Effect of atorvastatin and fluvastatin on the expression of plasminogen activator inhibitor type-1 in cultured human endothelial cells

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Abstract

Inhibitors of HMG-CoA reductase, namely statins, improve endothelial function independently of their cholesterol-lowering effects. Plasminogen activator inhibitor type-1 (PAI-1) plays a critical role in vascular pathophysiology both at the intra- and extravascular levels. We therefore investigated the effects of atorvastatin (ATOR) and fluvastatin (FLU) on PAI-1 and also tissue-type plasminogen activator (t-PA) synthesis in 20% fetal calf serum-cultured human umbilical vein endothelial cells (HUVEC) stimulated or not by recombinant human pro-inflammatory cytokines, i.e. tumor necrosis factor α (TNFα) and interleukin 1α (IL-1α). In non-stimulated HUVEC, ATOR and FLU significantly diminished (% at 2.0 mmol/l) the constitutive production of PAI-1 (mRNA level and protein secretion). This effect was prevented by addition of mevalonate (100 μmol/l). In HUVEC cultivated in 20% fetal calf serum, the t-PA antigen accumulation was not significantly altered, whereas in low serum concentration (1%) a significant stimulatory effect of ATOR (+30%) and FLU (+76%) was observed. In TNFα-stimulated cells, ATOR and FLU had a modest down-modulating effect (−17 and −20%, respectively) on TNFα-induced increase in PAI-1 synthesis. No effect of statins was observed in IL-1α-stimulated HUVEC, suggesting that statins do not interfere with the up-regulation of PAI-1 synthesis by pro-inflammatory cytokines. However, ATOR and FLU inhibited the TNFα-induced decrease in t-PA release. In conclusion, these results show that statins favorably modulate the expression of fibrinolytic factors produced by human endothelial cells. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Endothelial cells; Statins; PAI-1; Gene expression

1. Introduction

Statins were designed to specifically inhibit the enzyme of the rate-limiting step of hepatic cholesterol synthesis (HMG-CoA reductase) and consequently, by enhancing LDL-cholesterol uptake, to lower circulating LDL-cholesterol [1]. Their efficiency was demonstrated by several clinical trials univocally showing a significant reduction in cardiovascular-related morbidity and mortality [2].

However, there is accumulating evidences that the decrease in circulating LDL-cholesterol does not fully account for the beneficial therapeutic effect of statins on cardiovascular events, as cholesterol-independent mechanisms are also involved [3–8]. These cholesterol-independent mechanisms appear to be linked to the alteration of a large array of intracellular events regulated by metabolites downstream of mevalonate that is the immediate metabolite downstream of HMG-CoA [9,10]. For example, statins regulate the expression of proteins involved in vascular biology, such as MMP-9 in mouse macrophages [11], endothelin and NO synthase in vascular endothelial cells [12,13], tissue factor in monocytes [14,15] and adhesive molecules [16]. Also, statins reduce the platelet deposition on damaged vessel wall in a dyslipemic rabbit model [17].
Coronary thrombosis is well recognized as the precipitating event in acute ischemic heart diseases. In addition to disruption of atheromatous plaque, systemic thrombogenic factors may contribute to the initiation and progression of coronary thrombosis and its clinical sequel [18]. Impaired fibrinolytic activity is often the consequence of an increase in the circulating level of a major inhibitor of fibrinolysis, the plasminogen activator inhibitor type-1 (PAI-1). Increased levels of this inhibitor are considered to be a risk factor of myocardial infarction, particularly in the case of the syndrome of insulin resistance in obese subjects with high levels of circulating triglycerides [19,20]. Clinical studies performed so far have not demonstrated univocal effects of statins on PAI-1 plasma levels [21–26], although the different clinical protocol designs and type of statin may account for the differences observed. In addition, as PAI-1 binds to the extracellular matrix [27], changes in circulating PAI-1 concentration probably poorly reflect local changes in the vascular wall [28]. In addition to its function as a regulator of fibrinolysis at the luminal face of endothelial cells, PAI-1 also regulates proteolysis-dependent migration and adhesion processes in the vascular wall [29]. This may have important pathophysiological consequences in terms of vascular remodeling as demonstrated for example by retroviral administration of PAI-1 gene in a model of aneurysm in the rat [30] or after vascular injury in PAI-1 knock-out mice [31].

Works devoted to in vitro studies of the effects of statins on the fibrinolytic potential of endothelial cells are scarce. In a recent study, Essig et al. [32] showed that lovastatin decreased the constitutive PAI-1 expression in a transformed rat endothelial cell line whereas it increased the constitutive expression of t-PA in these cells and also in human umbilical vein endothelial cells (HUVEC). We therefore investigated the effect of statins on the synthesis of PAI-1 by HUVEC in non-stimulated conditions. TNF-α and IL-1β contribute to the inflammatory process in atherosclerosis and are major inducers of PAI-1 synthesis in cultured human endothelial cells [33,34]. We therefore studied if statins modulated the effects of pro-inflammatory cytokines on PAI-1 synthesis. To further investigate the modulation of the fibrinolytic balance of these cells by statins, we also included studies on t-PA synthesis. Two statins were studied: Atorvastatin (ATOR) and Fluvastatin (FLU).

Results showed that ATOR and FLU significantly decreased the synthesis of PAI-1 (mRNA and protein levels) in basal conditions, but modestly in HUVEC stimulated by pro-inflammatory cytokines. Both statins prevented the TNFα-induced decrease in t-PA production.

2. Materials and methods

2.1. Reagents

ATOR (calcium salt) and FLU (sodium salt) were provided as a powder by Parke-Davis and Novartis, respectively. Stock solutions (10 mM) of ATOR and FLU were made in dimethylsulfoxide (DMSO) and in pure absolute ethanol, respectively, and aliquots stored at −20°C. The volume of DMSO or ethanol never exceeded 0.1% (v/v) and did not affect the PAI-1 and t-PA synthesis. Special precautions were taken to avoid direct exposure of statins to light, before and during incubation procedures. Statins were diluted in warm culture medium just before incubation with HUVEC. Recombinant human TNFα was from Amersham Biotechnology and recombinant human IL-1β from R&D Systems. The Moloney-Murine Leukaemia Virus Reverse Transcriptase (M-MLV RT) and its appropriate buffer were purchased from Gibco BRL (Life Technologies). Taq polymerase and its appropriate buffer were from Bioprobe. All molecular biological products (dNTP, random hexaprimer, RNasin® and appropriate buffers) were from Promega. Monoclonal antibodies against PAI-1 were kindly provided by P. Declerck and R. Lijnen (Leuven, Belgium). Cytochalasin D, mevalonate and geranylgeranylpyrophosphate (GGPP) were from Sigma–Aldrich.

2.2. Cell culture and experimental conditions

HUVEC were isolated and cultivated in 20% fetal calf serum (FCS), or 1% when specified, as previously described [35]. They were used for experiments at the third passage in six-well plates (2 ml/well) unless otherwise indicated. Preconfluent HUVEC were preincubated overnight with indicated doses of statin. The culture medium was then eliminated and replaced by fresh culture medium with the same dose of statin and TNFα (20 U/ml) or IL-1β (10 ng/ml) was or was not added. Total RNA was extracted 14 h later and conditioned culture medium was collected 24 h later. It was centrifuged at 14 000 rpm at 4°C for 10 min and the supernatant was stored at −20°C pending PAI-1 and t-PA assays. In the experiments in which mevalonate or GGPP was added, HUVEC were first treated as above with statin, then mevalonate (100 μmol/l) or GGPP (15 μmol/l) was added and RNA was extracted at 14 h and culture medium collected at 24 h after addition of mevalonate.

2.3. RNA extraction and RT-PCR analysis

HUVEC were cultivated in 25 cm²-flasks. They were rinsed twice with PBS and total RNA extraction (extraction kit for total RNA, RNeasy from Quiagen) and
cDNA synthesis were performed as described [36]. The amplified fragment for human PAI-1 is of 284 bp, base position 10977–11260 (GenBank accession number X04744). Amplified fragment for eEF1α used as a house keeping gene [36] is of 289 bp, base position 382–670 (GenBank accession number X03558). PCR was performed on a Perkin-Elmer thermocycler (GeneAmp 2400). PCR conditions were calibrated in preliminary experiments, allowing determination of the cycle number for which there was a significant detectable spot of the amplified fragment. Twenty-five and 18 cycles were selected for PAI-1 and eEF1α, respectively. specificity of the amplified fragment was assessed by demonstrating that appropriate restriction enzymes generated the expected cleavage fragments. PCR started for 2 min at 95°C followed by cycles consisting of: 60 s at 58°C, 90 s at 72°C, and 45 s at 97°C. Amplification was terminated by 5 min at 72°C and products were visualized and photographed under UV radiation following gel agarose (2%) electrophoresis.

2.4. Protein assays

Total proteins of cell lysates were assayed according to specifications of the bicinechonic acid protein assay kit of Sigma. PAI-1 antigen assay was performed on supernatants from conditioned culture medium by ELISA as described by Declerck et al. [37]. t-PA antigen assay was performed on the same supernatants using the ELISA kit of Biopool International, according to the specifications of the manufacturer. Concentrations of PAI-1 and t-PA antigens in the culture medium were normalized with intracellular proteins. Lactate dehydrogenase (LDH) activity, released in the culture medium and used as an index of cell injury, was assayed using a clinical routine assay on an automatic analyzer (Hitashi).

2.5. Statistics

Because of the variability between the different HUVEC preparations, each determination was performed in triplicate and at least five different preparations of HUVEC were tested. Results are expressed as mean ± S.D. Comparison was analyzed by ANOVA test and significance calculated at $P < 0.05$ using the Fisher test.

3. Results

3.1. Effect of ATOR and FLU on PAI-1 synthesis in basal conditions

We first determined the effect of increasing doses of ATOR and FLU on HUVEC viability. We consistently observed that concentrations above 1.0 μmol/l of either ATOR or FLU induced increasingly marked changes in the morphological appearance of HUVEC characterized by some cell retraction and stretching (Fig. 1). For these reasons, we selected a range of concentration from 0.25 up to 2.0 μmol/l between which no significant increase in LDH release could be observed (Table 1).

As shown in Fig. 2(A), ATOR and FLU decreased the basal accumulation in culture medium of PAI-1 antigen measured at 24 h. The decrease became significant ($P < 0.05$) at 0.5 μmol/l with FLU (-46%) and at 1.0 μmol/l with ATOR (-30%). At 2 μmol/l the decrease was of 50% for the two statins. At the same concentration, the effects of ATOR and FLU were not statistically different ($P > 0.05$). To investigate the molecular level at which statins exerted their down-regulating effects, we analyzed the PAI-1 mRNA levels by semi-quantitative RT-PCR. As shown in Fig. 2(B), a significant reduction in PAI-1 mRNA levels was observed with either ATOR (1 and 2 μmol/l) or FLU (0.5–2 μmol/l). To further investigate the mode of action of statin, HUVEC were pretreated overnight with the statins at 2.0 μmol/l and then with mevalonate (100 μmol/l). Mevalonate alone did not alter basal PAI-1 synthesis (not shown). However, as shown in Fig. 2, mevalonate significantly reversed the inhibitory effect of both ATOR and FLU tested at 2.0 μmol/l. The levels of PAI-1 antigen that accumulated in the culture medium, as well as those of PAI-1 mRNA, measured 24 and 14 h after mevalonate addition, respectively, were similar to those of control untreated HUVEC. GGPP, which is a down-stream metabolite of mevalonate, was shown to reverse lovastatin-induced decrease in PAI-1 synthesis in cultured rat endothelial cells [32]. In HUVEC treated by ATOR or FLU (2 μmol/l), GGPP (15 μmol/l) significantly restored PAI-1 accumulation that attained 80 ± 9.8% ($n = 7$) of that of untreated HUVEC (not shown). We observed morphological changes (Fig. 1) of HUVEC treated by statins. This has also been previously reported and was attributed to disruption of actin filaments [32]. Cytochalasin D is a well-known disrupter of actin filaments and was therefore used to verify if alteration of the cytoskeleton may account for the statin-induced decrease in PAI-1 production. Cytochalasin D was used at 100 nmol/l, because higher concentrations (＞200 nmol/l) were cytotoxic. PAI-1 antigen accumulation after 24 h of treatment was not significantly changed (−10%, $P > 0.05$, $n = 6$). We then investigated the effects of ATOR and FLU on t-PA synthesis. Both statins exerted no significant effect on t-PA antigen accumulation in culture medium of HUVEC cultured in 20% FCS (Table 2). This result contrasts with previous studies performed in rat and human endothelial cells cultured in low serum concentration [32], that showed an in
crease in t-PA antigen following lovastatin treatment. We therefore cultured HUVEC in 1% FCS and incubated them with ATOR and FLU at 2 μmol/l. In these conditions, t-PA production was significantly increased by $30.0 \pm 3.5\%$ and $76.4 \pm 8.8\%$ ($n = 6$, $P < 0.05$), with ATOR and FLU, respectively (not shown).

3.2. Effect of ATOR and FLU on PAI-1 synthesis in TNFα-stimulated HUVEC

TNFα (20 U/ml) drastically enhanced (Fig. 3) PAI-1 accumulation (by a factor of 3.8) in the culture medium and mRNA levels, which is concordant with previous

![Phase contrast microphotography of HUVEC](image)

Fig. 1. Phase contrast microphotography of HUVEC: (A) control untreated cells; (B) ATOR 2.0 μmol/l; (C) FLU 2.0 μmol/l; (D) TNFα (20 U/ml); (E) ATOR 2.0 μmol/l + TNFα; (F) FLU 2.0 μmol/l + TNFα.

Table 1
Effect of ATOR and FLU on LDH release from HUVEC

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<th>ATOR (μmol/l)</th>
<th>FLU (μmol/l)</th>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>2.0</td>
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<tr>
<td>Control</td>
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<td>208.7 ± 2.5</td>
<td>205.7 ± 9.7</td>
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<tr>
<td>TNFα</td>
<td>192.0 ± 1.7</td>
<td>205.3 ± 2.0</td>
<td>201.7 ± 1.1</td>
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*HUVEC in 20% FCS were treated with statin (overnight) and then stimulated or not by TNFα (20 U/ml) still in the presence of statin for additional 24 h. Conditioned media were collected and assayed for LDH activity. Values (IU/l) are mean ± S.D. ($n = 6$) of two separate experiments each performed in triplicate.
Fig. 2. Effect of ATOR and FLU on basal synthesis of PAI-1 in HUVEC. (A): PAI-1 accumulation in the culture medium 24 h after addition of statins. (B): PAI-1 and eEF1α mRNA levels analyzed by RT-PCR 14 h after addition of statins. Values are means ± S.D. (n = 12) of four experiments each performed in triplicate. Differences are significant at *P < 0.05 (ANOVA, Fisher test) over control cells without treatment.

Data [33,34]. In the presence of ATOR or FLU, LDH release was not affected (Table 1). These statins modestly inhibited TNFα-induced PAI-1 accumulation in the culture medium. This inhibition did not exceed 17 and 25% for ATOR (at 1.0 μmol/l) and FLU (at 0.5 μmol/l), respectively, and was not reflected by signifi-

cant changes of PAI-1 mRNA levels. IL-1α is also a potent inducer of PAI-1 synthesis in HUVEC. In our conditions, IL-1α increased PAI-1 synthesis from 3.1 ±

Table 2 Efect of ATOR and FLU on t-PA release from HUVEC

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<th>None</th>
<th>ATOR (μmol/l)</th>
<th>FLU (μmol/l)</th>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
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<tr>
<td>Control</td>
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<tr>
<td>TNFα</td>
<td>0.13 ± 0.03**</td>
<td>0.17 ± 0.03*</td>
<td>0.21 ± 0.02</td>
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</table>

* HUVEC were treated as in described in Table 1. Conditioned media were collected and assayed for t-PA antigen. Values (ng t-PA/μg of total proteins) are means ± S.D. (n = 9) of three separate experiments each performed in triplicate.
* Differences are significant at *P < 0.05 (ANOVA, Fisher test) over control cells without treatment (none).
** Differences are significant at *P < 0.01 (ANOVA, Fisher test) over control cells without treatment (none).
0.5 up to 13.7 ± 1.3 ng PAI-1/μg of total proteins (n = 6). ATOR or FLU at 1.0 μmol/l did not significantly alter this IL-1α-induced increase in PAI-1 synthesis, as the decrease did not exceed 8% (data not shown).

We also investigated if ATOR or FLU altered the synthesis of t-PA. TNFα is known to decrease the basal synthesis of t-PA in HUVEC, which was also observed herein by a significant (P < 0.01) decrease in t-PA accumulation (−52%) when compared with unstimulated cells (Table 2). Pretreatment of HUVEC (cultivated in 20% FCS) with either FLU or ATOR reduced the inhibitory effect of TNFα on t-PA release. However, because of some wide dispersion of t-PA levels among the different HUVEC preparations, the only significant reversal effect of statins (i.e. no statistically significant decrease versus control; P > 0.05) was observed with ATOR at 2.0 μmol/l (−22%) and with FLU at 1.0 μmol/l (−15%).

4. Discussion

The beneficial effect of statins on cardiovascular diseases appears over and above the well established and proven decrease in circulating LDL-cholesterol. By interfering with prenylation of transducing proteins, statins may alter the expression of proteins implicated in vascular function [9,10]. The dysregulation of the fibrinolytic potential of endothelial cells is critically involved in the development and progression of atherothrombosis [38]. In this study, we analyzed whether the two statins ATOR and FLU altered the levels of PAI-1 in HUVEC placed in basal and proinflammatory conditions. Clearly, both statins significantly decreased the synthesis of PAI-1 in HUVEC cultivated in the presence of 20% serum. The effects of ATOR and FLU were not statistically different one from the other, although FLU tended to act at lower concentrations than ATOR, which may reflect their different hydrophobic properties. These results are in line with those of Essig et al. [32] who showed, in a rat transformed endothelial cell line, that lovastatin down-regulated PAI-1 expression, as regards both activity and mRNA levels. The inhibitory effect of FLU and ATOR on PAI-1 antigen release is reflected by a decrease in PAI-1 mRNA level and is prevented by addition of mevalonate. This strongly suggests that statins exerted their effect on PAI-1 gene expression through inhibition of the mevalonate pathway. Intermediate metabolites of this pathway, such as farnesylpyrophosphate or GGPP, are involved in the prenylation of transducing proteins, allowing proper interaction with the membrane and consequently an optimal transmission of the information through the cytoskeleton. In this way, it was demonstrated that prenylation of Rho GTPases by GGPP was inhibited by lovastatin [39]. Interestingly, in rat endothelial cells, GGPP was able to prevent the lovastatin-induced down-regulation of PAI-1 synthesis [32]. It was proposed that lovastatin induced a defect in the prenylation of Rho protein that in turn altered its interaction with the cytoskeleton [32]. Interestingly, we also observed with HUVEC that GGPP almost completely reversed the down-modulating effect of statins. However, in HUVEC, cytoskeletal reorganization itself does not seem to be responsible for the decrease in PAI-1 synthesis, as we observed that the disrupter of actin filaments, cytochalasin D, did not significantly alter PAI-1 synthesis. This result can be analyzed in the light of the results of Zohar et al. [40], who showed in NIH3T3 cells that Rho-effector molecules regulating actin structure are distinct from those signaling to the nucleus. The effect of statins on the activation of PAI-1 synthesis by proinflammatory cytokines (TNFα and IL-1α) was modest as the optimal decrease we observed (−17 to −20%) was with TNFα. This suggests that FLU or ATOR does not interfere with the proinflammatory cytokines-triggered pathway leading to PAI-1 gene activation. One may thus hypothesize that, in vivo, statins would significantly regulate PAI-1 production in normal or mildly activated endothelial cells rather than those lining an advanced inflamed atherosclerotic lesion. This, however, does not rule out the possibility that the signalling pathway triggered by other types of inducers of PAI-1 synthesis present in the atherosclerotic lesion can be down-regulated by statins. A different behavior was observed with t-PA synthesis in HUVEC. In basal conditions (i.e. 20% FCS), ATOR and FLU did not significantly alter t-PA release. However, in HUVEC and in rat endothelial cells incubated in low serum concentration and treated withLovastatin, Essig et al. [32] observed an increase in t-PA synthesis. In this study, we also observed that HUVEC incubated in 1% FCS increased the t-PA release in the presence of statin with a more marked effect of FLU. However, at this stage of investigation, the pathophysiological relevance of the relationships between statins, serum depletion and t-PA synthesis remains to be clarified. Interestingly, ATOR and FLU reduce the TNFα-induced decrease in t-PA antigen accumulation, suggesting that these statins interfere with some factors of the TNFα-triggered transduction pathway that down-regulate t-PA gene transcription. The differential regulation of PAI-1 and t-PA syntheses by ATOR and FLU in basal and TNFα-stimulated conditions underlines the complex regulation that statins exert on cell function and trafficking.

These results, taken together with those of the literature, emphasize the original cholesterol-independent effects of statins that tend to correct endothelial dysfunction.
5. Note added in proof

While this paper was in press, Bourcier and Libby (Arterioscler Thromb Vasc Biol, 2000;20:556–62) reported that simvastatin down-regulated PDGF- and TGFβ-induced PAI-1 synthesis in human endothelial cells.

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