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ISOPRENOID DEPLETION BY STATINS ANTAGONIZES CYTOKINE-INDUCED DOWN-REGULATION OF ENDOTHELIAL NITRIC OXIDE EXPRESSION AND INCREASES NO SYNTHASE ACTIVITY IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Endothelial dysfunction and atherosclerosis are associated with an inflammation-induced decrease in endothelial nitric oxide synthase (eNOS) expression. Based on the differences between hydrophobic and hydrophilic statins in their reduction of cardiac events, we analyzed the effects of rosuvastatin and cerivastatin on eNOS and inducible NO synthase (iNOS) expression and NOS activity in TNF- α -stimulated human umbilical vein endothelial cells (HUVEC). Both statins reversed down-regulation of eNOS mRNA and protein expression by inhibiting HMG-CoA reductase and isoprenoid synthesis. Cerivastatin tended to a more pronounced effect on eNOS expression compared to rosuvastatin. NOS activity - measured by conversion of [³H]-L-arginine to [³H]-L-citrulline - was enhanced under treatment with both drugs due to inhibition of HMG-CoA reductase. Statin-treatment reduced iNOS mRNA expression under normal conditions, but had no relevant effects on iNOS mRNA expression in cytokine-treated cells. Rosuvastatin and cerivastatin reverse the detrimental effects of TNF- α -induced down-regulation in eNOS protein expression and increase NO synthase activity by inhibiting HMG-CoA reductase and subsequent blocking of isoprenoid synthesis. These results provide evidence that statins have beneficial effects by increasing eNOS expression and activity during the atherosclerotic process.

Key words: *endothelial dysfunction, endothelial cells, atherosclerosis, HMG-CoA reductase inhibition, endothelial nitric oxide synthase, inducible nitric oxide synthase, NO synthase activity*

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INTRODUCTION

Endothelial cells maintain basal vascular tone and actively regulate vascular reactivity in physiological and pathological conditions. They respond to mechanical forces and neurohumoral mediators with the release of a variety of relaxing- and constricting-factors (1). Nitric oxide (NO) is one of the most important vasoactive substances released by the endothelium, not only acting as a vasodilator, but also by inhibiting inflammation of cells (1). In non-pathological conditions, NO is synthesized by enzymatic conversion of L-arginine, in the presence of molecular oxygen, by endothelial nitric oxide synthase (eNOS) which is expressed constitutively in endothelial cells (2). Inflammatory cytokines, such as tumor necrosis factor (TNF)- α , play a critical role in atherogenesis and cause endothelium to become dysfunctional as eNOS expression is down-regulated by TNF- α , resulting in decreased bioavailability of nitric oxide and development of endothelial dysfunction (3, 4).

Statins inhibit 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase and prevent the formation of mevalonic acid which is a precursor of cholesterol biosynthesis. Previous studies have shown that statins up-regulate eNOS mRNA and protein expression in human endothelial cells via inhibition of HMG-CoA reductase and subsequent depletion of the isoprenoid geranylgeranylpyrophosphate (GGPP) (5 - 8).

As inflammation is a condition that underscores many cardiovascular pathologies including endothelial dysfunction, and as there are differences between hydrophobic and hydrophilic statins in their reduction of cardiac events (9), we compared the effects of rosuvastatin (hydrophilic) and cerivastatin (hydrophobic) on the TNF- α -induced down-regulation of eNOS expression and NOS activity in human umbilical vein endothelial cells (HUVEC). As human endothelial cells also express inducible NO synthase (iNOS) under inflammatory conditions (10), we also quantified iNOS mRNA expression.

MATERIALS AND METHODS

TNF- α , mevalonic acid lactone (MEV), geranylgeranylpyrophosphate (GGPP), farnesylpyrophosphate (FPP), and monoclonal antibodies against human eNOS and glyceraldehyde-3-dehydrogenase (GAPDH) were purchased from Sigma-Aldrich, Germany. Rosuvastatin was kindly provided by AstraZeneca, United Kingdom; cerivastatin was kindly provided by Bayer Vital GmbH, Germany.

Cell cultures

Human endothelial cells (HUVEC) were obtained from collagenase type II-digested umbilical cords and cultured as described previously (11). The endothelial phenotype was confirmed using phase-contrast microscopy and staining for the endothelial-specific von Willebrand factor (data not

shown). HUVEC were treated with TNF- α (10 ng/ml) for 8 and 12 h; for simultaneous incubation with the statins, concentrations of 10^{-8} to 10^{-5} mol/l were used.

Cell viability

Cytotoxicity and cell proliferation was measured via metabolic activity through the chemical reduction of Alamar Blue by living cells. Assays were done in triplicate in 96-well flat-bottom microtiter plates as described previously (5).

Quantitative reverse transcription PCR

Oligonucleotide primers (forward 5'-CGGCATCACCAGGAAGAAGA-3', reverse 5'-TACAGGATTGTCGCCTTCACT-3') and fluorescently labeled TaqMan[®] probe (5'-JOE[™]-CCTCGCTCATGGGCACGGTGAT-TAMRA-3) for eNOS were designed using Primer Express 1.0 software (Applied Biosystems). For quantification of inducible NO synthase (iNOS) and GAPDH mRNA expression Assays-on-Demand[™] by Applied Biosystems (Germany) were used. Quantification of mRNA expression was performed on the Applied Biosystems ABI Prism 7700 Sequence Detection System. Total RNA isolation, cDNA synthesis and quantification were performed as described previously (5).

Protein isolation and quantification

HUVEC were homogenized in lysis buffer containing 10 mmol/l Tris (pH 7.4), 1 mmol/l sodium ortho-vanadate and 1% (w/v) SDS. Protein concentrations in the lysates were measured using the bicinchoninic acid Protein Assay Kit (Perbio Science, Germany). Proteins (50 μ g) were electrophoresed on 10% polyacrylamide gels according to standard procedures and transferred onto polyvinylidene difluoride membrane. Monoclonal antibodies against human eNOS, and GAPDH were used as primary antibodies in an enhanced chemiluminescence detection system from GE Healthcare, Germany. After scanning of the images, densities of ECL signals were quantified with TINA 2.09 g (raytest GmbH, Germany).

Determination of eNOS activity

500,000 HUVEC were seeded in a 9 cm plate and exposed to TNF- α (10 ng/ml) and statin for 12 h. Cells were harvested and total protein extracts were prepared after lysis of the cells in 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA supplemented with 1x protease inhibitor cocktail (Roche, Germany). Total protein concentration was determined by a bicinchoninic acid assay. Enzyme activity was determined by monitoring the conversion of [³H]-arginine to [³H]-citrulline with the NOS Assay Kit (Cayman Chemical, Ann Arbor, US) at 37 °C using 100 μ g of total proteins per reaction. After 60 min, the reaction was stopped and [³H]-citrulline was separated from [³H]-arginine by affinity chromatography using the provided equilibrated resin. NOS activity was quantified by counting the radioactivity of [³H]-citrulline in the eluate. Data are presented as pmol \bullet min⁻¹ \bullet μ g protein⁻¹.

Statistical analysis

Data are expressed as mean values \pm SEM for n preparations from different human umbilical cords. Effects of the indicated concentrations of the statins were analyzed using the Mann-Whitney U-test or 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

Cerivastatin and rosuvastatin have no relevant effects on cell viability of HUVEC in the presence of TNF- α

As presented in Fig. 1, cell viability of HUVEC was not significantly affected by exposure to increasing concentrations of either rosuvastatin or cerivastatin (10^{-8} to 10^{-5} mol/l) for up to 12 h in the presence of TNF- α (10 ng/ml), nor in the presence of mevalonate, GGPP or FPP.

Rosuvastatin and cerivastatin increase eNOS expression in TNF- α -treated cells

Inflammatory conditions, induced by addition of TNF α , lead to a decrease in eNOS expression. HUVEC were cultured with TNF- α (10 ng/ml) in the presence of increasing concentrations of either rosuvastatin or cerivastatin for 8 and 12 h. After 8 h, cytokine-decreased expression of eNOS mRNA was about ten-fold lower compared to expression in the absence of the cytokine ($p < 0.05$). Addition of the statins at 10^{-7} mol/l increased eNOS mRNA expression compared to medium-treated cells. Both rosuvastatin and cerivastatin significantly inhibited the TNF- α -induced down-regulation of eNOS mRNA (Fig. 2A) expression in a concentration-dependent manner. In TNF- α -treated cells, eNOS protein expression was up-regulated by cerivastatin in a concentration-dependent manner whereas no concentration-dependency was observed for rosuvastatin (Fig. 2B). High concentrations of cerivastatin tended to a somewhat higher increase of eNOS mRNA and protein expression in cytokine-stimulated HUVEC compared to the effects mediated by rosuvastatin.

To assess whether statin-mediated up-regulation of eNOS mRNA and protein levels was due to inhibition of HMG-CoA reductase, the medium was

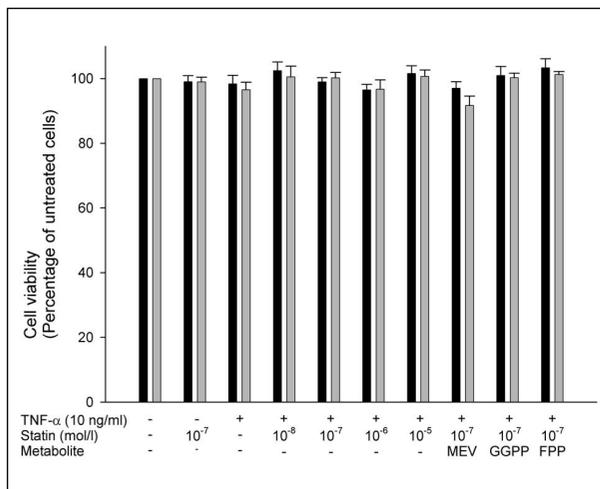


Fig. 1. Viability of human endothelial cells treated with statins in the presence of TNF- α . For 12 h, media were supplemented with rosuvastatin or cerivastatin in the absence or presence of the cytokine (10 ng/ml). Additionally, media were supplemented with 10^{-5} mol/l mevalonate, 10^{-4} mol/l GGPP, or 10^{-4} mol/l FPP, respectively. Cytotoxic effects were assessed by Alamar Blue assay. Cell viabilities are expressed as a ratio of absorbance of treated to untreated cells. Means \pm SEM of 4 (cerivastatin, grey bars) or 6 (rosuvastatin, black bars) experiments in triplicate are shown.

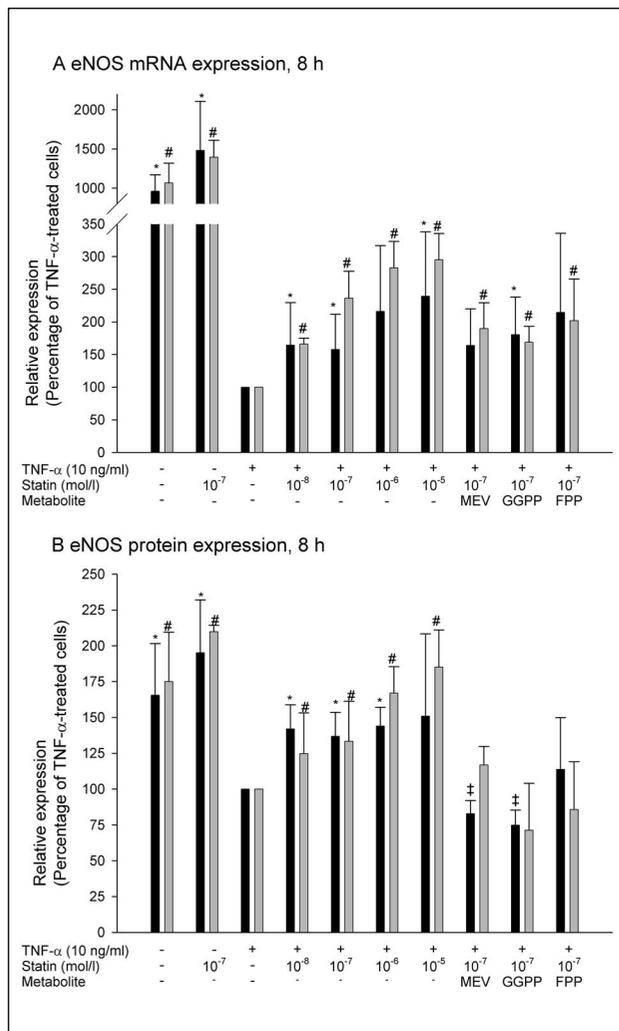


Fig. 2. The effects of the HMG-CoA reductase inhibitors rosuvastatin and cerivastatin and isoprenoids on eNOS expression in TNF- α -stimulated endothelial cells. For 8 h, HUVEC were stimulated with TNF- α (10 ng/ml) in the presence of increasing concentrations of either cerivastatin (grey bars) or rosuvastatin (black bars). Media were supplemented with 10⁻⁵ mol/l mevalonate, 10⁻⁴ mol/l GGPP, or 10⁻⁴ mol/l FPP, respectively. A. Total RNA was isolated and reverse transcribed. Expression data were obtained by quantitative reverse transcription PCR and are expressed as a percentage of TNF- α -treated cells. Data were obtained in 5 independent experiments in various cell preparations. B. Protein expression of eNOS was analyzed by Western blotting and relative densitometric band intensities were plotted. Data were obtained in 4 (rosuvastatin) and 6 (cerivastatin) independent experiments. *, # $p < 0.05$ compared to TNF- α -treated cells in the absence of statin, ‡ $p < 0.05$ vs. TNF- α /10⁻⁷ mol/l rosuvastatin-treated cells.

supplemented with mevalonic acid (10⁻⁴ mol/l), the immediate product of the reaction catalyzed by the enzyme. When added alone to the culture medium, mevalonate had no effect on eNOS mRNA and protein levels (data not shown). Mevalonate, GGPP, and FPP, had no relevant effects on eNOS mRNA expression in statin-treated (10⁻⁷ mol/l) cells in the presence of the cytokine (*Fig. 2A*). As shown in *Fig. 2B*, the stimulatory effects of rosuvastatin (10⁻⁷ mol/l) on eNOS protein expression in cytokine-treated cells were inhibited in the presence of mevalonate. Moreover, the downstream isoprenoid intermediate in the cholesterol biosynthesis, geranylgeranylpyrophosphate (10⁻⁵ mol/l), likewise inhibited the stimulatory effect of rosuvastatin on eNOS protein expression, which indicates that the observed effects of the statins were

dependent on HMG-CoA reductase inhibition and isoprenoid synthesis. Nevertheless, concomitant use of the isoprenoid derivative farnesylpyrophosphate (10^{-5} mol/l) failed to prevent the up-regulation of eNOS protein expression by rosuvastatin in the presence of TNF- α .

Increase in eNOS expression persists after longer incubation periods

To test, whether statin-treatment was still effective after longer incubation periods, we extended the exposure time to 12 h. Only high concentrations of rosuvastatin were able to reverse the TNF- α -induced down-regulation of eNOS mRNA whereas cerivastatin showed an optimal response at a

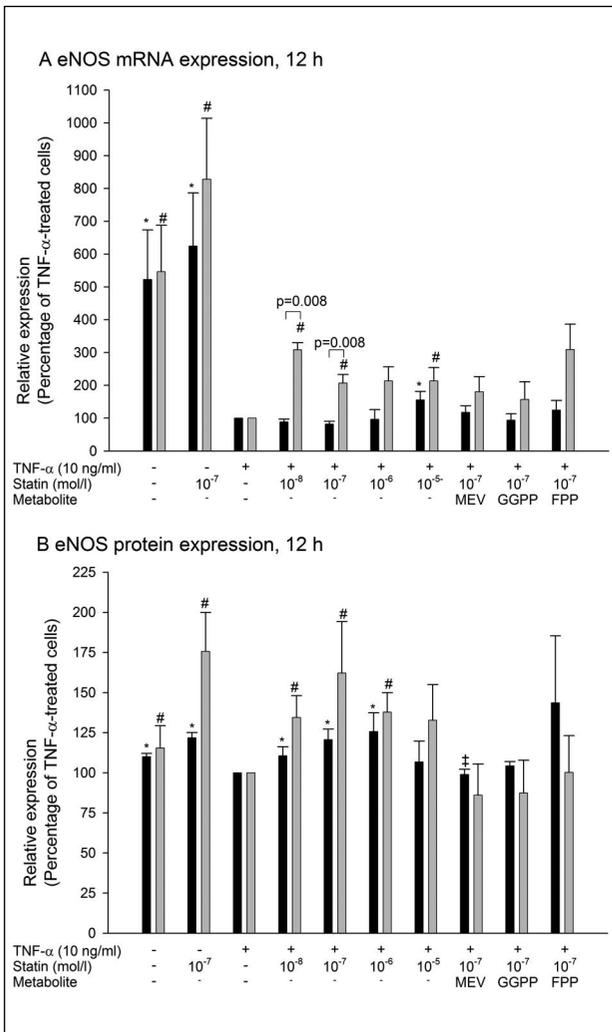


Fig. 3. The effects of rosuvastatin and cerivastatin on eNOS expression in TNF- α -stimulated endothelial cells after 12 h. For 12 h, HUVEC were stimulated with TNF- α (10 ng/ml) in the presence of increasing concentrations of either cerivastatin (grey bars) or rosuvastatin (black bars). Media were supplemented with 10^{-5} mol/l mevalonate, 10^{-4} mol/l GGPP, or 10^{-4} mol/l FPP, respectively. A. Total RNA was isolated and reverse transcribed. Expression data were obtained by quantitative reverse transcription PCR and are expressed as a percentage of TNF- α -treated cells. Data were obtained in 5 independent experiments in various cell preparations. B. Protein expression of eNOS was analyzed by Western blotting and relative densitometric band intensities were plotted. Data are from 4 - 5 (cerivastatin) and 5 (rosuvastatin) independent experiments. *, # P<0.05 compared to TNF- α -treated cells in the absence of statin; ‡ p=0.029 vs. TNF- α -treated cells in the presence of 10^{-7} mol/l rosuvastatin.

concentration of 10^{-8} mol/l (Fig. 3A). Supplementation of the media with either mevalonate or GGPP had marginal effects on the up-regulation of eNOS mRNA expression in cerivastatin-treated cells, but no effects on rosuvastatin-treated cells. In cytokine-treated HUVEC, the protein expression of eNOS (Fig. 3B) was up-regulated by rosuvastatin in a concentration-dependent manner (10^{-8} to 10^{-6} mol/l) whereas cerivastatin showed an optimal response at a concentration of 10^{-7} mol/l. In the presence of rosuvastatin (10^{-7} mol/l) and TNF- α , addition of mevalonate or GGPP reversed up-regulation of eNOS protein expression, but not addition of FPP. In cerivastatin/TNF- α -treated cells, mevalonate, GGPP, and FPP reversed the up-regulation of eNOS protein expression.

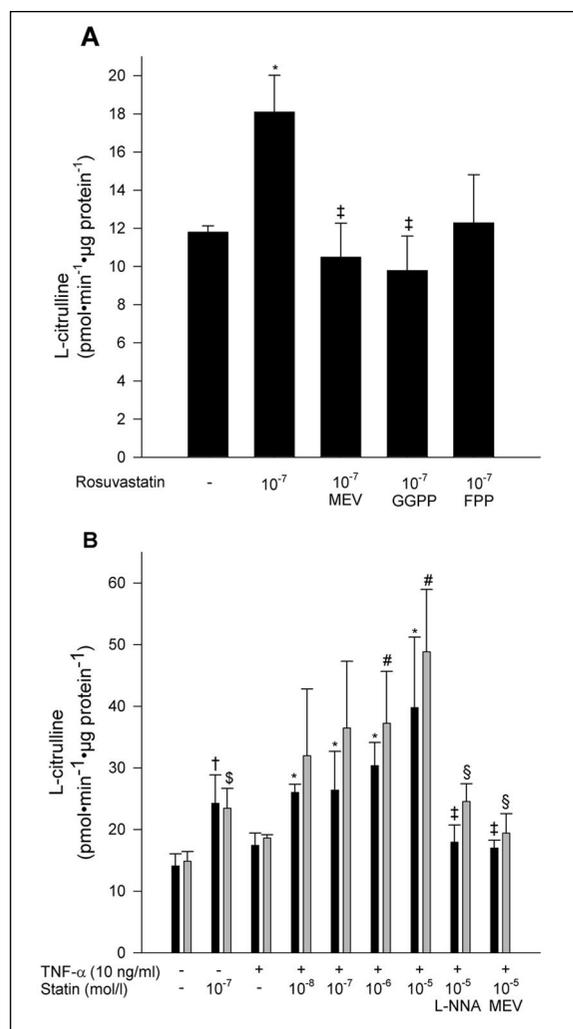


Fig. 4. Effects of statins on NOS activity in endothelial cells. For 12 h, HUVEC were treated with rosuvastatin (black bars) or cerivastatin (grey bars). NOS activity was determined by monitoring the conversion of [³H]-arginine to [³H]-citrulline after 60 min. [³H]-citrulline was separated from [³H]-arginine by affinity chromatography using the provided equilibrated resin. [³H]-citrulline was quantified by counting the radioactivity in the eluate. Data are from independent cell preparations and are presented as pmol • min⁻¹ • μg protein⁻¹. A. Rosuvastatin-mediated effects on NOS activity in the absence of TNF- α (n=4-5). * p<0.05 vs. control; ‡ p<0.05 vs. 10^{-7} mol/l rosuvastatin. B. Effects of statins on NOS activity in the presence of TNF- α (n=5). *, # p<0.05 vs. TNF- α -treated cells; ‡ p<0.05 vs. TNF- α / 10^{-5} mol/l rosuvastatin; § p<0.05 vs. TNF- α / 10^{-5} mol/l cerivastatin; § p<0.05 vs. untreated cells; † p=0.008 vs. untreated cells.

The statin-mediated upregulation of eNOS expression in TNF- α -treated HUVEC correlates with an increase in NOS activity

To determine whether the effects of rosuvastatin and cerivastatin on eNOS mRNA and protein expression resulted in an increase in enzyme activity, we measured NOS activity using total protein extracts from HUVEC which have been exposed to the statins for 12 h in the presence or absence of the cytokine. In the absence of inflammatory mediators (*Fig. 4A*), rosuvastatin (10^{-7} mol/l) enhanced NOS activity (18.1 ± 1.9 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$, $p < 0.05$) compared to cells treated in the absence of the drug (11.8 ± 0.3 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$). Addition of mevalonate (10.5 ± 1.8 , $p < 0.05$ vs. rosuvastatin-treated cells) or GGPP (9.8 ± 1.8 , $p < 0.05$ vs. rosuvastatin-treated cells) completely reversed, whereas addition of FPP (12.3 ± 2.5 , n.s.) partially reversed the rosuvastatin-mediated increase in NOS activity, implying isoprenoid depletion as the underlying mechanism. The increase in NOS activity induced by cerivastatin has been shown previously by Kalinowski and co-workers (12).

Inflammation plays a crucial part in many cardiovascular pathologies including endothelial dysfunction which can be defined as a reduced bioavailability of nitric oxide. Therefore, we repeated the experiments for rosuvastatin and cerivastatin in the presence of TNF- α (*Fig. 4B*). Treatment of

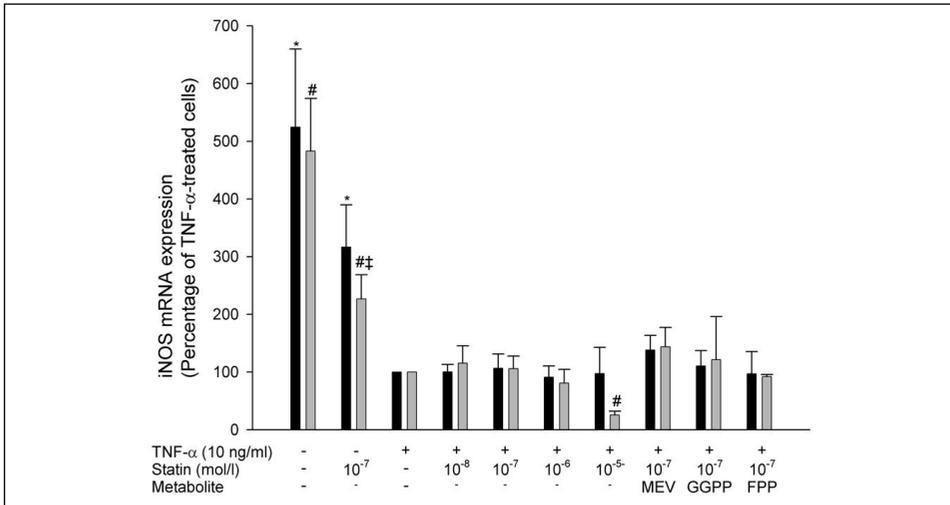


Fig. 5. The effects of rosuvastatin and cerivastatin on iNOS expression in TNF- α -stimulated endothelial cells after 12 h. For 12 h, HUVEC were stimulated with TNF- α (10 ng/ml) in the presence of increasing concentrations of either rosuvastatin (black bars) or cerivastatin (grey bars). Media were supplemented with 10^{-5} mol/l mevalonate, 10^{-4} mol/l GGPP, or 10^{-4} mol/l FPP, respectively. Total RNA was isolated and reverse transcribed. Expression data were obtained by quantitative reverse transcription PCR and are expressed as a percentage of TNF- α -treated cells. Data were obtained in 4 independent experiments in various cell preparations. *, # $p < 0.05$ vs. TNF- α -treated cells; ‡ $p < 0.05$ vs. untreated cells.

endothelial cells with 10^{-7} mol/l rosuvastatin (24.2 ± 4.6 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$, $p < 0.05$ vs. untreated cells) or 10^{-7} mol/l cerivastatin (23.4 ± 3.2 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$, $p < 0.05$ vs. untreated cells) for 12 h increased L-citrulline levels compared to cells treated with media in the absence of statin (14.1 ± 2.0 and 14.8 ± 1.6 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$). In contrast to the down-regulation of eNOS expression, NOS activity was slightly, albeit insignificantly, increased in cytokine-treated cells (~ 18 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$) compared to untreated cells. In the presence of TNF- α , treatment of HUVEC with rosuvastatin or cerivastatin resulted in a significant increase of L-citrulline levels compared to TNF- α -treated cells (17.4 ± 2.0 and 18.6 ± 0.5 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$). The increase was maximal at a concentration of 10^{-5} mol/l (rosuvastatin: 39.8 ± 11.5 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$; cerivastatin: 48.8 ± 10.1 pmol \cdot min $^{-1}$ \cdot μ g protein $^{-1}$; both $p < 0.05$ vs. TNF- α -treated cells). The increase in NOS activity was abolished in the presence of the NOS inhibitor L-N^G-nitroarginine (L-NNA, 1 mmol/l). Simultaneous incubation with mevalonate (10^{-4} mol/l) reversed the effects mediated by 10^{-5} mol/l cerivastatin or rosuvastatin on NOS activity in the presence of TNF- α indicating inhibition of HMG-CoA reductase as the underlying mechanism.

Statin treatment has no relevant effects on iNOS mRNA expression

The observed increase in NOS activity could be attributed to enhancement of the activity of inducible NO synthase (iNOS) in the presence of TNF- α . Therefore, we measured iNOS mRNA expression under the same conditions used for quantification of eNOS expression (Fig. 5). After 12 h, basal iNOS mRNA expression ($\sim 500\%$ of TNF- α -treated cells, $p < 0.05$) was reduced to $316.7 \pm 73.0\%$ in the presence of rosuvastatin and $227.0 \pm 41.5\%$ in the presence of cerivastatin. Treatment with statins in the presence of TNF- α had no relevant effects on iNOS mRNA expression. Supplementation of the media with mevalonate, GGPP, or FPP did not significantly change iNOS mRNA expression in the presence of the statins and the cytokine.

DISCUSSION

Our data confirm previous observations that TNF- α leads to a reduction of eNOS mRNA and protein expression (4). Most importantly, we show that two widely different HMG-CoA reductase inhibitors in terms of their physicochemical characteristics, rosuvastatin and cerivastatin, attenuate the down-regulation of eNOS mRNA and protein expression by depletion of isoprenoid intermediates. In non-cytokine-stimulated HUVEC, the long-term effects of cerivastatin, which are partly due to the up-regulation of eNOS by blocking isoprenoid biosynthesis, result in enhanced NO bioavailability (Kalinowski *et al.* 2000a, b). In our study, we show that in TNF- α -treated

HUVEC cerivastatin and rosuvastatin mediate their effects on eNOS mRNA and protein expression by depleting isoprenoid intermediates: The increase of eNOS protein expression was slightly inhibited in the presence of mevalonate and GGPP, but not FPP. This indicates that the process of geranylgeranylation in endothelial cells may negatively regulate eNOS expression. Inhibition of HMG-CoA reductase by rosuvastatin leads to the up-regulation of eNOS expression in bovine endothelial cells (13) and human endothelial cells (5). The increase in eNOS expression reported in our study was associated with an increase in NOS activity. A recent report describes that eNOS is activated by TNF- α in HUVEC (14). As this activation of eNOS by TNF- α is transient and the following increase in NO production is maximal at 30 min, we exclude the possibility that activation of eNOS by the cytokine was the cause for the observed increase in NOS activity in the experimental set-up used in our study. The increase in NOS activity might be attributed to enhancement of the activity of iNOS. It has been demonstrated that cerivastatin and fluvastatin are able to enhance the levels of iNOS mRNA expression if induced by IL-1 or IFN in vascular smooth muscle cells (15). Then again, Paz *et al.* were able to show in an experimental model of ischemia using isolated rat hearts that TNF- α does not lead to an increase in NO synthesis, but does lead to a decrease of eNOS mRNA expression (16). In this study, we were able to show that statin treatment decreased basal iNOS expression under normal conditions. Furthermore, under inflammatory conditions blockade of HMG-CoA reductase had no relevant effects on iNOS mRNA expression. Based on our data, we infer that the statin-mediated increase in NO activity, which correlated with an increase in eNOS expression, was due to enhanced eNOS expression and not iNOS expression.

Under all conditions, the drugs induced similar changes in eNOS expression and NOS activity suggesting that rosuvastatin and cerivastatin have similar effects on the transcriptional and post-transcriptional regulation of eNOS. Up-regulation of eNOS expression is, among other things, mediated by depletion of GGPP. Upon posttranslationally modification by geranylgeranylation, some members of the Rho GTPase family, e.g. RhoA and RhoB, are transferred to the membrane which causes activation of Rho (17 - 19). This translocation can be inhibited by statins (6). Consequently, Rho acts as a negative regulator of eNOS expression, a fact which is supported by the findings that treatment with mevalonate or GGPP alone did not affect eNOS expression. Under basal tissue culture conditions, Rho geranylgeranylation seems to be maximal.

A different mechanism how cerivastatin - independently of isoprenoid depletion - up-regulates eNOS expression was identified in bovine aortic endothelial cells (20). TNF- α leads to down-regulation of eNOS protein expression in bovine arterial endothelial cells *via* decreased eNOS mRNA stabilization. Cerivastatin prevents this down-regulation of eNOS protein expression by decreasing the binding activity of cytosolic proteins to the 3'-untranslated region of eNOS mRNA, leading to eNOS mRNA stabilization (20).

Conclusions

In summary, our data show that in human endothelial cells - at least under the basal and inflammatory conditions examined - rosuvastatin and cerivastatin improve eNOS expression and NOS activity but have no relevant effects on iNOS expression. As decreases in eNOS expression and activity contribute to the pathogenesis of atherosclerotic coronary artery disease, pharmacologically mediated increases in eNOS expression and activity might contribute to the beneficial effects of statins in cardiovascular diseases.

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