Pravastatin Attenuates Ceramide-Induced Cytotoxicity in Mouse Cerebral Endothelial Cells with HIF-1 Activation and VEGF Upregulation

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ABSTRACT: Ceramide is a pro-apoptotic lipid messenger that induces oxidative stress and may mediate apoptosis in cerebral endothelial cells (CECs) induced by TNF-α/cycloheximide, lipopolysaccharide, oxidized LDL, IL-1, and amyloid peptide. Exposure of CECs to C₂ ceramide for 12 h caused cell death in a concentration-dependent manner, with a LC₅₀ of 30 μM. Statins are inhibitors of 3-hydroxyl-3-methyl coenzyme A reductase which is the rate-limiting enzyme for cholesterol biosynthesis. Pretreatment with pravastatin at 20 μM for 16 h substantially attenuated ceramide cytotoxicity in mouse CECs. Increases in vascular endothelial growth factor (VEGF) expression were detected within 1–3 h after pravastatin treatment. This pravastatin action was accompanied by the activation of hypoxia-inducible factor–1 (HIF-1), a transcription factor known to activate VEGF expression. These results raise the possibility that pravastatin may protect CECs against ceramide-induced death via the HIF-VEGF cascade.

KEYWORDS: ceramide; reactive oxidative species; apoptosis; endothelium; HMG-CoA reductase inhibitors; pravastatin; vascular endothelial growth factor; hypoxia-inducible factor

INTRODUCTION

Ceramide is generated in endothelial cells in response to stress stimuli, such as TNF-α/cycloheximide, lipopolysaccharide, oxidized LDL, and IL-1. A variety

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of physiological signals, including those initiated by cytokines and growth factors, induce changes of ceramide levels. Once generated, the ceramide signal affects multiple aspects of cellular function, including apoptosis. We have previously shown that amyloid peptide increased the synthesis of ceramide via the neutral sphingomyelinase (nSMase) pathway, and we have established the causal role of the nSMase–ceramide cascade in amyloid-induced apoptosis of cerebral endothelial cells (CECs).7–10

The downstream mechanism by which ceramide causes endothelial dysfunction has yet to be determined. Several mechanisms, including generation of excessive reactive oxygen species (ROS) and inhibition of mitochondria complex III enzyme activity, have been implicated in the apoptotic processes.11,12

The atheroma-retarding properties of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, in both the coronary and carotid arterial beds are well established.13,14 Recently, a growing body of evidence suggests that statins possess important adjunctive properties that may confer additional benefit beyond the retardation of atherosclerosis.15 These include antioxidant activity, anti-inflammatory action, improved endothelial cell function, anti-platelet effect, plaque stabilization, and anti-thrombotic effect.16 Statins appear to be at least equipotent to vascular endothelial growth factor (VEGF) in promoting differentiation of endothelial progenitor cells.17 VEGF is an important trophic factor supporting the survival and proliferation of endothelial cells. VEGF has also been shown to protect endothelial cells against the apoptosis induced by ceramide.18 Upregulation of VEGF through hypoxia-inducible factor–1 (HIF-1) activation during tissue hypoxia has been well established.

In this study, we report pravastatin protection of CECs against ceramide-induced death. This pravastatin action was accompanied by HIF-1 activation and VEGF up-regulation, suggesting that the HIF-1–VEGF cascade may be involved in the protective action of pravastatin in CECs against ceramide cytotoxicity.

MATERIALS AND METHODS

Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. C2 ceramide was from Calbiochem (La Jolla, CA). Cell culture supplies were from Invitrogen Corporation (Carlsbad, CA). Pravastatin was a generous gift from Bristol-Myers Squibb Co. (New York, NY).

Cell Culture

Mouse CECs were prepared as previously described.2 In brief, mouse cerebral cortex was homogenized, filtered, and sequentially digested with collagenase B and collagenase/dispase (Roche Molecular Biochemicals, Indianapolis, IN). This was followed by centrifugation in a 40% Percoll solution. The second band containing microvessels was collected and plated onto collagen-coated dishes. Mouse CECs (4 to 15 passages, >95% purity based on expression of factor VIII and bradykinin receptor) were grown to 85–95% confluence before use.19
**Assessment of Mouse CEC Death and Apoptosis**

Cell survival was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.\(^2,20\) CECs grown on coverslips were washed with PBS and fixed in methanol/acetic acid (3:1) at 4°C for 5 min. After fixation, the cells were stained for 10 min with the fluorescent DNA-binding dye Hoechst 33258 at 8 µg/mL. Nuclear morphology was examined by fluorescence microscopy. Individual nuclei were visualized at 400× to distinguish the normal uniform nuclear pattern from the characteristic condensed, coalesced chromatin pattern of apoptotic cells.

**Western Blot Analysis**

Cytoplasmic and nuclear proteins were isolated from CECs as described previously.\(^20,21\) The cytoplasmic proteins were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto a polyvinylidene difluoride membrane according to standard protocols. The blot was incubated with the following primary antibodies for 1 to 2 h: mouse anti-VEGF antibody at 1:100 or mouse anti-actin antiserum at 1:500 (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then incubated with the goat anti-mouse IgG secondary antibody conjugated with alkaline phosphatase at 1:5000 (Promega, Madison, WI) at room temperature for 1 h.

**Reverse Transcription-Coupled Polymerase Chain Reaction (RT-PCR)**

Total RNA was isolated with RNaseasy Mini Kit (QIAGEN Inc., Valencia, CA). Equal amounts of total RNA (600 ng) were reverse-transcribed and the cDNAs amplified in 0.2 mM dNTP, 1 µM of each primer (forward: 5′-TCTTCAAGCCGTCTCTGTGTG-3′; reverse: 5′-AGGAACATTACACGTCTGC-3′), 1.5 mM MgCl₂, and 2.5 U of Taq polymerase (Roche Diagnostics Corporation). PCR was performed for 26 cycles with denaturation at 95°C for 20 s, primer annealing at 53°C for 30 s, and extension at 72°C for 1 min. Primers were designed based on the mouse VEGF sequences. The relative mRNA level of VEGF was normalized to the internal reference cyclophilin mRNA that was co-amplified in the same reaction for each sample. The RT-PCR was conducted within the linear ranges of PCR cycles and RNA input.\(^20\)

**Electrophoretic Mobility Shift Assay (EMSA)**

Gel shift assay for HIF-1 binding activity has been described in details elsewhere.\(^21,22\) The oligonucleotide with hypoxia-response element in the promoter of EPO gene (5′-agcttgccctagctgtctctcag-3′ and 5′-aattctgagacagcagtcgtaggcc-3′) was labeled with γ\(^{32}\)P]-ATP. Binding reaction was performed in a total volume of 20 µL containing 1× binding buffer, 0.0175 pmol of labeled probe (>10,000 cpm), 20 µg of nuclear proteins, and 1 µg of poly(dI-dC). After incubation for 20 min at room temperature, the mixtures were subjected to electrophoresis on a nondenaturing 6% polyacrylamide gel at 180 V for 2 h under low ionic strength conditions. The gel was dried and subjected to autoradiography.
FIGURE 1. Pravastatin attenuated C₂ ceramide-induced cytotoxicity in mouse CEC. (A) Mouse CECs pretreated with 20 μM pravastatin for 16 h were challenged with C₂ ceramide in serum-free medium for additional 12 h before MTT assay. (B) CECs treated with ceramide with or without pravastatin were stained with the fluorescent DNA-binding dye Hoechst 33258. Left panels: control cells without any treatment; middle panels: CECs treated with C₂ ceramide for 12 h; right panels: CECs pretreated with 20 μM pravastatin for 16 h followed by exposure to C₂ ceramide at 30 μM for additional 12 h. Upper panels: 400× magnification; lower panels, 100× magnification. The arrow indicates ceramide-induced condensed, coalesced, and fragmented nuclei. Data shown are representatives of three separate experiments with similar results. PS, pravastatin. *P < 0.05 as compared to the CECs without PS pretreatment.
**Statistical Analyses**

Data are expressed as mean ± SEM derived from triplicates of at least three separate experiments. Comparison between two experimental groups was based on the two-tailed Student’s t-test. A value of $P < 0.05$ was considered significant.

**RESULTS**

Exposure of mouse CECs to C$_2$ ceramide for 12 h caused cell death in a concentration-dependent manner, with a half-lethal concentration of approximately 30 µM as determined by MTT assay. Pretreatment with pravastatin at a concentration of 20 µM for 16 h significantly attenuated the cytotoxicity of C$_2$ ceramide at 30 to 50 µM in mouse CECs (Fig. 1A). Results based on Hoechst staining revealed that pravastatin pre-exposure at 20 µM for 16 h also markedly reduced the numbers of apoptotic cells (Fig. 1B).

VEGF, an endothelial cell–specific mitogen, promotes endothelial cell survival and angiogenesis. The anti-apoptotic effect of VEGF against ceramide-induced apoptosis has been shown in microvascular endothelial cells.$^{18}$ We demonstrated that pravastatin increased VEGF expression at the mRNA level after pravastatin treat-

![Figure 2](image-url)

**FIGURE 2.** Pravastatin enhanced VEGF expression at both mRNA and protein levels. (A) CECs had been treated with 20 µM of pravastatin for various periods of time before VEGF mRNA was quantified via RT-PCR. Lanes 1, 2, and 3, respectively, indicate control, 1 h, and 3 h after PS treatment. Cyclophilin cDNA was co-amplified as an internal standard to correct for the inherent variations of PCR. (B) The experimental condition was the same as described in (A) except that total proteins were prepared for Western analysis. Western blot for actin served as an internal control for equal loading of proteins in each lane. Results shown are representatives from three separate experiments with similar results.
ment for 1–3 h in mouse CECs (Fig. 2A). An in VEGF protein expression was also observed following pravastatin pretreatment based on the Western analysis (Fig. 2B). VEGF is known to be regulated by HIF-1 activation under an hypoxic environment. We therefore tested whether pravastatin-induced VEGF expression involves HIF-1 activation under a non-hypoxic condition. Results based on EMSA revealed that the HIF-1 binding activity was increased at 1 h, and then declined to basal level within 3 h, after pravastatin treatment (Fig. 3).

**DISCUSSION**

Ceramide is a key mediator of apoptosis in various death paradigms. Signaling of the stress response through ceramide appears to play a role in the development of human diseases, including amyloid-induced vascular changes, ischemia/reperfusion injury, insulin resistance in diabetes, atherogenesis, septic shock, and ovarian failure. Exogenous application of short-chain ceramide analogues can induce apoptosis in various types of vascular wall cells. In the present study, we showed that C2 ceramide induced cytotoxicity in CECs, in accordance with our earlier studies.

Clinical trials have demonstrated the important role of statins in reducing the incidence of coronary events and mortality in patients at risk for coronary disease. Meta-analysis of statin trials has shown a lower risk of ischemic stroke in patients with a history of coronary artery disease with average and/or elevated serum cholesterol levels. Subgroup analysis of the major statin trials suggests that some of the beneficial effects of statins may not be associated with cholesterol reduction. These potentially salutary actions of statins include antioxidant actions, anti-inflammatory action, improvement in endothelial cell function, anti-platelet aggregation, anti-thrombotic effect, and plaque stabilization. In the present study, we showed that pravastatin attenuated ceramide-induced cytotoxicity in mouse CECs. This favorable action of pravastain was accompanied by an increase in VEGF expression and
HIF-1 activation. VEGF is a well-known endothelial cell mitogen that promotes endothelial cell survival and angiogenesis. The anti-apoptotic effect of VEGF on starvation- and ceramide-induced apoptosis has been shown in human dermal microvascular endothelial cells. Statins stimulate VEGF expression in osteoblasts via reduction of protein prenylation and phosphatidylinositol-3 kinase pathway, which promotes osteoblastic differentiation. Circulating endothelial progenitor cells (EPCs) are derived from bone marrow and are mobilized in response to endogenous tissue ischemia or exogenous cytokine stimulation. Statins can augment the circulating population of EPCs through Akt signaling pathway. The statin effect appears to be at least equipotent to VEGF in increasing EPC differentiation. The augmentation of circulating EPC might contribute to the well-established beneficial effects of statins in patients with coronary artery disease. Pravastatin may attenuate the ceramide-induced CEC death via a similar pathway.

Transcriptional activation of VEGF by an increase in HIF-1 binding activity under the hypoxic condition is a well-known phenomenon. Evidence also supports that HIF-1α expression in many types of cancer cells may be hypoxia independent. Several mechanisms have been proposed for hypoxia-independent activation of HIF-1 in cancer cells, including loss of function in VHL, p53, PTEN, p14ARF, or gain of function in SRC. In addition, HIF-1 can also be induced under normoxic conditions by pharmacologic reagents such as cobalt ion and iron chelators. The molecular mechanisms underlying pravastatin-induced HIF-1 activation, however, remain to be fully delineated.

In conclusion, in the present study we present evidence to show that pravastatin attenuated ceramide-induced cytotoxicity in mouse CECs that may involve the HIF–VEGF cascade. Further investigations are needed to establish the causal role of HIF-1 and VEGF in statin-mediated cytoprotection in CECs.

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