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-1248 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<tr>
<td>F-795T 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-795C 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-774 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-763 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<tr>
<td>F-740 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-727 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-720 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<td>F-680 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<tr>
<td>F-466 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
</tr>
<tr>
<td>F-362 5’-AGCTACGAGGTTGCAATGACAACTCACTG-3’</td>
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<tr>
<td>Backward Primer:</td>
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<tr>
<td>5’-TACGAAGCTTTTATCTGTGCCACACTCTGCTGCT-3’</td>
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Underlined: Kpn I site  
Double-underlined: Hind III site  
(Four nucleotides were attached before the Kpn I or Hind III sites, so that the PCR products would be successfully digested by Kpn I or Hind III.)  
Bold letters in F-795T and F-795C: T/C polymorphism at nucleotide -786

levels⁴); however, statins can also have pleiotropic beneficial cardiovascular effects that are not explained by the reduction of serum LDL-cholesterol⁵). For instance, anti-inflammatory and anticoagulant activities of statins have been demonstrated⁶). Statins may also directly improve endothelial function and increase the expression and activity of eNOS⁷⁻⁹). Statins induce eNOS expression at least partly by stabilizing eNOS mRNA¹⁰), and these drugs may upregulate eNOS transcription. In fact, it has been reported that flavastatin induces human eNOS transcription and that the T/C genotype at position -786 of the human eNOS promoter influences the extent of this induction¹¹). We hypothesized that other regions of the eNOS promoter, in addition to -786 polymorphism, might also influence statin-induced eNOS transcription.

Methods

Cell Culture

A human endothelial cell line (EA.hy926 cells) and a human hepatoma cell line (HepG2 cells) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with antibiotics and 8% (v/v) heat-inactivated fetal bovine serum. Before each experiment, the culture medium was replaced by DMEM supplemented with antibiotics and 50 mg/mL lipoprotein-deficient fetal bovine serum (LPDS)¹²).

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tion of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed simultaneously using the Universal Probe Library Human GAPDH Gene Assay (Roche Applied Science), and the eNOS/GAPDH ratio was calculated.

**Plasmids**

Genomic DNA was isolated from EA.hy926 cells with a DNeasy kit (Qiagen, Tokyo, Japan), and was employed as a template for the following PCRs using KOD DNA polymerase (Toyobo, Osaka, Japan). One of the forward primers and the backward primer (Table 1) were used to amplify the human eNOS promoter fragment. PCR products were digested with Kpn I and Hind III, and then were ligated into the pGL4.10[luc2] vector (Promega) to create a human eNOS promoter-driven firefly luciferase gene. To construct the pGL4-SV40-luc2 plasmid, the SV40 promoter segment between the Sac I and Hind III sites of the pGL3 promoter vector (Promega) was inserted into the pGL4.10[luc2] vector. pSRE2-SV40-luc2, whose promoter contains two tandem typical sterol regulatory elements (SREs)13), and pF40-SV40-luc2 were constructed by inserting 5'-CGCGTG(AAAATCA CCCCCACTGCAAACCTCCTCCCCCTGC)2C-3' (antisense: 5'-TCTAAG(GCAGGGGGAGGATTT GCCGTTGGGTGTATTT)2A-3') and 5'-CGCGTG-ACCTGCAGCCCCGGAGGAGCGTGCCTACT-GAATGACGGGGTgcgac-3' (antisense: 5'-TCGAgtcgacCCCTTGCACTCGAGCAGCAGCTTCCC-GGGGCCGGCAGGTA-3'), respectively, into pSV40-luc2 between the Mlu I and Xho I sites (underlined: Mlu I site; double-underlined: Xho I site, lower case: Sal I site, which was attached for insert verification). For each plasmid, the presence of the correct DNA insert was confirmed by restriction analyses. The numbers indicating nucleotide positions relative to the transcription start site are consistent with those in a previous report11).

**Reporter Gene Assays**

One day before transfection, the culture medium was replaced by DMEM containing LPDS, which was used thereafter. On the day of transfection, a 10-cm dish of cells (~80% confluent) was trypsinized and transferred into a 96-well B&W IsoPlate (PerkinElmer, Waltham, MA, USA). Then, 0.4 μg of a firefly luciferase-expressing plasmid was transfected into each well using the transfection reagent FuGENE (Promega), along with 0.4 ng of pRL-CMV (an internal control plasmid expressing Renilla luciferase). Four hours later, the medium containing the transfection complex was replaced with fresh medium containing 9.0 μM pitavastatin calcium, 50 μM atorvastatin calcium, 50 μM fluvastatin sodium, or 0.1% (v/v) DMSO (vehicle). After 24 hours, cells were lysed for measurement of firefly and Renilla luciferase with the Dual-Luciferase Assay System (Promega), and normalized firefly luciferase activity (the ratio of firefly to Renilla luciferase luminescence) was calculated. In each experiment, 4 to 10 wells were allocated to one treatment, and the mean and standard deviation (SD) were calculated.

**Statistical Analysis**

When two treatment groups (DMSO or pitavastatin) were compared, statistical analysis was performed using Student’s t-test and p<0.05 was considered significant. When three or more treatment groups (DMSO, pitavastatin, atorvastatin or fluvastatin) were compared, analysis of variance (ANOVA) was performed, followed by a post hoc Tukey-Kramer test. All analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC). In the figures, significant differences are indicated by asterisks.

**Results**

First, we tested whether exposure to statins increased human eNOS mRNA levels in EA.hy926 cells. Quantitative real-time RT-PCR revealed that treatment with 9 μM pitavastatin, 50 μM atorvastatin, or 50 μM fluvastatin for 24 hours induced eNOS mRNA expression by 9.5-fold, 8.5-fold, and 8.3-fold, respectively (Fig. 1).
sterol regulatory element (SRE: with the consensus sequence of ATCACNCCAC13)), which is well known to mediate statin-induced transcriptional upregulation of other genes such as the LDL receptor in hepatocytes4), was found around nucleotide -1670 (Fig. 2, underlined nucleotides). To investigate whether this SRE-like sequence was involved in statin-induced transcription of human eNOS, we constructed peNOS-

![Fig. 2. Human eNOS promoter sequence.](image_url)

The bases indicated in bold (nucleotide positions are shown above) indicate the start of each promoter fragment used in this study, whereas the italicized “A” near the end of the sequence (ATG: start codon) shows the end of the fragments. The underlined segment is the putative SRE detected by the TFSEARCH program. The boxed nucleotide “C” (-786) indicates the T/C single nucleotide polymorphism (SNP). This SNP was analyzed previously11), and the nucleotide numbers used here are consistent with the previous report11). The double-underlined segment was ligated to pSV40-luc2 to construct pF40-SV40-luc2.

In order to investigate whether statins enhanced eNOS transcription, firefly luciferase reporter plasmids driven by various human eNOS promoter fragments were constructed. We searched the human eNOS promoter sequence for transcription factor-binding sites that might play a role in statin-induced transcription using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCH.html). As a result, a putative sterol regulatory element (SRE: with the consensus sequence of ATCACNNCCAC13), which is well known to mediate statin-induced transcriptional upregulation of other genes such as the LDL receptor in hepatocytes4), was found around nucleotide -1670 (Fig. 2, underlined nucleotides). To investigate whether this SRE-like sequence was involved in statin-induced transcription of human eNOS, we constructed peNOS-
Key Region for Statin-Induced eNOS

The activity of the human eNOS promoter fragment starting from -795 was enhanced by pitavastatin, while that of the fragment starting from -362 was not, suggesting that a DNA region somewhere between -795 and -362 mediates statin-induced transcription.

Further analyses of the human eNOS promoter region between positions -795 and -362 were performed using the reporter plasmids shown in Fig. 6. Firefly luciferase activity was elicited by pitavastatin in HepG2 cells that had been transfected with the plasmids from peNOS-740-luc2 to peNOS-720-luc2, while it was reduced by pitavastatin in cells that had been transfected with the plasmids from peNOS-720-luc2 to peNOS-466-luc2. In cells transfected with peNOS-720-luc2, firefly luciferase activity was unchanged by pitavastatin treatment, indicating that a DNA element involved in pitavastatin-induced transcription might exist around position -727.

We then tested the effect of pitavastatin treatment on the activity of reporter genes driven by shorter human eNOS promoter segments starting from position -1248 (peNOS-1248-luc2), position -795, or position -362 (peNOS-362-luc2) (Fig. 5). In order to specifically evaluate the influence of -786 T/C polymorphism, peNOS-795T-luc2 and peNOS-795C-luc2 (which respectively had T and C at nucleotide -786) were constructed. Firefly luciferase activity was increased by pitavastatin treatment of EA.hy926 cells that had been transfected with peNOS-1248-luc2, peNOS-795T-luc2, and peNOS-795C-luc2, while it was decreased by pitavastatin treatment of cells transfected with peNOS-362-luc2. Induction of firefly luciferase by pitavastatin was slightly greater in cells transfected with peNOS-795C-luc2 than in cells transfected with peNOS-795T-luc2, a finding which was consistent with the previous report that fluvastatin induces more human eNOS promoter activity in the presence of the -786C genotype than with the -786T genotype. Regardless of whether the nucleotide at -786 was T or C, the activity of the human eNOS promoter fragment starting from -795 was enhanced by pitavastatin, while that of the fragment starting from -362 was not, suggesting that a DNA region somewhere between -795 and -362 mediates statin-induced transcription.

We also tested the effects of atorvastatin and fluvastatin on firefly luciferase activity in EA.hy926 cells transfected with the plasmids from peNOS-763-luc2 to peNOS-705-luc2 (Fig. 7). Firefly luciferase activity was elicited by atorvastatin after transfection with peNOS-763-luc2 or peNOS-740-luc2, but was reduced by atorvastatin after transfection with peNOS-720-luc2 or peNOS-705-luc2. Fluvastatin

1695-luc2 with the SRE-like element and peNOS-1668-luc2 without it. When EA.hy926 cells were transfected with peNOS-1695-luc2, there was no induction of firefly luciferase activity after pitavastatin treatment compared with that in peNOS-1668-luc2-transfected EA.hy926 cells (Fig. 3), suggesting that the SRE-like segment around -1670 was not involved in statin-induced transcription. In addition, while pitavastatin elicited firefly luciferase transcription in HepG2 cells transfected with pSRE2-SV40-luc2 containing typical SREs, but not in HepG2 cells transfected with parental pSV40-luc2 lacking any SREs, the induction of firefly luciferase transcription by pitavastatin was similar in EA.hy926 cells transfected with either pSRE2-SV40-luc2 or pSV40-luc2 (Fig. 4). Thus, SRE-mediated transcriptional upregulation by pitavastatin was observed in HepG2 cells, but was not seen in EA.hy926 cells. Taken together, these results made it highly unlikely that the SRE-like sequence around position -1670 had a role in statin-induced human eNOS transcription.

Fig. 3. Pitavastatin-induced activation of the human eNOS promoter with or without the SRE-like sequence around -1670.

EA.hy926 cells were transfected either with peNOS-1695-luc2 (which contains the SRE-like element) or peNOS-1668-luc2 (which does not), and then treated with the vehicle (DMSO) or pitavastatin. Open and closed columns show the mean normalized firefly luciferase activity measured in cells treated with DMSO or pitavastatin, respectively. Error bars represent SDs for quadruplicate transfections. Asterisks indicate significantly (p < 0.05) higher normalized firefly luciferase activity than that in DMSO-treated cells. The mean value for DMSO-treated cells was defined as 1.0.
atorvastatin and fluvastatin suggested the existence of a statin-responsive region around position -727 of the human eNOS gene, consistent with the results obtained using pitavastatin (Fig. 6).

To isolate the DNA element responsible for transcriptional upregulation by statins, we investigated induced firefly luciferase activity in cells transfected with peNOS-763-luc2, but reduced it in cells transfected with peNOS-763-luc2, peNOS-727-luc2, peNOS-720-luc2, or peNOS-705-luc2. Thus, the response of each plasmid to atorvastatin and fluvastatin was slightly different. However, the overall results of these experiments using induced firefly luciferase activity in cells transfected with peNOS-1248-luc2, peNOS-795T-luc2, peNOS-795C-luc2, or peNOS-362-luc2 were treated with DMSO or pitavastatin. After the promoter segment starting from -795 was obtained by PCR, the forward primer bearing T or C at position -786 was used to construct peNOS-795T-luc2 or peNOS-795C-luc2, respectively. Open and closed columns show the mean normalized firefly luciferase activity for cells treated with the vehicle (DMSO) or pitavastatin, respectively. Error bars represent SDs for eight transfections. Asterisks indicate significantly ($p < 0.05$) different firefly luciferase activity from that in DMSO-treated cells. The mean value for DMSO-treated cells was defined as 1.0.

Fig. 4. Induction of SRE-mediated transcription by pitavastatin in HepG2 cells, but not in EA.hy926 cells.

HepG2 cells and EA.hy926 cells were transfected with either pSRE2-SV40-luc2 or pSV40-luc2 and then were cultured with or without pitavastatin. Open and closed columns indicate the mean standardized firefly luciferase activity in cells treated with the vehicle (DMSO) or pitavastatin, respectively. Error bars represent SDs for ten transfections. Asterisks indicate significantly ($p < 0.05$) higher firefly luciferase activity than that in DMSO-treated cells. The mean value for DMSO-treated cells was defined as 1.0.

Fig. 5. Pitavastatin-induced activation of human eNOS promoter fragments starting from positions -1248, -795, and -362.

EA.hy926 cells transfected with peNOS-1248-luc2, peNOS-795T-luc2, peNOS-795C-luc2, or peNOS-362-luc2 were treated with DMSO or pitavastatin. After the promoter segment starting from -795 was obtained by PCR, the forward primer bearing T or C at position -786 was used to construct peNOS-795T-luc2 or peNOS-795C-luc2, respectively. Open and closed columns show the mean normalized firefly luciferase activity for cells treated with DMSO and pitavastatin, respectively. Error bars represent SDs for eight transfections. Asterisks indicate significantly ($p < 0.05$) different firefly luciferase activity from that in DMSO-treated cells. The mean value for DMSO-treated cells was defined as 1.0.
whether the 40-bp sequence from -751 to -712 (F40, double-underlined in Fig. 2) influenced the statin responsiveness of the SV40 promoter-driven reporter plasmid (Fig. 8). Although pitavastatin weakly induced firefly luciferase activity in EA.hy926 cells transfected with pF40-SV40-luc2, similar induction was observed in cells transfected with the parental plasmid pSV40-luc2, suggesting that F40 did not mediate pitavastatin-induced transcription when ligated to the SV40 promoter. As was the case with pitavastatin, neither atorvastatin nor fluvastatin induced significantly more firefly luciferase activity in EA.hy926 cells transfected with pF40-SV40-luc2 than in cells transfected with pSV40-luc2 (data not shown). Thus, we were unable to identify a statin-responsive DNA element that could function even in a heterologous promoter.

**Discussion**

We constructed reporter genes driven by 5’-deleted human eNOS promoter regions of various lengths, and attempted to narrow down the segment that was critical for the induction of human eNOS transcription by statins. Pitavastatin induced firefly luciferase activity to a similar extent in EA.hy926 cells transfected with a reporter gene driven by the promoter segment with or without the SRE-like sequence around -1670, and the typical SRE that mediates the induction of gene transcription by statins in HepG2 cells did not function in EA.hy926 cells. Thus, it would be safe to conclude that the SRE-like segment around -1670 is not important.

Consistent with the report that fluvastatin elicited more extensive human eNOS transcription in the presence of the -786C/C genotype than with the -786T/T genotype, pitavastatin induced slightly more firefly luciferase activity in EA.hy926 cells transfected with peNOS-795C-luc2 than in cells transfected with peNOS-795T-luc2; however, there seemed to be another crucial determinant located more proximal to the transcription start site. In subsequent analyses, we found that a pitavastatin-responsive region may exist around position -727. This region also seemed to be responsible for transcriptional activation
However, because cells transfected with the plasmids from peNOS-720-luc2 through peNOS-362-luc2 exhibited a decrease of firefly luciferase transcription after treatment with pitavastatin, we concluded that a statin-responsive element does exist around nucleotide -730.

It would be reasonable to assume that one or more transcription factor-binding sites are located around -727; however, the TFSEARCH program did not reveal any consensus sites in this region. At this stage, it seems impossible to detect the binding of any proteins to this region, because we could not isolate the statin-responsive DNA element for use in gel-shift assays and other analyses.

It is necessary to keep in mind the following limitations of our study. Although EA.hy926 is a human endothelial cell line that is widely used for in vitro experiments, it may not be possible to directly extrapolate our results to the endothelial cells of patients on statin treatment. It should be noted that our observations were only obtained in a particular experimental setting using one cell line.

In conclusion, we found that pitavastatin, atorvastatin, and fluvastatin all induce eNOS transcription by atorvastatin and fluvastatin as well as pitavastatin, indicating that it was responsive to various statins; however, we were unable to isolate a statin-responsive element that could function even in a heterologous promoter.

Because statins induced firefly luciferase activity, albeit weakly, even in EA.hy926 cells transfected with the negative control plasmid pSV40-luc2 (Fig. 4 and 8), there is a possibility that the SV40 promoter contains an unidentified statin-responsive element. Nonetheless, it can be safely concluded that neither the typical SRE nor F40 is involved in the response of EA.hy926 cells to statins, because neither of them augmented statin responsiveness after ligation to pSV40-luc2. Another possibility is that there might be an unidentified statin-responsive sequence somewhere in the pGL4 plasmid backbone, other than the SV40 promoter region. This possibility leads us to suspect that statin-induced firefly luciferase transcription observed in EA.hy926 cells transfected with the plasmids from peNOS-1695-luc2 through peNOS-740-luc2 might not be ascribed to the 740-bp human eNOS promoter sequence, but to such an unidentified statin-responsive sequence in the pGL4 backbone.

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in EA.hy926 cells. There seems to be a key statin-responsive segment around -730 which functions in the human eNOS promoter, but not in the heterologous SV40 promoter. Further investigations might elucidate precisely how statins enhance human eNOS promoter activity, which would help to unveil a novel aspect of the pleiotropic effects of statin therapy.

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Conflicts of Interest

None.

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Fig. 8. Effect of the region from -751 to -712 on pitavastatin-induced gene transcription.