3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Upregulate Inducible NO Synthase Expression and Activity in Vascular Smooth Muscle Cells

Alexey Y. Kolyada, Alexandre Fedtsov, Nicolaos E. Madias

Abstract—Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase ameliorate atherosclerosis by both cholesterol-dependent and cholesterol-independent mechanisms. We examined whether HMG-CoA reductase inhibitors affect the expression and activity of inducible NO synthase (iNOS) in cultured rat aortic vascular smooth muscle (VSM) cells. Atorvastatin (34 to 68 μmol/L) markedly increased nitrite production, an increase that was essentially abrogated by the NO synthase inhibitor N\text{G}-monomethyl-L-arginine (500 μmol/L). Activity of iNOS, determined by the conversion of L-arginine to L-citrulline, increased 9-fold after atorvastatin treatment. Western blot and semiquantitative reverse transcriptase–polymerase chain reaction revealed that atorvastatin (34 to 68 μmol/L) strongly upregulated iNOS protein and mRNA levels, respectively. These concentrations of atorvastatin did not cause cytotoxicity, as judged by the cell survival rate. Similarly, simvastatin and lovastatin (34 μmol/L) caused robust upregulation of the iNOS protein level. Transfection experiments demonstrated that the −1034- to 88-bp human iNOS promoter was strongly induced by atorvastatin (34 μmol/L). Electromobility and supershift assays using a nuclear factor-κB (NF-κB) consensus oligonucleotide and nuclear extracts from VSM cells as well as transfection studies using an NF-κB reporter plasmid suggested that the transcriptional activation of the iNOS gene by atorvastatin is not mediated via the NF-κB pathway. We conclude that HMG-CoA reductase inhibitors potently upregulate iNOS expression and activity in VSM cells, at least in part, by transcriptional mechanisms that do not depend on transcription factor NF-κB. (Hypertension. 2001;38:1024-1029.)

Key Words: nitric oxide synthase ■ transcription ■ cholesterol ■ atherosclerosis ■ lipids

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are potent cholesterol-lowering compounds. These agents ameliorate atherosclerosis and improve cardiovascular outcome in humans with elevated or average serum cholesterol levels.\(^1\)\(^2\)\(^3\)

The mechanisms through which HMG-CoA reductase inhibitors ameliorate atherosclerosis remain under investigation. Available evidence points to a number of mechanisms, including improvement in endothelium-dependent vasomotion, reduction in the prothrombotic state, inhibition of platelet thrombus formation, decreased vascular smooth muscle (VSM) cell proliferation, and plaque stabilization.\(^4\)\(^5\) The decrease in serum cholesterol level effected by these agents is widely acknowledged as a crucial variable underlying these mechanisms. Indeed, the improved survival of patients with coronary artery disease treated with HMG-CoA reductase inhibitors is correlated with the reduction in serum cholesterol levels. A strong correlation exists between elevated serum cholesterol level and abnormal endothelium-dependent vasomotion, an early abnormality of atherosclerosis.\(^6\)

Oxidized LDL causes endothelial dysfunction, in part by downregulating the expression and activity of endothelial nitric NO synthase (eNOS) and the availability of endothelial NO.\(^7\) Furthermore, oxidized LDL promotes thrombus formation and VSM cell proliferation.\(^8\)\(^9\)

These data notwithstanding, considerable information has been produced supporting cholesterol-independent effects of HMG-CoA reductase inhibitors in ameliorating atherosclerosis. Thus, these agents exert a potent antiproliferative effect on VSM cells.\(^10\) In addition, HMG-CoA reductase inhibitors upregulate the expression and activity of eNOS in vascular endothelial cells and prevent the downregulation of these properties by oxidized LDL.\(^11\) This upregulation of eNOS activity reflects an increase in eNOS mRNA stability.\(^11\)

In the present study, we have considered the possibility that HMG-CoA reductase inhibitors affect the expression and activity of inducible NO synthase (iNOS) in VSM cells. We demonstrate for the first time that HMG-CoA reductase
inhibitors activate iNOS in unstimulated VSM cells at least in part by transcriptional mechanisms and that this upregulation results in a substantial increase in NO production.

Methods

Materials

Atorvastatin was a gift from Parke-Davis (Morris Plains, NJ), and lovastatin and simvastatin were gifts from Merck (West Point, Pa). Recombinant mouse interleukin (IL)-1β was obtained from R&D Systems. N²-monomethyl-L-arginine (L-NMMA) was purchased from Calbiochem.

Cell Culture

We used VSM cells from rat aortas (250- to 300-g, 2-month-old, male Sprague-Dawley rats from Harlan Sprague Dawley, Indianapolis, Ind) obtained by enzymatic dissociation with the use of standard methods. Cells were maintained in DMEM supplemented with 10% FCS and penicillin/streptomycin.

Nitrite Assay

Nitrite was measured by a standard method with the use of Griess reagent.

NOS Activity

NO synthase (NOS) activity in homogenates of VSM cells was measured as the conversion of L-arginine to L-citrulline by using the NOSdetect kit (Stratagene) with [14C]arginine (Amersham) according to the manufacturer’s protocol.

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was isolated by Trizol reagent from GIBCO-BRL according to the manufacturer’s protocol. RNA (1 μg) was reverse-transcribed with 200 U Superscript II reverse transcriptase (RT, GIBCO-BRL). As a primer for the RT reaction, we used the gene specific primer 5’-TGG CCG ACC TGA TGT TGC CA-3’ and 5’-ACT TGC AAG AGA TAT CCG AG-3’. The amplification primers for rat iNOS were 5’-TGG AAG TTT CTC TTC AGA GT-3’ and 5’-ACT TGC AAG AGA TAT CCG AG-3’.

Plasmids

Isolation and cloning of the human iNOS promoter (−1034 to 88 bp) in pGL2 plasmid (Promega) has been described previously. The promoter for plasmid pHrNOS-1034NF-κBLuc, which featured complete deletion of the nuclear factor-κB (NF-κB) site at position −115 to −106 bp, was obtained by polymerase chain reaction (PCR) using the pHrNOS-1034Luc plasmid as template and appropriate primers (coding primer 5’-AGC TAA CTG TAC ACA AGA AGC TTT GTA AAC AAG AAA A3’). The mutant promoter was then cloned into the pGL2 vector. The pPGK-βGAL plasmid, expressing β-galactosidase under the control of the phosphoglycerate kinase promoter, was a generous gift of Dr V. Zannis (Boston University School of Medicine, Boston, Mass). The pNF-κBLuc plasmid was purchased from Clontech. The control plasmid pNN-Luc was created from the pNF-κBLuc plasmid through excising the 8 NF-κB sites.

Cell Transfection and Reporter Gene Assay

Cells were transfected by electroporation with the use of Bio-Rad Gene Pulsar apparatus. We used a mixture of one of the human iNOS promoter constructs described above and the pPGK-βGAL plasmid (in a ratio of 10:1) as an internal control of transfection efficiency. After 16 hours, the media were changed, and atorvastatin was added. Cells were harvested 24 hours later, and the luciferase and β-galactosidase activities were determined as described.

Western Blot Analysis

The assay was performed with antibodies against iNOS (sc-650) and secondary antibodies obtained from Santa Cruz Biotechnology. Monoclonal antibody against α-tubulin was obtained from Sigma Chemical Co.

Electrophoretic Mobility Shift and Competition Assay

The electrophoretic mobility shift and competition assay (EMSA) was performed as described previously. Antibodies against NF-κB p50 (sc-1192) and NF-κB p65 (sc-109) were obtained from Santa Cruz Biotechnology. The sequence of NF-κB oligonucleotide probe was 5’TGA GAG GGG AGT TTC CGA GAG-3’. Nuclear extracts from untreated or atorvastatin-treated VSM cells were prepared as described.

Cytotoxicity Assays

Cytotoxicity assays with crystal violet were performed as described.

Results

Statins Increase iNOS Activity and Expression in Cultured VSM Cells

We measured nitrite accumulation in the culture medium as an index of NO production in Sprague-Dawley rat aortic VSM cells treated with variable concentrations (8.5 to 68 μmol/L) of the HMG-CoA reductase inhibitor atorvastatin for 24 or 48 hours. No change in nitrite production was observed after treatment for 24 hours at any concentration (Figure 1A). By contrast, incubation with 34 or 68 μmol/L of atorvastatin for 48 hours resulted in a significant increase in nitrite production (P<0.05) (Figure 1A). The cholesterol precursor, mevalonate, completely abrogated the upregulation of nitrite production by atorvastatin (data not shown). To demonstrate that the upregulation of nitrite production observed in VSM cells after atorvastatin treatment reflected increased iNOS activity, we performed experiments using the NOS inhibitor L-NMMA (500 μmol/L). We found that this inhibitor essentially abrogated the upregulation of nitrite production by atorvastatin treatment at concentrations of 34 or 68 μmol/L for 48 hours in VSM cells (Figure 1B).

No eNOS or neuronal NOS isoform was detected in these VSM cells with RT-PCR or Western blot, respectively (data not shown). This information coupled with the fact that L-NMMA is a competitive inhibitor of NOS activity leads us to conclude that the observed increase in nitrite production after atorvastatin treatment was mediated by an increase in iNOS activity. Indeed, iNOS activity measured by conversion of l-arginine to l-citrulline increased 9-fold after treatment with atorvastatin and was blocked by mevalonate (Figure 1C).

We then examined the iNOS protein level in these cells by using Western blot analysis. We observed a strong upregulation of iNOS protein in VSM cells after treatment with atorvastatin at 34 or 68 μmol/L for 48 hours (Figure 1D). This upregulation of iNOS protein was entirely blocked by mevalonate (Figure 1E). Similar upregulation of iNOS protein level was obtained with simvastatin and lovastatin (34 μmol/L) (Figure 1F). Furthermore, using semiquantitative RT-PCR, we found a strong upregulation of the iNOS mRNA level in VSM cells after treatment with 34 or 68 μmol/L of atorvastatin for 24 hours (Figure 1G).

There was no evidence that concentrations of atorvastatin ranging from 3.4 to 68 μmol/L resulted in cell toxicity when
applied for 24 or 48 hours, as judged by the cell survival rate (data not shown).

Atorvastatin Increases the Transcriptional Activity of the Human iNOS Promoter in VSM Cells

In previous studies, we have demonstrated competence of the 5′ upstream region, −1034 to 88 bp, of the human iNOS gene to promote transcription of the reporter luciferase gene in VSM cells and macrophages. In transient transfections of VSM cells with the human iNOS promoter −1034 to 88/ luciferase construct (phiiNOS-1034Luc plasmid), we have now found a substantial (4.5-fold) upregulation of luciferase activity after atorvastatin treatment at 34 µmol/L for 24 hours (Figure 2A). Mevalonate blocked such iNOS promoter up-regulation (Figure 2B). We conclude that the upregulation of iNOS protein and activity by atorvastatin in VSM cells occurs at least in part at the transcriptional level.

Transcriptional Activation of Human iNOS Promoter by Atorvastatin in VSM Cells Does Not Depend on Transcription Factor NF-κB

To determine whether transcription factor NF-κB is involved in the activation of the human iNOS promoter by atorvastatin, we transiently transfected VSM cells with either the wild-type phiNOS-1034Luc plasmid or the mutated phiNOS-1034ΔNF-κBLuc plasmid, which features complete deletion of the proximal NF-κB site at position −115 to −106 bp. We found that deletion of the NF-κB site decreased the level of induction of the human iNOS promoter by atorvastatin (34 or 68 µmol/L for 24 hours) by ≈50% (Figure 2C).

Nevertheless, when we performed EMSA with an NF-κB consensus oligonucleotide used as a probe and nuclear extracts from VSM cells treated with 68 µmol/L of atorvastatin for 1, 2, or 6 hours, we did not find any changes in the pattern of DNA-protein complexes compared with the pattern produced by nuclear extracts from untreated cells (Figure 2E). On the other hand, treatment of VSM cells with 0.6 nmol/L of IL-1β for 30 minutes (in the presence or absence of atorvastatin) strongly upregulated the DNA-p50/p65 complex formation. The specificity of this complex was indicated by the fact that its formation was competed out by excess cold oligonucleotide NF-κB. The presence of the p50 and p65 subunits of transcription factor NF-κB in this complex was verified by supershift assays using specific antibodies. Furthermore, we did not find differences in DNA-protein complex formation between nuclear extracts from VSM cells

**Figure 1.** Effect of HMG-CoA reductase inhibitors on nitrite production, NOS activity, and iNOS protein and mRNA expression in cultured VSM cells. A and B. Nitrite production in VSM cells. In panel A, atorvastatin (ATOR) was added to the culture medium for 24 or 48 hours. Histograms and error bars represent mean±SEM of 6 independent experiments performed in duplicate. *P<0.05 vs control. In panel B, ATOR was added to the culture medium in the presence or absence of L-NMMA (500 µmol/L) for 48 hours. Histograms and error bars represent mean±SEM of 4 independent experiments performed in duplicate. *P<0.05 vs corresponding ATOR concentration plus L-NMMA. C. NOS activity in VSM homogenates, measured as the conversion of l-arginine to l-citrulline at 22°C. ATOR and mevalonate (100 µmol/L) were added to the culture medium for 48 hours. Histograms and error bars represent mean±SEM of 4 independent experiments performed in duplicate. *P<0.01 vs control. D through F, Western blot analysis of iNOS protein expression after statin treatment of cultured VSM cells. In panel D, ATOR was added to the culture medium for 48 hours. Concentrations of ATOR are indicated at the top of the figure. Filter was stained with an antibody against iNOS and then stripped and stained for a second time with an antibody against α-tubulin to control for variability in loading. Molecular size markers (in kDa) are shown on the left. Lane M indicates molecular size markers, HaeIII-digested X-174 (1353, 1078, 872, 603, and 310 bp). Lanes 1 to 5 represent control cells and cells treated with different concentrations of ATOR. Lane 6 represents VSM cells treated with IL-1β (10 ng/mL) for 24 hours.
treated with 68 μmol/L of atorvastatin for 24 or 48 hours and corresponding nuclear extracts from untreated cells (data not shown).

To bolster our EMSA results, we transiently transfected VSM cells with either the pNF-κBLuc plasmid, which is composed of a tyrosine kinase promoter and 8 binding sites for transcription factor NF-κB, or the pNN-Luc plasmid, which is essentially the same as the pNF-κBLuc plasmid but lacks the NF-κB binding sites. Twenty-four hours after completion of the transfection procedure, experimental cultures were treated with 34 μmol/L of atorvastatin or 0.6 nmol/L of IL-1β, with each applied for 24 hours. Figure 2D presents the difference in transcriptional activity between the 2 transfection settings. As can be seen, whereas treatment with IL-1β upregulated the luciferase activity obtained by the pNF-κBLuc plasmid by 5.6-fold compared with that of the untreated cells, no induction was discerned after treatment with atorvastatin. These results add support to our findings that atorvastatin does not activate transcription factor NF-κB in VSM cells.

Taken together, these results indicate that the transcriptional activation of the human iNOS promoter by atorvastatin...
in VSM cells does not depend on transcription factor NF-κB. The suppression of such activation by the deletion of the proximal NF-κB site at position −115 to −106 bp (Figure 2C) likely reflects abrogation of binding to this site of transcription factors other than NF-κB.

Discussion

The central findings of the present study are that HMG-CoA reductase inhibitors activate iNOS in VSM cells, with such an activation occurring, at least in part, at the transcriptional level, and that this upregulation of iNOS results in a substantial increase in NO production. This is the first demonstration indicating that inhibitors of HMG-CoA reductase induce upregulation of iNOS expression and NO production in unstimulated VSM cells.

The mechanism through which statins upregulate iNOS expression and activity in VSM cells involves inhibition of HMG-CoA reductase, because these effects of atorvastatin on iNOS expression were prevented in the presence of mevalonate. Mevalonate alone did not induce any changes in iNOS expression, suggesting that the basal intracellular mevalonate level is sufficient to maintain iNOS expression at a very low level (data not shown).

What is the magnitude of the atorvastatin-induced increase in NO production? As shown in Figure 1A, this increase appeared to be ≈4.5-fold. However, this estimate is confounded by the background production of nitrate by non-iNOS-mediated pathways. Complete blockade of iNOS activity in VSM cells by L-NMMA, an approach depicted in Figure 1B, can be used to correct for this confounding variable. Application of this correction yielded an iNOS-mediated production of nitrate by unstimulated VSM cells of only 0.04 nmol per 5 × 10^5 cells at 48 hours that reached a level of 1.53 nmol per 5 × 10^5 cells at 48 hours after atorvastatin treatment. This approach uncovers a robust, nearly 40-fold, increase in NO production by atorvastatin in VSM cells. We were also able to demonstrate that atorvastatin results in a 9-fold increase in iNOS activity, as measured by l-arginine to l-citrulline conversion.

The pathophysiological significance of our observations remains uncertain. Extrapolation of in vitro rates of NO production to the in vivo setting is a vexing problem. Furthermore, the concentrations of atorvastatin that induced activation of iNOS and increased NO production in VSM cells in the present study far exceed the anticipated tissue levels from clinically prescribed pharmacological dosages of the agent (on the order of 2 μmol/L). Nevertheless, prolonged exposure to the much lower tissue levels of atorvastatin, as they obtain in chronically treated patients, might yield clinically significant stimulation of NO production in VSM cells. Indeed, the effect of low levels of locally produced NO might mimic the impact on VSM cells of much greater rates of NO production originating from the overlying endothelial cells.

Previous studies have shown that the HMG-CoA reductase inhibitor lovastatin (2 to 10 μmol/L) downregulates the lipopolysaccharide (LPS)-stimulated induction of iNOS in macrophages. In accord with these results, we have found that 68 μmol/L of atorvastatin reduces the induction of iNOS by IL-1β in VSM cells by 50% and the induction by LPS in the macrophage-like cell line RAW 264.7 by 55% after 48 hours of incubation (data not shown). Such an effect is probably mediated by reduction of the activation of transcription factor NF-κB, which occurs after IL-1β or LPS stimulation and might reflect IκB stabilization. By contrast, others have shown that fluvastatin upregulates iNOS expression and NO synthesis in IL-1β–stimulated rat VSM. Such a discrepancy might be attributed to the use of different statins. Our EMSA findings as well as the results of our transfection studies using an NF-κB reporter plasmid suggest that the observed activation of iNOS by atorvastatin in VSM cells is not mediated via the NF-κB pathway (Figures 2D and 2E). Therefore, the available data suggest disparate effects of HMG-CoA reductase inhibitors on iNOS induction. In the presence of strong inducers of iNOS, such as cytokines and LPS, certain HMG-CoA reductase inhibitors downregulate this induction via the NF-κB pathway; by contrast, in the absence of cytokines, the HMG-CoA reductase inhibitors activate iNOS by mechanisms that do not involve the NF-κB pathway and currently remain undefined. A host of other transcription factors bind to the human iNOS promoter (eg, CAAT box/enhancer-binding protein, Oct, and cAMP-responsive element–binding protein) and represent candidate molecules for this activation.

The downregulation of cytokine-stimulated activation of NF-κB effected by at least certain HMG-CoA reductase inhibitors might exert important salutary effects on the atherosclerotic process. Indeed, a beneficial impact might be anticipated on pernicious phenomena such as early response gene expression, macrophage activation, inhibition of eNOS, endothelial dysfunction, VSM cell proliferation, peroxynitrite formation, and plaque rupture. On the other hand, the “direct” activation of iNOS by HMG-CoA reductase inhibitors in VSM cells might also impart benefit by generating relatively small amounts of NO in the absence of concomitant NF-κB activation. Our data may offer insight into yet another cholesterol-independent mechanism through which HMG-CoA reductase inhibitors ameliorate atherosclerosis.

Acknowledgment

These studies were supported in part by an unrestricted grant from Parke-Davis.

References

5. Lacoste L, Lam JY, Hung J, Letchacovski G, Solymoss CB, Waters D. Hyperlipidemia and coronary disease: correction of the increased throm-
3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors Upregulate Inducible NO Synthase Expression and Activity in Vascular Smooth Muscle Cells
Alexey Y. Kolyada, Alexandre Fedtsov and Nicolaos E. Madias

Hypertension. 2001;38:1024-1029
doi: 10.1161/hy1101.093103

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/38/5/1024

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/