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Biochemical and Biophysical Research Communications 336 (2005) 1005–1009
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Blockade of geranylgeranylation by rosuvastatin upregulates eNOS expression in human venous endothelial cells

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Received 16 August 2005
Available online 7 September 2005

Abstract

Endothelial dysfunction is associated with a reduction in nitric oxide (NO) bioavailability. Positive effects of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) on the improvement of endothelial dysfunction have been shown. We investigated the effects of rosuvastatin and isoprenoid metabolites on endothelial NO synthase (eNOS) mRNA and protein expression in human umbilical venous endothelial cells after exposure to 10^{-8}–10^{-5} mol/l rosuvastatin for 8 and 12 h. Cell viability was not significantly altered after exposure to the statin for 12 h. In a concentration-dependent manner, rosuvastatin upregulated eNOS mRNA and protein expression. The effects on eNOS expression mediated through rosuvastatin could be reversed by treatment with mevalonate indicating inhibition of HMG-CoA reductase as the underlying mechanism. Treatment with geranylgeranylpyrophosphate, but not farnesylpyrophosphate, reversed the increase of eNOS expression induced by rosuvastatin. Rosuvastatin may have beneficial effects on endothelial dysfunction associated with cardiovascular diseases beyond its effects on lowering cholesterol.

Keywords: Endothelial dysfunction; eNOS; HMG-CoA reductase inhibition; Statin; Endothelial cells

Inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by statins effectively reduces cardiovascular events in patients with coronary artery disease [1,2]. Besides the cholesterol-lowering effect of this drug class there seem to be additional beneficial effects, e.g., atherosclerotic plaque stabilization [3,4] or anti-inflammatory [5] and -angiogenic effects [6]. In vitro studies have shown positive effects of statins on the improvement of endothelial dysfunction [7]. Simvastatin produces relaxation of conductance in small arteries through a mevalonate-sensitive pathway [8] whereas cerivastatin potentiates nitric oxide (NO) release and endothelial NO synthase (eNOS) expression through inhibition of isoprenoid biosynthesis [9]. Protection by statins was completely abolished in eNOS knock-out mice [10] or after inhibition of eNOS with L-NAME [11]. Statins inhibit the synthesis of isoprenoid intermediates such as geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) [12]. Several proteins, such as the small G proteins Ras and Rho GTP-binding proteins, are post-translationally modified by isoprenylation with FPP or GGPP to facilitate lipid attachment [13] with resultant functional activation.

As endothelium-derived nitric oxide is a pivotal mediator of vasodilation and represents a fundamental physiological mechanism to maintain the functional and structural integrity of blood vessels, the amelioration of eNOS expression by statins remains an important observation. Hydrophobic statins such as atorvastatin and simvastatin can enter endothelial cells by penetrating the lipid bilayers of the cell membranes. However, the uptake of the hydrophilic rosuvastatin into hepatic cells is facilitated by organic anion transporters [14,15].

In cultured human endothelial cells, rosuvastatin, at high concentrations, increases heme oxygenase-1 expression in

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.225

endothelial cells by uncoupling the effects from HMG-CoA reductase or eNOS activity [16]. On the other hand, inhibition of HMG-CoA reductase by rosuvastatin leads to the upregulation of eNOS expression in bovine endothelial cells [17]. As nothing is known about the effects of rosuvastatin on eNOS expression in human endothelial cells, we investigated whether rosuvastatin upregulates eNOS expression by prevention of isoprenoid intermediate formation in human umbilical venous endothelial cells (HUVEC).

Materials and methods

Cell culture. Human venous endothelial cells (HUVEC) were isolated from umbilical cords and cultured according to standard procedures. The endothelial phenotype was confirmed using phase-contrast microscopy and staining for the endothelial-specific von Willebrand factor. Confluent cell layers were exposed to rosuvastatin concentrations ranging from 10^{-8} to 10^{-5} mol/l for 8 or 12 h. In some experiments, the media were supplemented with mevalonic acid (MEV, 10^{-4} mol/l), geranylgeranylpyrophosphate (GGPP, 10^{-5} mol/l), and farnesylpyrophosphate (FPP, 10^{-5} mol/l). MEV, GGPP, and FPP were purchased as aqueous solution from SigmaAldrich Chemie, Taufkirchen, Germany. Rosuvastatin was kindly provided by AstraZeneca, Macclesfield, United Kingdom.

Cell viability. The Alamar Blue assay measures cytotoxicity and cell proliferation via metabolic activity through the chemical reduction of Alamar Blue by living cells. The assays were done in triplicate in 96-well flat-bottomed microtiter plates. After a 12-h incubation with rosuvastatin in the presence or absence of MEV, GGPP, or FPP, 1/10 volume of Alamar Blue (Biosource, Solingen, Germany) solution was added and cells were incubated at 37 °C for an additional 2 h. The absorbance of the wells was measured at 560/595 nm using a Wallac Victor (Perkin-Elmer, Rodgau-Jüggesheim, Germany) fluorometer. Results are expressed as a percentage of relative cell numbers of control cells that were not exposed to rosuvastatin.

Quantitative reverse transcription PCR. Quantification of eNOS and GAPDH mRNA expression, as an endogenous control to normalize for differences in the amount of total RNA in each sample, was performed using pre-designed TaqMan Gene Expression Assays (Applera Deutschland GmbH, Darmstadt, Germany) on the Applied Biosystems ABI Prism 7700 sequence-detection system (TaqMan). Total RNA was isolated using standard procedures including digestion with DNAse according to the manufacturer's protocol (Qiagen, Hilden, Germany). After reverse transcription of 500 ng RNA, PCRs for all samples were performed in duplicate in 96-well optical plates according to the manufacturer's protocol. Analysis of relative gene expression data was performed using the $2^{-\Delta\Delta C_t}$ method [18].

Immunoblot analysis. HUVEC were washed twice with media and then homogenized in lysis buffer (10 mM Tris/HCl, pH 7.4, 1 mM sodium orthovanadate, and 1% [w/v] SDS). Protein concentrations were measured using the bicinchoninic acid (BCA) Protein Assay Kit (Perbio Science, Bonn, Germany). Fifty micrograms of protein was electrophoresed on 10% polyacrylamide gels according to standard procedures and transferred onto polyvinylidine difluoride membrane. Monoclonal antibodies against human eNOS and GAPDH (SigmaAldrich Chemie, Taufkirchen, Germany) were used as primary antibodies in an enhanced chemiluminescence detection system from Amersham Biosciences, Freiburg, Germany. After scanning of the images, densities of ECL signals were quantified with TINA 2.09 g (raytest GmbH, Straubenhardt, Germany).

Statistical analysis. Results are expressed as mean values ± SEM for n preparations from different human umbilical cords. Effects of the indicated concentrations of rosuvastatin were analyzed using the Mann–Whitney U-test or nonparametric analysis of variance with post hoc analysis (Kruskal–Wallis one-way analysis of variance on ranks followed by Student–Newman–Keuls testing). A p value <0.05 was considered statistically significant.

Results

Effects of rosuvastatin on cell viability of HUVEC

As presented in Fig. 1, after 12 h exposure to rosuvastatin (10^{-8}–10^{-5} mol/l) viability of human endothelial cells was only marginally affected. As this was not concentration-related it may reflect variability in the assay. Addition of the isoprenoid intermediates mevalonic acid, GGPP or FPP in the presence of the statin did not change cell viability.

Rosuvastatin increases eNOS mRNA and protein expression in a concentration-dependent manner

Treatment of endothelial cells with rosuvastatin for 8 h increased eNOS mRNA and protein expression in a concentration-dependent manner. As shown in Fig. 2A, rosuvastatin concentration-dependently increased eNOS mRNA expression when compared to cells treated for 8 h in the absence of the compound. This effect was maximal ($188.8 \pm 14.8\%$, $p < 0.05$) in the presence of 10^{-5} mol/l rosuvastatin. At a concentration of 10^{-8} mol/l rosuvastatin, eNOS mRNA expression increased to $131.2 \pm 12.5\%$ ($p < 0.05$) compared to basal expression. The effect of rosuvastatin on upregulation of eNOS protein expression in endothelial cells was also concentration-dependent at the same concentration range as for mRNA expression. As shown in Fig. 2B, significant changes in the protein level of eNOS were detected in immunoblots after treatment with rosuvastatin. These changes were maximal ($200.6 \pm 23.6\%$; $p < 0.05$) with the concentration of 10^{-5} mol/l and already detectable at 10^{-8} mol/l ($127.5 \pm 10.0\%$; $p < 0.05$).

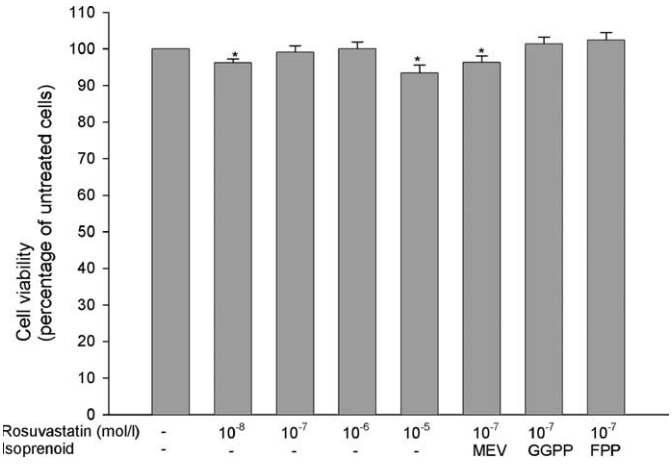

Fig. 1. Viability of human endothelial cells after exposure to rosuvastatin. HUVEC were treated with 10^{-8}–10^{-5} mol/l rosuvastatin for 12 h. Cytotoxic effects were assessed by Alamar Blue assay; cell viabilities are expressed as a ratio of absorbance of treated to untreated cells. Means ± SEM of six experiments in triplicate are shown. *$p < 0.05$ vs. cells treated in the absence of the statin.