Statin enhances cytokine-mediated induction of nitric oxide synthesis in vascular smooth muscle cells

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Abstract

Objective: We investigated the effects of the statins, cerivastatin and lovastatin, on the induction of nitric oxide (NO) production in vascular smooth muscle cells (VSMC) stimulated by interleukin-1β (IL-1) or in combination with interferon-γ (IFN). Methods: We measured NO release, inducible NO synthase (iNOS) mRNA and protein levels, iNOS gene transcription rates, and iNOS mRNA stabilities in cytokine-activated VSMC. We also evaluated nuclear factor (NF)-κB activity and tetrahydrobiopterin (BH4) synthesis. Results: NO production induced by cytokines was dose-dependently enhanced by both statins. Incubating VSMC with IL-1/IFN stimulated iNOS mRNA and protein expression. Both statins significantly upregulated IL-1/IFN-stimulated iNOS mRNA and protein expression, and enhanced iNOS gene transcription as shown by nuclear run-on assays. However, they did not alter the stability of iNOS mRNA. Both statins slightly modulated IL-1/IFN-induced NF-κB activation, which was not associated with their effect on NO production. Cytokines induce the de novo synthesis of BH4 in VSMC. This event is essential for the induction of NO synthesis, which requires transcriptional induction of the genes that encode not only iNOS but also guanosine triphosphate cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in de novo BH4 synthesis. The synthesis of BH4 and GTPCH mRNA induced by IL-1/IFN were enhanced by both statins. Exogenous mevalonate significantly prevented and geranylgeranylpyprophosphate reversed the stimulatory effect of both statins. Furthermore, the geranylgeranyltransferase I inhibitor GGTI-298 significantly increased IL-1/IFN-induced NO production. Conclusion: Our data demonstrated that statins enhance immunostimulants-induced NO production by increasing iNOS gene expression at the transcriptional level via a NF-κB-independent pathway. The effect of statins on NO production is due at least partly through blocking the biosynthesis of mevalonate, which prevents isoprenoid biosynthesis. In addition to augmenting iNOS expression, statins potentiate GTPCH gene expression and BH4 synthesis, thereby preventing a relative shortage of BH4 which may shift the balance between NO-catalyzed generation of protective NO and deleterious reactive oxygen species. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Immunostimulants trigger vascular smooth muscle cells (VSMC) to express the inducible isoform of nitric oxide synthase (iNOS) [1–3]. Induction of iNOS and the over-production of nitric oxide (NO) in VSMC are implicated in the genesis of septic and cytokine-induced circulatory shock [4,5]. Therefore, elucidating the factors that control iNOS activity should provide basis upon which to design therapeutics that will limit NO overproduction. On the other hand, NO may also be synthesized by iNOS expressed in VSMC under specific pathological conditions such as atherosclerosis [6,7]. Although the contribution of iNOS to atherosclerotic changes awaits elucidation, several lines of evidence indicate that iNOS expression is valuable in the treatment or limitation of atherosclerosis [8–10].

Large clinical trials have shown that the inhibition of cholesterol biosynthesis by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors or statins improves clinical outcomes in patients with atherosclerosis [11,12].
Statins exert anti-atherosclerotic effects, beyond the normalization of hypercholesterolemia, by directly acting on endothelial cells, macrophages and VSMC [13–15]. Statins reduce cardiovascular events even in patients without hypercholesterolemia [16], and inhibit the synthesis of isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpiphosphate (GGPP). Both FPP and GGPP are important lipid attachments for the post-transcriptional modification of several proteins, including small G proteins Ras and Rho GTP-binding proteins [17,18]. The involvement of small G proteins in iNOS induction in various cell types is complex [19–21].

The present study examines the effects of statins on immunostimulants-induced NO production in VSMC. Because the activation of NO formation by cytokines in VSMC requires the induction of iNOS gene expression, which is thought to be regulated predominantly at the level of transcription and to be dependent on transcription factor NF-κB [22,23], we investigated the effects of statins on this process in cytokine-stimulated VSMC. Since the intracellular concentration of tetrahydrobiopterin (BH4), an essential cofactor of NO synthase(s) [24], could be rate limiting for NO synthesis, we also investigated the effects of statins on biopterin biosynthesis in cytokine-stimulated VSMC.

2. Methods

2.1. Cell culture and RNA extraction

VSMC were isolated by elastase and collagenase digestion of thoracic aortae from male Wistar rats [25]. Cultures were fed twice weekly with Dulbecco’s modified minimal essential medium (DMEM) containing 10% fetal bovine serum and antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Cells in passages 10–15 were used for experiments. Total RNA was extracted from confluent VSMC after passages 10–15 using a modified guanidinium isothiocyanate method [26].

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1985).

2.2. Nitrite assay

Nitrite accumulation, an indicator of NO synthesis, was measured in the cell culture medium of confluent VSMC [27]. Nitrite was quantified colorimetrically after adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100-μl samples. Absorbance at 550 nm was determined using a microplate reader ( Molecular Devices, Richmond, CA, USA). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

2.3. iNOS and GTPCH mRNA analysis

Standard Northern blotting was used to investigate the mRNA expression for iNOS and GTPCH as previously described [28]. Probes were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers for iNOS [29] and GTPCH [29], labeled with [α-32P]dCTP by random priming and used for Northern blot analysis of mRNA expression. After probing for iNOS expression, membranes were stripped and reprobed for 18S mRNA. Radioactivity on the blots was quantified using a BAS2000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

To examine the stability of iNOS mRNA, the mRNA levels were increased by stimulating VSMC under certain conditions. After blocking further transcription with actinomycin D (5 μg/ml), the rate of disappearance of iNOS mRNA was evaluated by Northern blotting.

2.4. Assay of biopterin

Biopterin (BH4 and more oxidized species) were measured essentially as described [30]. Cells were treated with 0.2 M perchloric acid and oxidized by exposure for 1 h at room temperature in the dark to 0.2 M perchloric acid containing 0.2% I2 and 0.4% KI. Ascorbate (2%) was added to remove residual free I2, and the mixture was then centrifuged for 10 min at 10 000×g. Biopterin in the supernatant was quantified by reversed-phase high-performance liquid chromatography on a C18 column with fluorescence detection using authentic biopterin as the standard. Protein concentration was measured by the method of Lowry et al. [31] using bovine serum albumin as standard.

2.5. NF-κB activation

To study NF-κB activation, the cells were stably transfected with a cis-reporter plasmid containing a luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-Luc: Stratagene, La Jolla, CA, USA) [28]. For this, the pNFκB-Luc plasmid was transfected together with a pSV2neo helper plasmid (Clontech, Palo Alto, CA, USA) into rat VSMC using the FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were cultured in the presence of G418 (Clontech) at a concentration of 500 μg/ml with medium replacement at 2- to 3-day intervals. Approximately 3 weeks later, G418-resistant clones were isolated using a cloning cylinder and analyzed individually for expression of luciferase activity. Several clones were selected for analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).
Fig. 1. (A) Effect of cerivastatin or fluvastatin on nitrite production in IL-1- or IL-1/IFN-stimulated rat VSMC. Cells were incubated with IL-1 (gray bars) or a combination of IL-1 and IFN (black bars) in the presence of cerivastatin (5 μM) or fluvastatin (25 μM) for 24 h, then nitrite accumulation in media was measured. Inset: Cells were incubated with IL-1 in the presence of various concentrations of cerivastatin (triangles) or fluvastatin (squares) for 24 h, after which nitrite accumulation in media was measured. (B) Time course of nitrite production in rat VSMC stimulated with IL-1/IFN. Nitrite accumulation in culture medium was measured at times indicated in the absence (open bars) and presence of cerivastatin (hatched bars) or fluvastatin (gray bars). Data for nitrite are means±S.E. (n=6). (C) Effect of cerivastatin (triangles) or fluvastatin (squares) on nitrite production in TNF/IFN-stimulated rat VSMC. Cells were incubated with TNF and IFN in the presence of various concentrations of cerivastatin or fluvastatin for 24 h, then nitrite accumulation in culture media was measured. Data are means±S.E. (n=6). *P<0.05 and **P<0.01 compared with control (IL-1/IFN only).
2.6. Nuclear run-on assay

Isolation of nuclei and in vitro transcription proceeded as described [32]. Linearized plasmids containing target cDNAs dissolved in 0.4 M NaOH were immobilized onto Hybond-N+ nylon membranes (Amersham Pharmacia) and hybridized with the labeled RNA as described. Blots were exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) and signal intensity was quantified using a FUJIX bioimaging analyzer (BAS2000II).

2.7. Western blot of iNOS

Monolayer VSMC were lysed using electrophoresis sample buffer. The protein concentration of the samples was measured using a Bio-Rad detergent-compatible protein assay. Subsequently, β-mercaptoethanol was added at a final concentration of 1%, and samples were denatured by boiling for 5 min. Samples containing 10 μg of protein were resolved by electrophoresis on 7% SDS–polyacrylamide gels and transferred to PVDF membranes (Bio-Rad) that were incubated with a mouse monoclonal IgG1 antibody against rat iNOS (1:800) (Wako Pure Chemical Industries, Osaka, Japan). The binding of iNOS antibody was detected using sheep anti-mouse IgG horseradish peroxidase (1:20 000) and the ECL Plus system (Amersham, Buckinghamshire, UK).

2.8. Statistical analysis

Data are presented as means±S.E.M. Multiple comparisons were evaluated by ANOVA followed by Fisher’s protected least significant difference test. A value of P<0.05 was considered statistically significant.

3. Results

Interleukin-1β (IL-1, 10 ng/ml) activates rat VSMC to synthesize and release nitrite. IL-1-induced nitrite release was markedly potentiated by interferon-γ (IFN, 100 U/ml). In contrast, VSMC treated with IFN alone (at concentrations of up to 1000 U/ml) did not produce detectable levels of nitrite. We examined the effect of two statin compounds with lipophilic properties (cerivastatin and fluvastatin), on nitrite accumulation in IL-1- or IL-1/IFN-stimulated VSMC (Fig. 1A). Both cerivastatin and fluvastatin potentiated IL-1- or IL-1/IFN-stimulated nitrite production in a concentration-dependent manner when added to VSMC before cytokine(s). As shown in the inset of Fig. 1A, cerivastatin and fluvastatin potentiated nitrite production by 50% above the IL-1-induced response at concentrations of 1 and 5 μM, respectively. On the other hand, the hydrophilic drug pravastatin had no effect on IL-1-induced nitrite production even at 100 μM (data not shown). Fig. 1B shows the time course of the effect of IL-1/IFN and cerivastatin or fluvastatin on nitrite accumulation in the culture medium. A lag phase of 6–8 h preceded the induction, followed by a progressive increase in nitrite synthesis for at least 24 h. The amount of nitrite that accumulated 24 h after stimulation was 40.3±0.7 μM. Cerivastatin and fluvastatin produced a significant increase in cumulative nitrite production in VSMC (Fig. 1B). The amount of nitrite that accumulated in the presence of cerivastatin (5 μM) or fluvastatin (25 μM) at 24 h after stimulation with IL-1/IFN was 68.4±0.9 and 74.6±1.2 μM, respectively. Similarly, tumor necrosis factor-α (TNF) dose-dependently increased nitrite synthesis in the presence of IFN, but TNF alone had little effect on nitrite production by VSMC. Studies with different concentrations of cerivastatin or fluvastatin showed a concentration-dependent increase in TNF/IFN-stimulated nitrite production in the concentration range of 0.25–2.5 μM for cerivastatin and 1.0–10 μM for fluvastatin (Fig. 1C).

We examined whether or not statins directly affect VSMC iNOS activity or expression by time-course studies using a final concentration of 5 μM cerivastatin or 25 μM fluvastatin (Fig. 2). When statins were added at increasing intervals after the stimulation of VSMC with IL-1/IFN, the potentiation of nitrite production decreased as the interval lengthened.

NF-κB activation was detected by measuring NF-κB-dependent transcription in rat VSMC stably transfected with a luciferase reporter construct. Cells were incubated with various concentrations of cerivastatin or fluvastatin, then stimulated with IL-1/IFN for 3 h. In the absence of

![Graph showing nitrite production over time](https://example.com/nitrite_production.png)

Fig. 2. Addition of cerivastatin or fluvastatin after stimulation of rat VSMC with IL-1/IFN. Cerivastatin (5 μM, black bars) or fluvastatin (25 μM, gray bars) was added at the indicated times after stimulation. Nitrite accumulation was measured 24 h after IL-1/IFN. Data are means±S.E. (n=6). *P<0.05 and **P<0.01 compared with control (time=0).
the statins, NF-κB was activated by IL-1/IFN. No significant correlation was observed in the effect of the statins on NF-κB (Fig. 3). Cerivastatin slightly increased the NF-κB activity at concentrations of 1.25–12.5 μM, while fluvastatin modestly decreased with increasing concentrations.

To identify the product of the HMG-CoA reductase reaction necessary for the effect of cerivastatin or fluvastatin, mevalonate, FPP, or GGPP was added to VSMC stimulated with IL-1/IFN in the presence of the statins. Mevalonate is a cholesterol precursor, and FPP and GGPP are, respectively, involved in farnesylation, and geranylgeranylation of proteins. Mevalonate (0.1 mM) completely reversed the statin-induced increase in nitrite production (Fig. 4A). Similarly, GGPP (10 μM) completely reversed the statin-induced increase in nitrite production, whereas FPP (10 μM) did not block this effect of the statins (Fig. 4A). Mevalonate, FPP, and GGPP alone did not affect the basal or IL-1/IFN-induced nitrite production (data not shown). To determine whether the blockade of prenyltransferases is related to modulation of the induction of NO synthesis, we incubated cells with the geranylgeranyltransferase I inhibitor GGTI-298 or the farnesyltransferase inhibitor FTI-227. Fig. 4B shows that GGTI-298 (10 μM) significantly increased IL-1/IFN-induced nitrite. In contrast, FTI-227 (10 μM) inhibited IL-1/IFN-induced nitrite production.

We investigated whether or not the upregulation of transcriptional activity by statins impacts gene and protein expression by evaluating iNOS mRNA and protein levels by Northern and Western blotting. While iNOS mRNA levels approached the detection limit in unstimulated VSMC, IL-1/IFN stimulated iNOS mRNA expression (Fig. 5A) and this was clearly increased by the statins. Fig. 5B shows that iNOS protein, which were very low levels in unstimulated cells, was substantially induced by IL-1/IFN.

The transcription rate of the iNOS gene assessed by nuclear run-on assay was substantially increased 8 h after
Fig. 5. (A) Time course of iNOS mRNA induction in rat VSMC. Cells were incubated with IL-1/IFN in the absence (white bars) and presence of cerivastatin (5 μM, hatched bars) or fluvastatin (25 μM, gray bars) for indicated times. Total RNA was isolated, Northern hybridized with a rat iNOS-specific probe. Signals of iNOS were quantified and normalized to those of 18S using a bioimaging analyzer. Data represent means of duplicate determinations from each of two RNA preparations. (B) iNOS protein expression in rat VSMC. Cells were incubated with IL-1/IFN in the presence of indicated concentrations of cerivastatin or fluvastatin for 24 h, then iNOS protein was detected by Western blotting as a band with a molecular mass of ~125 kDa.
exposure to IL-1/IFN, and potentiated by the statins (Fig. 6). We further examined whether or not the increased stability of iNOS mRNA could serve as the basis for the altered iNOS mRNA induction by the statins. Fig. 7A shows that the rate of decay of iNOS mRNA induced by incubating cells with IL-1/IFN was not altered by the statins, with a half-life approximating 2.5 h. In another experiment, we evaluated iNOS mRNA stability under conditions where VSMC had been incubated with IL-1/IFN in the absence and presence of the statins. Although the statins enhanced the induction of iNOS mRNA, the rate of decay of iNOS mRNA thereafter was not altered by the presence of statins during the iNOS induction process (Fig. 7B).

Unstimulated VSMC contained negligible amounts of biotyperin. However, incubating the cells with IL-1/IFN substantially increased the cellular biotyperin content. We examined the effects of statins on the IL-1/IFN-induced increase in the cellular biotyperin content. Both cerivastatin and fluvastatin dose-dependently enhanced the IL-1/IFN-induced increase in cellular biotyperin levels (Fig. 8A). To evaluate how the statins induced BH4, we investigated the effect of statins on the IL-1/IFN-induced increase in GTPCH mRNA. Whereas GTPCH mRNA levels are very low in untreated VSMC, IL-1/IFN increased the amount of the transcript after 24 h (Fig. 8B). The statins substantially upregulated the IL-1/IFN-induced GTPCH mRNA levels.

4. Discussion

The present findings demonstrated that statins enhanced the induction of NO synthesis by cytokines in VSMC, confirming a previous report and extending our understanding of the mechanisms of statin action [33]. Statins added after the induction of iNOS did not significantly increase nitrite production, indicating that the effects of these drugs are associated with the potentiation of iNOS induction rather than its catalytic activity. Statins enhanced the levels of iNOS mRNA induced by IL-1/IFN without affecting iNOS mRNA stability. Indeed, statins potentiated the transcriptional activity of the iNOS gene driven by IL-1/IFN, which was not associated with NF-κB activity. We therefore concluded that statins enhanced immunostimulant-induced NO production by increasing iNOS gene expression at the transcriptional level via an NF-κB-independent pathway.

Another important finding of this study is that statins increase the cellular content of BH4, the essential cofactor for NO formation. Cytokines induce the de novo synthesis of BH4 in VSMC, which is essential for the induction of NO synthesis [27]. Activation of NO formation by cytokines in VSMC requires transcriptional induction of the genes that encode not only iNOS but also GTPCH, the first and rate-limiting enzyme in de novo BH4 synthesis [29]. Unstimulated VSMC express low levels of GTPCH mRNA. IL-1/IFN induced the expression of GTPCH mRNA in VSMC, and this was enhanced in the presence of statins. Parallel with the changes in GTPCH mRNA levels, cellular BH4 concentrations were increased to detectable levels by IL-1/IFN, and were further increased by statins. In the presence of BH4, NOS dimers secrete small amounts of O2 or H2O2 and instead couple their heme and O2 reduction to NO synthesis [34]. Significant amounts of O2 may be produced due to heme-catalyzed reduction when BH4 concentrations fall below those required to saturate the enzyme [34]. Indeed, subsaturating levels of BH4 can lead to endothelial dysfunction as a result of decreased NO production in endothelial cells, and insufficient BH4 can also result in the NOS-uncoupled production of reactive oxygen intermediates, such as superoxide anion and hydrogen peroxide [35,36]. NOS may generate both NO and O2− when concentrations of BH4 are low [37–40]. When the steady-state flux of O2− is high, evidence indicates the formation of ONOO−, which may be the reactive species responsible for many of the toxic effects of NOS [41,42]. Under most physiological conditions, the vasculature is regulated by NO synthesized by endothelial (e)NOS present in the vascular endothelium,

\[\text{iNOS} \quad \text{GTPCH} \]

\[
\text{BASE} \quad \text{IL1/IFN} \quad +\text{Ceriva} \quad +\text{Fluva}
\]

Fig. 6. iNOS gene transcription in IL-1/IFN stimulated rat VSMC. Nuclear run-on analysis was conducted 8 h after incubating rat VSMC with IL-1/IFN in the absence or presence of cerivastatin (5 μM) or fluvastatin (25 μM). Transcription of the isolated nuclei was analyzed by hybridizing 32P-labeled RNA to iNOS and GAPDH cDNAs immobilized on nylon membranes. Representative of three experiments carried out with similar results (top). Signals of iNOS were quantified and normalized to those of GAPDH using a bioimaging analyzer (bottom). Intensities of iNOS are expressed as a fold increase over that of IL-1/IFN. Data represent means of triplicate determinations (±S.E.).
where the endothelium-derived NO exerts important antiatherosclerotic effects [43]. However, under specific pathological conditions such as atherosclerosis, NO may also be synthesized by iNOS expressed in VSMC. Under such circumstances, the increased induction of BH4 may be important for statin-induced iNOS overexpressed in VSMC to be antiatherogenic because a relative shortage of BH4 may cause a shift between NOS-catalyzed generation of protective NO and deleterious reactive oxygen species.

The stimulatory effect of statins on iNOS parallels their ability to inhibit HMG-CoA reductase, since the effect of cerivastatin was greater than that of fluvastatin when evaluated with EC_{50}. The hydrophilic drug pravastatin has no effect. Thus, the lipophilic properties of the compounds govern their effects at the cellular level. The present study found that mevalonate prevented the stimulatory effect of the statins on IL-1/IFN-induced NO production. Furthermore, we found that GGPP reversed the effect of the statins. These results raise the notion that the decrease in GGPP levels secondary to mevalonate depletion is related to the stimulatory effect of statins on iNOS expression. Moreover, using a specific inhibitor of geranylgeranyltransferase I, IL-1/IFN-induced NO production was suppressed, as it was by statins. On the other hand, a blockade of protein farnesylation by a specific farnesyltransferase inhibitor resulted in decreased NO production. Thus, Rho proteins, which need to be geranylgeranylated, appeared to inhibit iNOS induction in VSMC, whereas the activation of Ras proteins, which need to be farnesylated, may be required for iNOS induction.

The present study demonstrated that cerivastatin and fluvastatin significantly upregulate iNOS expression and NO synthesis in cytokine-treated VSMC at concentrations of 0.2–10 μmol/l and of 1–50 μmol/l, respectively. Thus, this effect, which occurred nearly at concentrations that are reached after administration in conventional doses [44,45], may represent an important mechanism by which these drugs provide protection against atherosclerosis.

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