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Atorvastatin prevents hypoxia-induced inhibition of endothelial nitric oxide synthase expression but does not affect heme oxygenase-1 in human microvascular endothelial cells

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Abstract

Beneficial cardiovascular effects of statins, the inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are particularly assigned to the modulation of inflammation. Endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) are listed among the crucial protective, anti-inflammatory genes in the vasculature. Here we show that atorvastatin at pharmacologically relevant concentration (0.1 μ M) enhanced the expression of eNOS in human microvascular endothelial cells (HMEC-1). Moreover, atorvastatin prevented hypoxia-induced decrease in eNOS expression. However, in the same cells atorvastatin was ineffective in modulation of HO-1 protein level. Therefore, we suggest that the protective effect of statins at their pharmacological concentrations is not mediated by enhancement of HO-1 activity, but may involve eNOS.

Keywords

Angiogenesis; Atherosclerosis; Statins; Hypercholesterolemia

Statins, the competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase are the most widely prescribed drugs for treatment of cardiovascular diseases (for reviews see: 1,2). Importantly, their beneficial effects often appear before the clinically detectable changes in cholesterol level and constitute the so-called pleiotropic effects of statins. In fact, the improvement in cardiovascular functions can be demonstrated already after 2 weeks of statin treatment (for Refs. see: 1,2). It is supposed that these effects are to a significant extent dependent on the enhancement of endothelial nitric oxide synthase (eNOS) expression, which results in decrease in platelet activation, attenuation of the expression of adhesion molecules, reduction in the production of inflammatory cytokines, and diminishment in the generation of reactive oxygen species (for Refs. see: 1,2).

Heme oxygenase-1 (HO-1) expression appears also to be beneficial for endothelial cell function (for a review see: 3). HO-1 degrades heme to carbon monoxide, iron and biliverdin. Products of HO-1 activity have recently attracted significant attention due to their apparent

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involvement in modulation of the inflammatory response. Recently, it has been demonstrated that several pharmacological compounds, such as aspirin [4], rapamycin [5] and probucol [6] effectively enhance HO-1 expression in vascular wall cells.

Hypoxia is a significant modulator of gene expression, and its effect on expression of protective genes, such as eNOS or HO-1 may add to the pathological changes in cardiovascular diseases. So far, however, limited data are available on the hypoxic regulation of these genes in human microvascular endothelial cells and the effects of statins on regulation of their expression. Therefore, we investigated, whether atorvastatin can modulate the expression of eNOS and HO-1 in human microvascular endothelial cells (HMEC-1) both under normoxic and hypoxic conditions.

1. Materials and methods

1.1. Cell culture and incubation experiments

Human microvascular endothelial cells (HMEC-1) were used and cultured as described previously [7]. Cells were cultured either in normoxic (21% O₂) or hypoxic conditions (1% O₂) for 6 or 24 h. Hypoxia was created using a Modular Incubator Chamber (Billups-Rothenberg Inc., Del Mar, CA, USA) by putting the cells into the chambers which were tightly closed and aired for 20 min with the gas mixture containing 1% O₂, 5% CO₂ and 94% N₂.

Atorvastatin (provided by Pfizer) was dissolved in DMSO to obtain a stock of 10 mM and added to the cells at indicated concentrations for the whole incubation. Diluent was added to the control cells at the relevant concentrations.

1.2. Reverse transcription-polymerase chain reaction (RT-PCR)

Isolation of RNA, synthesis of cDNA and PCR was performed as described [7]. The primers recognizing HO-1: (5'-GTG GAG ACG CTT TAC GTA GTG C and 5'-CTT TCA GAA GGG TCA GGT GTC C), eNOS (5'-GTG ATG GCG AAG CGA GTG AA and 5'-CCG AGC CCG AAC ACA CAG AA) and EF2 (5'-GCG GTC AGC ACA ATG GCA TA and 5'-GAC ATC ACC AAG GGT GTG CAG) as a reporter gene have been used. The products lengths were: HO-1, 250 bp; eNOS, 421 bp; EF2, 218 bp.

1.3. Real-time RT-PCR

Exons overlapping primers and minor groove binder (MGB) probes labeled with 6-carboxyfluorescein (FAM) used for real time RT-PCR were purchased as Assay-on-Demand from Applied Biosystems: HO-1 [Hs00157965] and eNOS [Hs00167166]. Endothelial cell marker CD31 [Hs00169777] was used as a housekeeping gene. TaqMan real-time PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems) using the following cycling conditions: 2 min at 50 °C, 10 min at 95 °C, and 45 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. As controls, RNA samples not subjected to reverse transcriptase were analyzed to exclude non-specific signals arising from genomic DNA. These samples showed no amplification signals. All PCR reactions were carried out in triplicates. Relative quantification of gene expression was calculated based on the comparative C_T (threshold cycle value) method ($\Delta C_T = C_{T \text{ gene of interest}} - C_{T \text{ housekeeping gene}}$). Comparison of gene expressions in different samples was performed based on the differences in ΔC_T of individual samples ($\Delta \Delta C_T$).

1.4. ELISA assays

eNOS and HO-1 protein were measured in cell lysates using commercial kits (R&D Systems, Abingdon, UK and Stressgen, Victoria, Canada, respectively).

1.5. HO activity assay and Western blotting

Western blotting for HO-1 was done as described previously [8]. Activity of HO enzyme was measured by bilirubin generation as described previously [9].

1.6. Statistical analysis

All experiments were performed in duplicates or triplicates and were repeated 2–6 times. Data are presented as mean $\pm$ S.D. Statistical evaluation was done with Student's *t*-test. Differences were accepted as statistically significant at $p < 0.05$.

2. Results

Culturing of HMEC-1 in 1% oxygen down-regulated the expression of eNOS, as shown by real-time RT-PCR (Fig. 1(A) and (B)), visualised by qualitative RT-PCR (Fig. 1(C)) and confirmed by measurement of eNOS protein in cell lysates using ELISA (Fig. 1(D)).

Treatment with atorvastatin reverted the inhibitory effect of hypoxia on eNOS. As shown by real-time RT-PCR (Fig. 1(A) and (B)), qualitative RT-PCR (Fig. 1(C)) and ELISA (Fig. 1(D)), atorvastatin at pharmacologically relevant concentration of 0.1 μ M moderately enhanced eNOS expression in normoxia and completely prevented its decay in hypoxia, as clearly demonstrated for eNOS protein (Fig. 1(D)).

In contrast to eNOS, the HO-1 mRNA expression increased about two-fold in hypoxia (Fig. 2(A)–(C)), however, the HO-1 protein level was not significantly affected, as demonstrated by ELISA (Fig. 2(D)) and Western blotting (not shown). Also, the activity of HO, as determined by measurement of bilirubin formation did not change in hypoxia ($94 \pm 54\%$ of the value observed in normoxia). On the other hand, treatment of HMEC-1 with hemin (10 μ M), an inducer of HO-1, significantly augmented HO-1 protein in these cells (Fig. 2(D)). Therefore, it allowed us to claim, that the absence of HO-1 response under hypoxia is not due to ill-responsiveness of the HMEC-1 cells.

Effect of atorvastatin on HO-1 expression is different than on eNOS. Real-time RT-PCR (Fig. 2(A) and (B)) and qualitative RT-PCR (Fig. 2(C)) did not reveal any significant stimulatory effect of atorvastatin on HO-1 either in normoxia or in hypoxia at 6–24 h after treatment (Fig. 2(A)–(C)). Rather, a decrease in HO-1 mRNA expression in hypoxic HMEC-1 treated with atorvastatin may be observed (Fig. 2(B)). Moreover, the lack of effect of atorvastatin was found at the protein level, as demonstrated by ELISA (Fig. 2(D)).

Similarly, also at higher, 1 μ M concentration of atorvastatin, no changes in HO-1 protein production could be detected what was also reflected in no changes of the HO activity (not shown). On the contrary, hemin at 10 μ M was highly capable of inducing HO-1 production both in normoxic and hypoxic HMEC-1 (Fig. 2(D)).

One can presume that 0.1 or 1 μ M concentrations of atorvastatin were too low to affect the expression of HO-1. Therefore, we treated HMEC-1 cells with higher, up to 10 μ M concentrations of atorvastatin. An enhanced cytotoxicity of atorvastatin on HMEC-1 has been noted already at the 3 μ M concentration, an effects which was aggravated at 10 μ M (not shown). Diluent (DMSO) itself did not induce such an effect. Also, prolonged, i.e. 48–72 h incubation of HMEC-1 with even 1 μ M atorvastatin significantly decreased cell viability (not shown).

3. Discussion

Enhancement of eNOS activity and prevention of decrease in eNOS expression has been suggested to play a significant role in cardioprotective effects of statins. So far, these effects have been studied in macrovascular endothelial cells only [10,11]. Here we reveal a similar activity of atorvastatin in human microvascular endothelial cells. Our second finding is that atorvastatin does not affect significantly the expression of HO-1, the other gene implicated in statin-based cardioprotection. We believe that these results elucidate some of the discrepancies regarding the effects of statins on HO-1.

The observed lack of the effect of atorvastatin on HO-1 expression is in contrast to some recent reports demonstrating enhancement of HO-1 expression in cells treated with different statins. Thus, recently four papers have been published in which the investigators reported the induction of HO-1 by statins [12]. In a study by Lee et al. simvastatin, at the concentrations of 1–10 μ M up-regulated HO-1 expression in human vascular smooth muscle cells, but, interestingly, neither in human macrophages nor in human umbilical vein endothelial cells (HUVEC) [13]. The effect of simvastatin was concentration dependent, and although the highest expression was attained at 10 μ M, already at 1 μ M of simvastatin HO-1 was induced. On the other hand Uchiyama et al. reported recently that 500 nM simvastatin enhanced HO-1 in human aortic endothelial cells, although the effect was moderate and no data on HO activity were shown [14].

In two other studies, Grosser et al. demonstrated the induction of HO-1 expression by lovastatin and simvastatin [12] as well as by rosuvastatin [15]. They observed that treatment of ECV304 and EaHy296 cells with extremely high concentrations of statins, i.e. 100–300 μ M, augmented HO-1 expression. However, both the concentrations of statins and the cell types used in these reports raise some concerns about the physiological relevance of experiments. Moreover, in both studies the authors employed ECV304 cells, a cell line that has been considered as being of endothelial origin. However, recent studies convincingly demonstrated that they are derived from epithelial cells of bladder cancer [16].

Importantly, all studies in which concentrations of statins above 10 μ M are used have to be interpreted with caution. In fact, plasma concentrations of statins in patients is kept at the steady state level of 0.1–0.3 μ M for lovastatin [17] and about 0.002–0.2 μ M for atorvastatin [18]. It has been convincingly demonstrated that micromolar concentrations of all statins except pravastatin and rosuvastatin, induced apoptosis of endothelial cells [19,20], while nanomolar concentrations of statins protected endothelial cells from apoptosis and enhanced their angiogenic effect [21,22].

Although the overall effect of atorvastatin on HO-1 protein expression and activity is negligible, some influence has been observed at the mRNA level, as this statin decreased hypoxia-induced HO-1 mRNA. It can be hypothesized that this is due to the inhibition of the activity of inflammatory transcription factors, such as NF κ B or AP-1, by statins [23].

HO-1 mRNA was moderately enhanced in hypoxia, but the protein level did not change. Apparently, the induction of mRNA is not sufficiently high to be translated to protein synthesis. Indeed, it is well known for many cell types and genes that mRNA levels alone are unreliable indicators of the corresponding protein abundance [24] and this explanation can be also extended to our study. However, the cell-specific effects have to be also considered, as hypoxia was reported to inhibit the HO-1 expression in human umbilical vein endothelial cells [25], while it enhanced HO-1 in human keratinocytes [26].

Ischemia/reperfusion injury represents the significant clinical problem, which may result in tissue damage during e.g. myocardial infarction and transplantation. Statins have been

reported to protect the cells from the oxidative stress. Also induction of HO-1 appears to be beneficial in many experimental systems. Therefore, it was reasonable to believe that the protective effects of statins might be mediated via HO-1. However, it appears to be not a case.

Overall, protective functions of HO-1 in the cardiovascular system are very much similar to those exerted by eNOS (for reviews see: 27,28). Therefore, one can suppose that compounds which are effective in reducing the cardiovascular morbidity and mortality mediate such effects via enhancement of those protective systems. In the light of our research it seems that pleiotropic effects of statins involve upregulation of eNOS but not induction of HO-1 in endothelial cells. Further studies are required to elucidate the apparent cell-specific differences of statin actions and their physiological relevance.

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Fig. 1.

Effect of atorvastatin on eNOS expression in normoxic and hypoxic HMEC-1. Atorvastatin moderately augmented eNOS expression in normoxia and reverted inhibitory effect of hypoxia on eNOS mRNA measured by real-time RT-PCR both after 6 h (A) and 24 h (B) as well as by conventional PCR (C). The similar effect was observed at a protein level (D). (A and B) Mean of two independent experiments; (C) representative RT-PCR (one of six experiments); (D) mean of three independent experiments. * $p < 0.05$ vs. control in normoxia, # $p < 0.05$ vs. control in hypoxia.

Fig. 2.

Effect of atorvastatin on HO-1 expression in normoxic and hypoxic HMEC-1. Atorvastatin stimulated HO-1 mRNA expression neither after 6 h (A) nor after 24 h (B–D) incubation. Results from two independent real-time RT-PCR (A and B), qualitative RT-PCR [(C) representative data from six independent experiments] and ELISA [(D) experiments performed three times in duplicates). * $p < 0.05$ vs. control in normoxia, # $p < 0.05$ vs. control in hypoxia.