Statin Treatment Upregulates Vascular Neuronal Nitric Oxide Synthase Through Akt/NF-κB Pathway


Objective—Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are known to enhance vascular expression of endothelial (eNOS) and inducible nitric oxide synthase (iNOS). In this study, we examined whether statins also upregulate vascular expression of neuronal NOS (nNOS).

Methods and Results—In cultured rat aortic smooth muscle cells, treatment with atorvastatin significantly increased nNOS expression, associated with activation of Akt and NF-κB. Inhibition of Akt by dominant-negative Akt suppressed atorvastatin-induced nNOS expression as well as Akt and NF-κB activation. Inhibition of NF-κB by dominant-negative IkB also attenuated atorvastatin-induced nNOS expression and NF-κB activation, but not Akt activation. We further examined whether atorvastatin also enhances nNOS expression in isolated mouse aorta, and if so, how much nNOS-derived NO accounts for atorvastatin-induced NOx production. In isolated aortas of wild-type mice, atorvastatin significantly increased all three NOS isoform expression and NOx production. In isolated aortas of doubly i/eNOS−/−, n/eNOS−/−, and n/iNOS−/− mice, which express only nNOS, iNOS, and eNOS, respectively, atorvastatin-induced NOx production was approximately 25%, 25%, and 50% to that of wild-type mice, respectively, suggesting that nNOS accounts for 25% of the atorvastatin-mediated NOx production.

Conclusions—These results indicate that atorvastatin upregulates vascular nNOS through Akt/NF-κB pathway, demonstrating a novel nNOS-mediated vascular effect of the statin. (Arterioscler Thromb Vasc Biol. 2007;27:92-98.)

Key Words: Akt ■ neuronal nitric oxide synthase ■ nitric oxide ■ nuclear factor-κB ■ statins

Nitric oxide (NO) that is synthesized by three different NO synthase (NOS) isoforms contributes to the maintenance of vascular homeostasis through multiple mechanisms. Although the roles of endothelial NOS (eNOS) and inducible NOS (iNOS) in the pathogenesis of arteriosclerosis have been extensively investigated, little is known about those of neuronal NOS (nNOS). We have recently demonstrated that nNOS also exerts important vasculoprotective effects in vivo. In a carotid artery ligation model, nNOS−/− mice exhibited accelerated neointimal formation and constrictive vascular remodeling. In a rat balloon injury model, selective inhibition of nNOS activity enhanced vasoconstrictor responses to calcium-mobilizing stimuli, and exacerbated neointimal formation. In these models, nNOS was upregulated in vascular lesions, predominantly in the neointima and medial vascular smooth muscle cells (VSMCs), and to a lesser extent, in endothelial cells. Others have also reported that nNOS is functionally upregulated in the coronary endothelium of eNOS−/− mice, causing nNOS-dependent coronary dilation in response to shear stress or acetylcholine. These findings provide an important concept that nNOS can be regarded as an “inducible” enzyme in the vascular system, in contrast to its constitutive role in the nervous system. However, the regulatory mechanism(s) for vascular nNOS expression remains to be fully elucidated.

Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are potent blockers of cholesterol biosynthesis, and widely used in the treatment of hypercholesterolemia. A number of large clinical trials have demonstrated their clinical usefulness for preventing cardiovascular events, such as myocardial infarction, stroke, and sudden cardiac death. Although statins appear to exert these vasculoprotective effects mainly through an improvement of plasma lipid profile, accumulating evidence has suggested that they also have several non–lipid-lowering actions. These include enhancement of eNOS expression in endothelial cells and that of iNOS expression in...
Upregulation of Vascular nNOS by Atorvastatin

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However, no study has ever addressed the potential effect of statins on vascular nNOS expression. It has been reported that statins activate the serine/threonine protein kinase Akt in a phosphatidylinositol 3 kinase (PI3K)-dependent manner.\(^\text{20}\) It also has been shown that the nuclear transcriptional factor NF-\(\kappa\)B is one of the important downstream targets of Akt.\(^\text{21}\)

Thus, the present study was designed to examine whether atorvastatin upregulates vascular nNOS expression, and if so, whether Akt/PI3K pathway is involved.

Methods

This study was approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan, and was carried out according to the Institutional Guidelines for Animal Experimentation and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Materials

Atorvastatin was provided from Pfizer (New York, NY). Rabbit polyclonal antibodies against nNOS, iNOS, and eNOS were purchased from BD Biosciences (San Jose, Calif). Rabbit polyclonal antibodies against total- and phospho-Akt were from Cell Signaling Technology (Beverly, Mass). A rabbit polyclonal antibody against NF-\(\kappa\)B-alpha and eNOS kinase-beta were generous gifts of Dr Thomas F. Franke (Department of Pharmacology, Columbia University, New York, NY)\(^\text{22}\) and Dr. Aming Lin (Ben May Institute for Cancer Research and the Committee on Cancer, University of Chicago, Chicago, IL),\(^\text{23}\) respectively. Angiotensin II, endothelin-1, l- mevalonate, and a mouse monoclonal antibody against vinculin were from Sigma (Saint Louis, Mo).

Cultured Rat Aortic VSMCs

VSMCs were isolated from the thoracic aortas of 8-week-old male Sprague-Dawley rats by enzymatic dissociation. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a CO\(_2\) incubator. When phosphorylation of Akt and nuclear translocation of NF-\(\kappa\)B p65 was examined, the concentration of FBS in DMEM was reduced to 0.5%. Passages between 3 and 8 were used.

Cultured Human Umbilical Vein Endothelial Cells

Human umbilical vein endothelial cells (HUVECs; BioWhittaker, Walkersville, Md) were grown in phenol red-free EBM media in a CO\(_2\) incubator. Passage 4 was used.

Isolated Mouse Aorta

Eight- to 10-week-old male wild-type (C57BL/6) (Charles River Japan Inc, Yokohama, Japan), doubly n/iNOS\(^\text{+/−}\), n/eNOS\(^\text{+/−}\), i/eNOS\(^\text{+/−}\), and triply n/i/eNOS\(^\text{+/−}\) mice were used.\(^\text{24}\) The mice were euthanized by an overdose intraperitoneal injection of ketamine, and the aortas were excised aseptically.

Reverse Transcription-Polymerase Chain Reaction

Nested polymerase chain reaction for nNOS was performed, as we previously reported.\(^\text{25}\) In nucleic acid sequence analysis, there were 98% of identities between the nNOS RT-PCR product and the previously reported rat nNOS sequence (supplemental Figure I, available online at http://atvb.ahajournals.org).

Western Blot Analysis

Western blot analyses for nNOS, iNOS, eNOS, and phospho Akt were performed as we previously reported.\(^\text{10}\)

Plasmid Transfection

Four \(\mu\)g of each plasmid was transfected into cultured rat aortic VSMCs using the cationic liposome-mediated transfection method (Lipofectamine 2000, Invitrogen Corp, Carlsbad, Calif). In the experiments with dominant-negative mutants of Akt, I\(\kappa\)B, or I\(\kappa\)B kinase, atorvastatin was administered 6 hours after plasmid transfection and was incubated for 24 to 48 hours.

Immunofluorescence

Immunofluorescent staining for NF-\(\kappa\)B p65, nNOS, or vinculin was performed as we previously reported.\(^\text{23}\) Activation of NF-\(\kappa\)B was assessed by nuclear translocation of NF-\(\kappa\)B p65, and nuclear immunofluorescence intensity of NF-\(\kappa\)B p65 was quantified by Axiovision version 4.3 (Carl Zeiss, Jena, Germany).

Statistical Analysis

Results are expressed as mean±SEM. Statistical analyses were performed by one-way ANOVA followed by Fisher post-hoc test. A value of \(P<0.05\) was considered to be statistically significant.

Results

Effects of Atorvastatin on NOS Expression in Cultured Rat Aortic VSMCs

We first examined whether treatment with atorvastatin modulates expression of each NOS isoform in cultured rat aortic VSMCs. Atorvastatin significantly increased nNOS expression at both mRNA and protein levels in time- and concentration-dependent manners (Figure 1A through 1C). At both mRNA and protein levels, a significant increase in the nNOS expression was observed from day 1 to day 5 of the atorvastatin treatment (Figure 1B) and at 0.1 to 30 \(\mu\)mol/L of the statin (Figure 1C). Based on these results, in the following experiments regarding nNOS expression, the cells were exposed to 10 \(\mu\)mol/L atorvastatin for 2 days. Atorvastatin (10 \(\mu\)mol/L) also significantly enhanced iNOS protein expression in a time-dependent manner (1 to 5 days, \(P<0.05\); Figure 1A). By contrast, no eNOS protein expression was detected after the atorvastatin treatment (Figure 1A).

Effects of Atorvastatin on Akt and NF-\(\kappa\)B Activity in Cultured Rat Aortic VSMCs

We next investigated the signal transduction for nNOS expression induced by atorvastatin. Activation of Akt and NF-\(\kappa\)B was assessed by Akt phosphorylation\(^\text{22}\) and nuclear translocation of NF-\(\kappa\)B p65,\(^\text{23}\) respectively. Atorvastatin (10 \(\mu\)mol/L) significantly increased Akt phosphorylation, but not total Akt protein levels, in cultured rat aortic VSMCs in a time-dependent manner (Figure 2A). Atorvastatin (10 \(\mu\)mol/L) simultaneously enhanced nuclear translocation of NF-\(\kappa\)B p65 (Figure 2B) in a time-dependent manner. In both signals, significant changes were seen from day 1 to day 5 of the atorvastatin treatment (Figure 2A and 2B). From these results, in the following experiments concerning Akt...
and NF-κB activity, the cells were treated with atorvastatin for 1 day. Low concentration of atorvastatin (0.1 μmol/L) also significantly enhanced Akt phosphorylation (supplemental Figure IIA), nuclear translocation of NF-κB p65 (supplemental Figure IIB), and nNOS protein expression (supplemental Figure IIC) in cultured rat aortic VSMC.

Effects of Akt Blockade on Atorvastatin-Induced Activation of Akt and NF-κB and nNOS Expression in Cultured Rat Aortic VSMCs

Gene transfer of dominant-negative Akt (DN-Akt) significantly reduced Akt phosphorylation (Figure 3A), nuclear translocation of NF-κB p65 (Figure 3B), and nNOS protein expression (Figure 3C) induced by atorvastatin (10 μmol/L).

Effects of NF-κB Blockade on Atorvastatin-Induced Activation of Akt and NF-κB and nNOS Expression in Cultured Rat Aortic VSMCs

Gene transfer of either dominant-negative IkB (DN-IkB) or dominant-negative IkB kinase (DN-IkBK) did not affect the atorvastatin-induced Akt phosphorylation (Figure 4A), but significantly suppressed the atorvastatin-induced nuclear translocation of NF-κB p65 (Figure 4B) and nNOS protein expression (Figure 4C).

Effects of Akt Overexpression on Atorvastatin-Induced iNOS Expression in Cultured Rat Aortic VSMCs

Even in the absence of atorvastatin, gene transfer of wild-type Akt per se elicited Akt phosphorylation (Figure 5A), nuclear translocation of NF-κB p65 (Figure 5B), and nNOS protein expression (Figure 5C), as did atorvastatin. Gene transfer of either DN-IkB or DN-IkBK again abolished nuclear translocation of NF-κB p65 (Figure 5B) and nNOS protein expression (Figure 5C), but not Akt phosphorylation (Figure 5A), induced by gene transfer of wild-type Akt.
Effects of Mevalonate on Atorvastatin-Induced nNOS and iNOS Expression in Cultured Rat Aortic VSMCs

Mevalonate is the first product that is converted from the precursor HMG-CoA by HMG-CoA reductase. In cultured rat aortic VSMCs, supplementation of mevalonate (100 μmol/L, 2 days) alone did not significantly induce any protein expression of nNOS (92±13%) or iNOS (98±6%). By contrast, supplementation of mevalonate significantly reversed atorvastatin-induced increases in nNOS protein expression (294±7% with atorvastatin versus 123±15% with atorvastatin plus mevalonate) and those in iNOS protein expression (329±18% with atorvastatin versus 104±4% with atorvastatin plus mevalonate; both P<0.05, n=5 to 7).

Effects of Angiotensin II or Endothelin-1 on nNOS Expression and NF-κB Activity in Cultured Rat Aortic VSMCs

Treatment with either angiotensin II (100 nmol/L, 1 day) or endothelin-1 (100 nmol/L, 1 day) significantly enhanced nuclear translocation of NF-κB p65 (supplemental Figure IVA) and nNOS protein expression (supplemental Figure IVB) in cultured rat aortic VSMC (P<0.05 each, n=6 to 7).

Effects of Mevalonate on Atorvastatin-Induced nNOS and iNOS Expression in Cultured Rat Aortic VSMCs

Intracellular Localization of nNOS in Cultured Rat Aortic VSMCs

Immunofluorescence for nNOS showed that the enzyme was upregulated predominantly in the cellular membrane, the cytoplasm, and the nuclear membrane in cultured rat aortic VSMCs after the atorvastatin treatment (10 μmol/L, 2 days; n=6; supplemental Figure VA). Costaining with nNOS and vinculin, a marker of the membrane, revealed positive double immunofluorescence in the plasma membrane after the atorvastatin treatment (n=6; supplemental Figure VB).

Effect of Atorvastatin on nNOS Expression in Cultured HUVECs

Treatment with atorvastatin (10 μmol/L, 2 days) significantly enhanced nNOS protein expression in cultured HUVECs (P<0.05, n=6 each; supplemental Figure VI).

Effects of Atorvastatin on NOS Expression and NOx Production in Isolated Mouse Aortas

To further clarify the action of atorvastatin, we next performed organ culture experiments using NOS−/− mouse aortas with endothelium. NOx accumulation in culture medium was used as an indirect indicator of vascular NO bioactivity, and a difference in NOx accumulation with and

Figure 3. Effects of gene transfer of dominant-negative Akt (DN-Akt) on atorvastatin-induced Akt phosphorylation, nuclear translocation of NF-κB p65, and nNOS protein expression in cultured rat aortic VSMCs. Gene transfer of DN-Akt significantly attenuated Akt phosphorylation (A), nuclear translocation of NF-κB p65 (B), and nNOS protein expression (C) induced by atorvastatin (10 μmol/L; n=5). *P<0.05 vs control; †P<0.05 vs atorvastatin treatment.

Figure 4. Effects of gene transfer of dominant-negative IκB (DN-IκB) or dominant-negative IκB kinase (DN-IκB kinase) on atorvastatin-induced Akt phosphorylation, nuclear translocation of NF-κB p65, and nNOS protein expression in cultured rat aortic VSMCs. Gene transfer of either DN-IκB or DN-IκB kinase significantly inhibited atorvastatin (10 μmol/L)-induced nuclear translocation of NF-κB p65 (B) and nNOS protein expression (C), but not Akt phosphorylation (A; n=5 to 6). *P<0.05 vs control; †P<0.05 vs atorvastatin treatment.

Intracellular Localization of nNOS in Cultured Rat Aortic VSMCs

Immunofluorescence for nNOS showed that the enzyme was upregulated predominantly in the cellular membrane, the cytoplasm, and the nuclear membrane in cultured rat aortic VSMCs after the atorvastatin treatment (10 μmol/L, 2 days; n=6; supplemental Figure VA). Costaining with nNOS and vinculin, a marker of the membrane, revealed positive double immunofluorescence in the plasma membrane after the atorvastatin treatment (n=6; supplemental Figure VB).

Effect of Atorvastatin on nNOS Expression in Cultured HUVECs

Treatment with atorvastatin (10 μmol/L, 2 days) significantly enhanced nNOS protein expression in cultured HUVECs (P<0.05, n=6 each; supplemental Figure VI).

Effects of Atorvastatin on NOS Expression and NOx Production in Isolated Mouse Aortas

To further clarify the action of atorvastatin, we next performed organ culture experiments using NOS−/− mouse aortas with endothelium. NOx accumulation in culture medium was used as an indirect indicator of vascular NO bioactivity, and a difference in NOx accumulation with and
without atorvastatin treatment was regarded as an indirect indicator of atorvastatin-induced vascular NO bioactivity. In isolated aortas of the wild-type mice, atorvastatin (10 \( \mu \)mol/L, 2 days) significantly enhanced protein expression of all three NOSs (Figure 6A) and NOx accumulation in culture medium (Figure 6B). Significant increases in atorvastatin-induced NOx accumulation in culture medium were also seen in isolated aortas of doubly i/eNOS/×/×, n/eNOS/×/×, and n/iNOS/×/× mice (Figure 6B). In addition, low concentration of atorvastatin (0.1 to 0.3 \( \mu \)mol/L) slightly but significantly caused increases in nNOS protein expression in the wild-type mouse aortas (supplemental Figure VIIA and VIIB) and NOx accumulation in culture medium in the wild-type mouse aortas and those three kinds of doubly NOS/×/× mouse aortas (Figure VIIC and VIID). The difference in NOx accumulation with and without atorvastatin treatment in isolated aortas of those doubly NOS/×/× mice was ≈25% (nNOS alone), 25% (iNOS alone), and 50% (eNOS alone) as compared with that of the wild-type mice, respectively (Figure 6C). On the other hand, no significant difference in NOx accumulation in culture medium with and without atorvastatin treatment was seen in isolated aortas of triply n/i/eNOS/×/× mice (0.28 ± 0.01 without versus 0.30 ± 0.10 with atorvastatin, n = 5 each).

Endothelium removal significantly reduced the atorvastatin-induced vascular NOx production in the wild-type mice and the mice expressing either nNOS or eNOS only, but not in the mice expressing iNOS only (supplemental Figure VIII).

**Discussion**

The novel findings of the present study were as follows: (1) in rat VSMC, atorvastatin enhanced nNOS expression; (2) this action was suppressed by inhibition of either Akt or
NF-κB; (3) in mouse aortas, atorvastatin also elicited nNOS expression as well as NOx production; and (4) ≈25% of the vascular NOx production was estimated to be derived from nNOS. To the best of our knowledge, these results provide the first evidence that statin treatment substantially upregulates vascular nNOS through activation of Akt/NF-κB pathway.

**Effect of Atorvastatin on Vascular nNOS Expression**

Atorvastatin increased nNOS expression in rat VSMCs, human endothelial cells, and mouse aortas. The stimulatory effects of atorvastatin were noted at mRNA levels as well as protein levels in rat VSMCs, suggesting the expression regulation at the transcriptional level. Inhibition of HMG-CoA reductase appears to be involved in the atorvastatin-induced nNOS expression in rat VSMCs, as evidenced by the fact that the enhancing effects of atorvastatin on nNOS expression were reversed by addition of mevalonate.

In humans, plasma concentration of atorvastatin attained by high-dose atorvastatin therapy (80 mg/d) is ≈0.2 μmol/L.26 Thus, the concentration of 10 μmol/L atorvastatin that we used in the main part of this study is very high when compared with that in humans. However, we also demonstrated that clinically relevant low concentrations of atorvastatin (0.1 to 0.3 μmol/L) modestly but significantly caused Akt and NF-κB activation, vascular nNOS expression, and vascular NOx production.

**Roles of Akt and NF-κB in Atorvastatin-Induced Vascular nNOS Expression**

Atorvastatin caused Akt phosphorylation (without affecting Akt protein synthesis) and nuclear translocation of NF-κB p65 in rat VSMCs. Blockade of either Akt or NF-κB signaling suppressed the atorvastatin-induced nNOS expression. Moreover, overexpression of Akt gene per se mimicked the effect of atorvastatin to induce nNOS expression and NF-κB activation, both of which were blunted by NF-κB blockade. These results indicate that both Akt and NF-κB plays a pivotal role in the atorvastatin-induced nNOS expression in VSMCs.

Activation of NF-κB is associated with inflammatory injury and is induced by a variety of molecules, such as angiotensin II or endothelin-1.27 In this study, either angiotensin II or endothelin-1 enhanced NF-κB activity and nNOS expression in rat VSMCs. It has been reported that activation of NF-κB leads to generation of reactive oxygen species, including superoxide.28 It has also been revealed that superoxide reacts with NO, causing loss of NO bioavailability and subsequent formation of the strong oxidant peroxynitrite.2 Therefore, there might be a possibility that potential atorvastatin-induced generation of reactive oxygen species via NF-κB activation, concomitant with nNOS upregulation, might result in vascular injury rather than vasculoprotection.

On stimulation with atorvastatin, inhibition of Akt decreased NF-κB activity, whereas inhibition of NF-κB had no effect on Akt activity. In addition, overexpression of Akt gene led to NF-κB activation. Thus, it is likely that Akt is located upstream of NF-κB.

**Induction of Vascular iNOS Expression**

Treatment with atorvastatin, as well as overexpression of Akt gene, increased iNOS expression, but not eNOS expression, in rat VSMCs. This action of atorvastatin was inhibited by either Akt or NF-κB blockade. Thus, atorvastatin-induced iNOS expression also appears to be mediated by the Akt/NF-κB signaling axis, as was the nNOS expression.

**Induction of Vascular eNOS Expression**

Atorvastatin induced eNOS expression in mouse aortas with endothelium; however, it was not the case in rat VSMCs. This discrepancy may be attributable to the presence or absence of the endothelium in those preparations, because statins are reported to increase expression of eNOS located in endothelial cells.16,17 Although eNOS, as well as nNOS, was originally reported to be expressed constitutively, the present study and previous studies16,17 indicate the induction of eNOS expression. In addition, although iNOS was historically introduced as an inducible enzyme, the constitutive expression of iNOS under physiological conditions has also been shown.29 Thus, all the NOS isoform appear to have both the constitutive and the inducible modes of expression.

**Vascular NOx Production**

We finally studied whether atorvastatin also enhances nNOS expression in mouse aorta, and if so, to what extent nNOS-derived NO accounts for the atorvastatin-induced increase in NO bioactivity. The present study with doubly NOS−/− mice suggested that nNOS, iNOS, and eNOS accounted for ≈25%, 25%, and 50% of the atorvastatin-induced NOx production, respectively. These results provide the first evidence that in addition to eNOS and iNOS, nNOS also substantially contributes to the atorvastatin-induced increase in NO bioactivity. Endothelium removal diminished the atorvastatin-induced vascular NOx production in the mice expressing either nNOS or eNOS only, suggesting that the NO is partly coming from endothelial nNOS and eNOS.

**Limitations of the Present Study**

We and others have shown that nNOS exerts vasculoprotective actions.10,11,30 Thus, the present findings may explain, at least in part, why statin therapy is beneficial for the primary and secondary prevention of human cardiovascular diseases. However, (1) clinically relevant concentrations of atorvastatin caused only a modest upregulation of vascular nNOS, (2) atorvastatin induced NF-κB activation and iNOS induction, both of which might lead to the promotion of arteriosclerosis, and (3) proinflammatory and atherogenic endothelin-1 and angiotensin II mimicked the effects of atorvastatin to upregulate vascular nNOS through NF-κB pathway. Thus, it remains to be clarified whether or not overall atorvastatin effects in the present study can indeed explain the beneficial cardiovascular actions of the statin in humans. This point remains to be examined in future studies.

In summary, we were able to demonstrate that statin treatment potently upregulates vascular nNOS via Akt/NF-κB signaling pathway, demonstrating a novel nNOS-mediated vascular effect of the statin.
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Disclosures
None.

References

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Figure Legends of Online Data Supplements

Figure I
Nucleic acid sequence of the product for nNOS RT-PCR. There were 98% (157/159) of identities between the RT-PCR product sequence and the previously reported rat nNOS sequence.

Figure II
Effects of 0.1µmol/L atorvastatin on Akt phosphorylation and nuclear translocation of NF-κB p65 in cultured rat aortic VSMC. Treatment with 0.1µmol/L atorvastatin significantly enhanced Akt phosphorylation (A), nuclear translocation of NF-κB p65 (B), and nNOS protein expression (C) in cultured rat aortic VSMC. -, without atorvastatin treatment; +, with atorvastatin treatment; *P<0.05 vs. without atorvastatin treatment.

Figure III
Effects of atorvastatin and Akt gene transfer on iNOS protein expression in cultured rat aortic VSMC. Treatment with atorvastatin (10 µmol/L, 2 days) significantly increased iNOS protein expression, which effect was prevented by gene-transfer of dominant-negative Akt (DN-Akt) (A) (n=6 each). Atorvastatin-induced iNOS protein expression was also significantly suppressed by gene transfer of dominant-negative IκB (DN-IκB) or dominant-negative IκB kinase (DN-IκBK) (B) (n=6-7). Gene transfer of wild-type-Akt (WT-Akt) per se elicited iNOS protein expression, which effect was again inhibited by gene transfer of DN-IκB or DN-IκBK (C) (n=6-7). *P<0.05 vs.
control; †P<0.05 vs. atorvastatin treatment or WT-Akt gene transfer.

**Figure IV**

Effect of angiotensin II or endothelin-1 on nuclear translocation of NF-κB p65 and nNOS protein expression in cultured rat aortic VSMC. Treatment with angiotensin II (100 nmol/L, 1 day) significantly increased both nuclear translocation of NF-κB p65 and nNOS protein expression (both P<0.05, n=6-7 each). Treatment with endothelin-1 (100 nmol/L, 1 day) also significantly caused both changes (both P<0.05, n=6-7). Ang II, angiotensin II; ET-1, endothelin-1; *P<0.05 vs. control.

**Figure V**

Immunofluorescence for nNOS and vinculin in cultured rat aortic VSMC. Following the treatment with atorvastatin (10 µmol/L, 2 days), nNOS (green) was upregulated predominantly in the cytoplasm, the cellular membrane, and the nuclear membrane in cultured rat aortic VSMC (A) (n=6). Co-staining with nNOS (green) and vinculin (red), a marker of the membrane, revealed positive double immunofluorescence (yellow) in the plasma membrane after treatment with atorvastatin (B) (n=6).

**Figure VI**

Effect of atorvastatin on nNOS expression in cultured human umbilical vein endothelial cells (HUVEC). Treatment with atorvastatin (10 µmol/L, 2 days) significantly enhanced nNOS protein expression (P<0.05, n=6 each). *P<0.05 vs. control.

**Figure VII**
Effects of low concentrations of atorvastatin on NOx production in isolated mouse aortas. Treatment with 0.1-0.3 µmol/L atorvastatin for 2 days significantly caused increases in nNOS protein expression in the wild-type mouse aortas (A,B) and NOx accumulation in culture medium in the wild-type mouse aortas and those three kinds of doubly NOS−/− mouse aortas (C,D) (P<0.05 each, n=6-10). -, without atorvastatin treatment; +, with atorvastatin treatment; *P<0.05 vs. without atorvastatin treatment.

Figure VIII
Effect of endothelium removal on atorvastatin-induced vascular NOx production in wild-type and NOS−/− mouse aortas. Endothelium removal significantly reduced atorvastatin (10 µmol/L, 2 days)-induced NOx accumulation in culture medium in the wild-type mice and the mice expressing either nNOS or eNOS only (P<0.05 each), but not in the mice expressing iNOS only (n=5 each). NS, not significant; *P<0.05 vs. aortas with endothelium.
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**Figure I**
Figure II

A

Phosphorylation of Akt (% increase)

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0.1 μmol/L atorvastatin

B

Nuclear fluorescence intensity (Arbitrary unit, x10^5)

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0.1 μmol/L atorvastatin

C

nNOS Protein (% increase)

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0.1 μmol/L atorvastatin
Figure III
Figure IV

A

B
Figure V

A

Control

Atorvastatin

B

nNOS

Vinculin

nNOS+Vinculin

10 μM
Figure VI

nNOS Protein (% increase)

Control  Atorvastatin

*
Figure VII
Figure VIII

Atorvastatin-induced NOx accumulation in culture medium (mmol/L/g)

- Wild-type
- i/eNOS<sup>-/-</sup> (nNOS alone)
- n/eNOS<sup>-/-</sup> (iNOS alone)
- n/iNOS<sup>-/-</sup> (eNOS alone)

* indicates significance compared to the control group.

NS indicates no significant difference.

Bar graph showing the accumulation of NOx with and without endothelium for each group.