

Fluvastatin Inhibits Basal and Stimulated Plasminogen Activator Inhibitor 1, but Induces Tissue Type Plasminogen Activator in Cultured Human Endothelial Cells

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Key words

Plasminogen activator, endothelial cells, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, isoprenoids

Summary

The effects of fluvastatin, a synthetic hydroxymethylglutaryl coenzyme A (HMG-CoA) inhibitor, on the biosynthesis of tissue plasminogen activator (t-PA) and of its major physiological inhibitor (plasminogen activator inhibitor type 1, PAI-1) were investigated in cultured human umbilical vein endothelial cells (HUVEC). Fluvastatin (0.1 to 2.5 μM), concentration-dependently reduced the release of PAI-1 antigen by unstimulated HUVEC, subsequent to a reduction in PAI-1 steady-state mRNA levels and *de novo* protein synthesis. In contrast, it increased t-PA secretion.

The drug also reduced PAI-1 antigen secreted in response to 10 μg/ml bacterial lipopolysaccharide (LPS), 100 U/ml tumour necrosis factor α (TNFα) or 0.1 μM phorbol myristate acetate (PMA).

Mevalonate (100 μM), a precursor of isoprenoids, added to cells simultaneously with fluvastatin, suppressed the effect of the drug on PAI-1 both in unstimulated and stimulated cells as well as on t-PA antigen. Among intermediates of the isoprenoid pathway, all-*trans*-geranylgeraniol (5 μM) but not farnesol (10 μM) prevented the effect of 2.5 μM fluvastatin on PAI-1 antigen, which suggests that the former intermediate of the isoprenoid synthesis is responsible for the observed effects.

Introduction

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (statins) are competitive inhibitors of cholesterol synthesis by blocking the conversion of HMG-CoA to mevalonate, the rate-limiting step in cholesterol biosynthesis. Besides their efficacy in the treatment of hypercholesterolemia, these drugs have been shown to reduce cardiovascular mortality and morbidity (1–3). However, the observation that the benefit in terms of cardiovascular and cerebrovascular events is partly independent of baseline lipid levels (4, 5), suggests that cholesterol-independent mechanisms may be responsible for these effects. Moreover, the reductions in atherosclerotic plaque progression as well as in intimal-medial thickness of carotid arteries induced by statins are small in size and do not fully account for the beneficial effects of these drugs (6–7). Arterial plaque stabilization as well as changes in their proinflammatory content has been proposed as potential mechanisms (8, 9). In addition statins have been shown to have antithrombotic effects (8, 10). They reduce *in vivo* platelet activation (11) and platelet deposition on mildly damaged vessel walls (12), prevent endothelial dysfunction (13–15) as well as macrophage tissue factor expression (16).

Vascular thrombosis results from a complex interaction between vessel wall and blood constituents, which leads to fibrin deposition. The lysis of fibrin deposits within the vascular tree is brought about by plasmin, which binds to fibrin-rich thrombi and gives rise to fibrin degradation products. The generation of plasmin within the circulation is regulated by tissue plasminogen activator (t-PA) and by its major inhibitor, tissue plasminogen activator inhibitor type 1 (PAI-1) (17). Elevated levels of PAI-1 in plasma have been shown to be predictive of cardiovascular events in free-living populations and in survivors of myocardial infarction (17–20). Clinical data on the effects of statins on plasma fibrinolytic parameters are still conflicting (10).

Recently it has been shown that the HMG-CoA reductase inhibitors lovastatin and simvastatin *in vitro* increase t-PA mRNA expression and reduce PAI-1, thereby favouring fibrinolysis in endothelial cells (21).

Fluvastatin ([R,S-(E)]-(±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indole-2-yl]-3,5-dihydroxy-6-heptenoic acid monosodium salt) is a synthetic HMG-CoA reductase inhibitor that has been shown to effectively reduce plasma total and LDL cholesterol levels (22). In this study we have investigated the effects of this drug on the biosynthesis of PAI-1 antigen by cultured human umbilical vein endothelial cells (HUVEC) in relation to that of t-PA and the molecular mechanism underlying the effects.

Methods

Materials

All disposable materials were from Costar (Cambridge, MA); Hybond N^+ filters, Rediprime DNA labeling system and [^{32}P]-labeled dCTP were from Amersham Corp, Buckinghamshire, UK. All the culture media, antibiotics and aminoacids were from GIBCO Mascia Brunelli, Milan, Italy. All-*trans*-geranylgeraniol was purchased from American Radiolabeled Chemicals Inc; phorbol myristate acetate (PMA), phenylmethylsulfonyl fluoride (PMSF), tumor necro-

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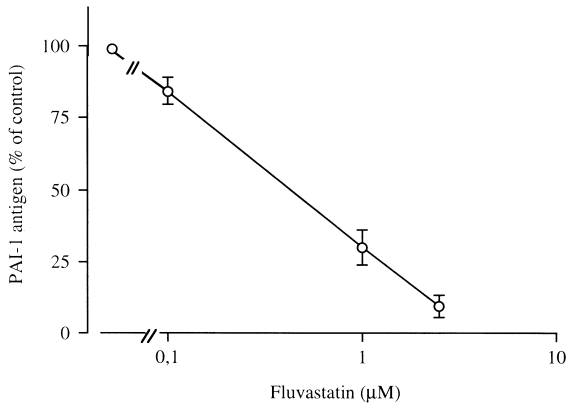


Fig. 1 Effect of fluvastatin on PAI-1 secretion by HUVEC. Monolayers of confluent HUVEC were preincubated for 24 h with fluvastatin (0-2.5 μM) or vehicle in Medium 199 containing 0.1 mg/ml heparin, 0.1 mg/ml ECGF, 15% FCS. The medium was then removed and cells were exposed for 16 h to freshly prepared medium containing 2.5% FCS in the presence of the same concentrations of fluvastatin or vehicle. PAI-1 antigen levels were determined in the conditioned medium as described in "Methods". Results are expressed as mean percentages of vehicle treated cells taken as control (100%). Data are given as arithmetic means (with vertical lines showing SEM of five individual experiments performed in duplicate)

sis factor α (TNFα), aprotinin, farnesol and mevalonate (as mevalonolactone) were from Sigma Chemical Co. Protein-A Sepharose was from Pharmacia Fine Chemical. LPS (Escherichia coli 011:B4) was obtained from Difco Labs. Fluvastatin as sodium salt (Novartis, Basel, Switzerland) was dissolved in ethanol immediately before use.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from umbilical cord using the method of Jaffe (23) and grown to confluence in 25 cm^2 tissue culture flasks (Costar, Cambridge, Mass.). The cells were cultured in medium 199 (M199) containing 20% fetal calf serum (FCS), 2 mM L-glutamine, 50 UI/ml penicillin, 50 μg/ml streptomycin, 0.1 mg/ml of porcine intestinal heparin and 0.1 mg/ml crude extract of endothelial cell growth factor (ECGF). The cells used in these studies were between the first and second passages and had been derived from single cords.

Experimental Procedures

Confluent cells were split 1:1 into 12 well cluster plates (4 cm^2) and grown until confluency. Cells were then incubated for 24 h in M199 containing 15% FCS, heparin 0.1 mg/ml, ECGF 0.1 mg/ml and different concentrations of fluvastatin (0–2.5 μM). Mevalonate and isoprenoids were present through the incubation where indicated. The cells were then exposed for 16 h to freshly prepared medium containing 2.5% FCS, in the presence of fluvastatin (0–2.5 μM) and/or mevalonate and isoprenoids and an appropriate stimulus: LPS (10 μg/ml), PMA (0.1 μM) or TNFα (100 U/ml). After incubation the conditioned medium (CM) was collected and centrifuged to remove cell debris and stored at –20° C.

Quantitation of t-PA and PAI-1 Antigen

PAI-1 and t-PA antigen were measured by means of a commercial ELISA kit (FI-5 Monozyme, Copenhagen, Denmark for PAI-1 and Imulyse, Biopool AB, Umea, Sweden for t-PA) in accordance with the manufacturer's instructions. Data were expressed as ng antigen/μg cell protein by comparison with a standard curve. Total protein concentrations of cell lysates were determined by the Bradford method (24).

Quantitation of t-PA Activity

t-PA activity was determined in conditioned medium by a chromogenic plasmin substrate assay. Different amounts of CM were incubated in a 96-well microtest plate in the presence of pPAR/L (Biopool, Sweden), a reaction mixture containing Glu-plasminogen, poly-D-lysine and a chromogenic substrate for plasmin (D-But-CHT-Lys-pNA). Absorbance was measured at 405 nm, in a spectrophotometric plate reader, after overnight incubation at 37° C. Data were expressed as International Unit of t-PA in comparison with a curve of standard t-PA run in parallel.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was obtained from cells seeded in a 25 cm^2 flask according to Chomczynski (25). 10–20μg of total RNA, determined spectrophotometrically, was subjected to electrophoresis in formaldehyde-agarose gel and transferred overnight to nylon membranes (Hybond N, Amersham). Membranes were prehybridized (4 h) and hybridized overnight at 42°C, in 50% formamide, SSPE 5× (0.75 mol/L NaCl, 0.05 mol/L $NaH_2PO_4 \cdot H_20$, 5 mmol/L EDTA), Denhardt's solution 4×, salmon-sperm DNA 100 μg/ml and bakers yeast t-RNA 200 μg/ml. Hybridization was performed with human cDNA PAI-1 or t-PA and human cDNA GAPDH labeled with ^{32}P d-CTP by the random primer method, as described by the manufacturer (Rediprime DNA labelling system, Amersham). Membranes were then washed under stringently controlled conditions at 42° C (two washes with 5×SSPE, one wash with 1×SSPE, 0.1% SDS and one with 0.1×SSPE, 0.1% SDS). Filters were air-dried and exposed to autoradiography film (Hyperfilm MP, Amersham) with intensifying screens at – 80° C. Autoradiography bands were quantified (density × area) using the NIH Image and results expressed in arbitrary units.

Labeling of HUVEC with [^{35}S]-methionine

Endothelial cells were preincubated for 24 h with the appropriate compounds in M199 containing 15% FCS, 0.1 mg/ml of heparin and ECGF. After three washings with medium alone, cells were then incubated in methionine-free Minimum Essential Medium (MEM) in the presence of 20 μCi/well [^{35}S]-methionine (350 Mbq/ml, Amersham), containing 2.5% FCS, 0.1 mg/ml heparin, 0.1 mg/ml ECGF, and the appropriate compounds or stimulus. At the end of the incubation, CM was collected and centrifuged at 10,000 g for 5 min to remove cell debris. Cells were washed three times with PBS and extracted in 500 μl of PBS pH 8.0, containing 0.5% Triton X-100, for 5 min at 37° C. The extra cellular matrix was then washed extensively with bidistilled water

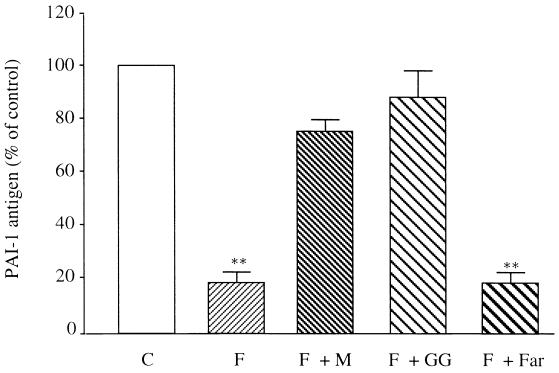


Fig. 2 Effect of mevalonate (M), all-trans-geranylgeraniol (GG) and farnesol (Far) on PAI-1 secretion exerted by fluvastatin (F). Cells were exposed to F (2.5 μM) and the different isoprenoids (M 100 μM; GG 5 μM; Far 10 μM) for preincubation and incubation times (24 + 16 h) as specified in legend to figure 1. Data represent percent of vehicle treated cells and are given as arithmetic means (with vertical lines showing SEM of 7 individual experiments performed in duplicate). ** $p < 0.01$ versus vehicle treated cells (C)

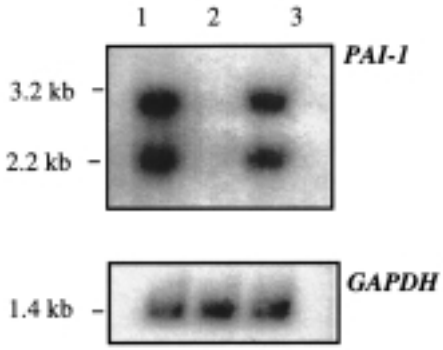


Fig. 3 Effect of fluvastatin and geranylgeraniol on PAI-1 mRNA levels. Confluent HUVEC were preincubated and then incubated with vehicle or fluvastatin (2.5 μM) and fluvastatin plus geranylgeraniol (5 μM) as described in legend to figure 1. Total RNA was extracted and analyzed by Northern blotting with radiolabeled human-PAI-1 and human-GAPDH cDNA probes. A representative autoradiogram is shown. Lane 1: vehicle-treated cells; lane 2: fluvastatin-treated cells; lane 3: cells treated with fluvastatin plus geranylgeraniol

and removed with 500 μl PBS containing 0.1% SDS. The absence of cells and cell debris in extra cellular matrix was verified by light microscopy. Samples divided into aliquots were stored at –70° C until use.

Immunoprecipitation of PAI-1

Immunoprecipitation was performed, after metabolic labeling of cells with [^{35}S] methionine, as previously described (26) with slight modifications as follows. Radiolabeled conditioned medium (100 μl), cell extracts (25 μl) or extracellular matrix (10 μl) were incubated with 15 μg goat polyclonal antibody against human PAI-1 (Product 379 American Diagnostica Inc.) or with 15 μg goat nonimmune IgG for 16 h at 4° C in the presence of PMSF (1 mM). Immune complexes were conjugated to Protein-A Sepharose suspension (50 μl of packed beads) for 1 h at 4° C. Beads were precipitated at 10,000g for 15 min, washed 3 times with PBS pH 7.4 containing 1 mM PMSF, 100 IU/ml aprotinin, 0.1% SDS, 0.1% Nonidet P-40 and twice with cold PBS. Immunoprecipitated proteins were solubilized in 55 μl of Laemmli sample buffer (27), heated to 100° C for 3 min and subjected to SDS-PAGE (12% acrylamide) under reducing condition. After electrophoresis, gels were fixed and subjected to autoradiography on Hyperfilm-MP (Amersham) at –80° C.

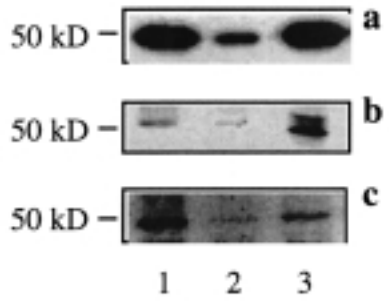


Fig. 4 Effect of fluvastatin and geranylgeraniol on PAI-1 biosynthesis in conditioned medium, intracellular and extracellular compartments. Confluent HUVEC, preincubated for 24 h with vehicle, fluvastatin (2.5 μM) or fluvastatin plus geranylgeraniol (5 μM), and then incubated for further 16 h in methionine-free MEM in the presence of 20 μCi/well [^{35}S]-methionine (350 Mbq/ml, Amersham). Labeled PAI-1 was detectable in a band of approximately 50 kD in: (a) conditioned medium (100 μl) or (b) cell extracts (25 μl) or (c) extracellular matrix (10 μl) after immunoprecipitation as described in "Methods". Lane 1: vehicle-treated cells; lane 2: fluvastatin-treated cells; lane 3: cells treated with fluvastatin-plus geranylgeraniol

Statistical Analysis

All experiments were conducted in duplicate with individual preparations of HUVECs. The results are expressed as mean ± SEM. Comparisons among treatment conditions were carried by two-tail variance analysis followed by Dunnett's test. The accepted level of significance was $p < 0.05$.

Results

Effect of Fluvastatin on PAI-1 Synthesis by Unstimulated HUVEC

Monolayers of confluent HUVEC were incubated for periods ranging from 3 to 24 h with the appropriate concentration of fluvastatin or vehicle and then cultured for 16 h with freshly prepared media containing 2.5% FCS and supplemented with the statin or vehicle alone. Fluvastatin (2.5 μM) reduced PAI-1 antigen secretion in conditioned medium and this effect was dependent upon the time of preincubation, there being almost complete suppression at 24 h (data not shown). The experiments described below were then performed in cells preincubated with the drug for 24 h.

Fluvastatin, concentration-dependently, reduced the amounts of PAI-1 by the cells from 1.69 ± 0.21 ng PAI-1 antigen/μg protein (vehicle) to 1.45 ± 0.24, 0.4 ± 0.09 and 0.28 ± 0.09 ng PAI-1 antigen/μg protein, respectively at the concentration of 0.1, 1 and 2.5 μM (the IC_{50} being 0.34 μM ± 0.037) (Fig. 1).

Mevalonate (100 μM) added together with fluvastatin (2.5 μM) fully prevented the effect of the drug on PAI-1 release, which indicates that fluvastatin reduces PAI-1 release as a consequence of inhibition of the HMG-CoA reductase enzyme (Fig. 2). Serum, a source of exogenous cholesterol, was present during all incubations, thus, the possibility that the effect of fluvastatin on PAI-1 is due to depletion of intracellular cholesterol can be ruled out. Therefore, intermediates of the mevalonate pathway, other than cholesterol, are responsible for the observed effects. Among the early derivatives of the isoprenoid pathway, geranylgeraniol and farnesol are of particular importance in view of

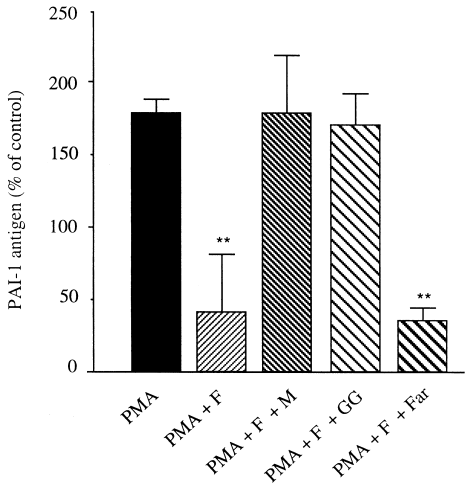


Fig. 5 Effect of fluvastatin, mevalonate and geranylgeraniol on PAI-1 release in PMA-stimulated cells. Monolayers of HUVEC were incubated for 24 h with fluvastatin (2.5 μM) and the different isoprenoids (M 100 μM; GG 5 μM; Far 10 μM), and for an additional 16 h with fresh medium containing fluvastatin, isoprenoids and PMA (0.1 μM). At the end of the incubation the medium was removed and processed to measure PAI-1 antigen levels. Results are expressed as mean percentages of vehicle treated cells taken as control (100%) and are mean ± SEM of 6 experiments performed in duplicate. ** $p < 0.01$ versus PMA-treated cells

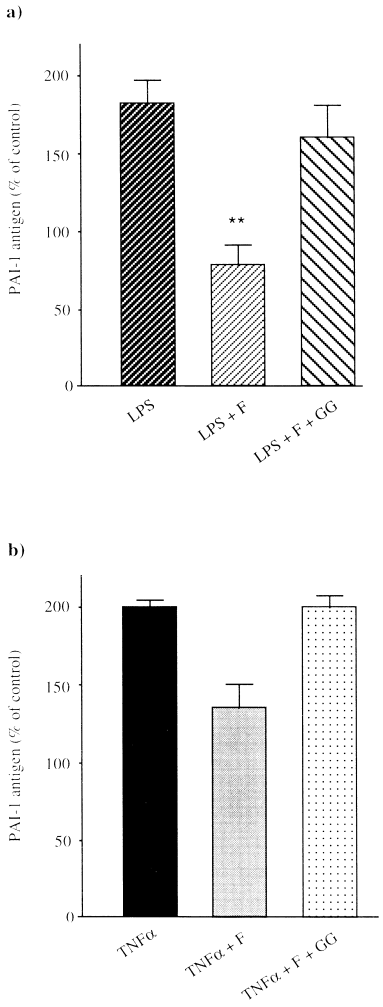


Fig. 6 Effect of fluvastatin and geranylgeraniol on PAI-1 secretion in untreated and LPS or TNFα-treated cells. HUVEC were incubated for 24 h with fluvastatin (F) (2.5 μM) and geranylgeraniol (GG) (5 μM) and for an additional 16 h with fresh medium containing fluvastatin, geranylgeraniol and LPS (10 μg/ml) (a) or TNFα (100 U/ml) (b). At the end of the incubation time the medium was removed and processed to measure PAI-1 antigen levels. Results are expressed as mean percentages of vehicle treated cells taken as control (100%) and are mean ± SEM of five (a) and three (b) experiments performed in duplicate. ** $p < 0.01$ versus LPS-treated cells

their role in the modifications of several proteins that regulate cell function. To determine whether the inhibition of PAI-1 biosynthesis induced by fluvastatin was mediated by depletion of isoprenoid intermediates, all-trans-geranylgeraniol (5 μM) and farnesol (10 μM) were added to HUVEC together with 2.5 μM fluvastatin. Farnesol did not modify the effect of fluvastatin on PAI-1 release, whereas all-*trans*-geranylgeraniol completely prevented the inhibitory effect of fluvastatin on PAI-1 secretion, which suggests a role of geranylgeranylated proteins in PAI-1 regulation (Fig. 2). Northern blot analysis showed that 2.5 μM fluvastatin almost abolished the steady-state levels of both the 3.2 and 2.2 kb PAI-1 mRNA transcripts compared with vehicle-treated cells (Fig. 3), thus suggesting that fluvastatin inhibits PAI-1 protein synthesis as the result of inhibition of PAI-1 mRNA accumulation. All-*trans*-geranylgeraniol (5 μM) completely prevented the reduction of PAI-1 mRNA exerted by fluvastatin, indicating that this effect is consequent to the depletion of geranylgeraniol (Fig. 3).

Effect of Fluvastatin on the Biosynthesis of PAI-1 in Unstimulated HUVEC

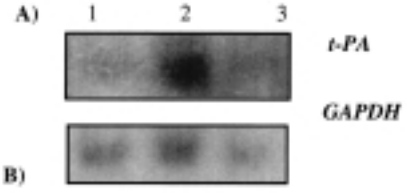
To determine whether changes in the accumulation of PAI-1 protein in conditioned medium reflected changes in the rate of PAI-1 synthesis, we performed metabolic labeling of HUVEC with [^{35}S]-methionine. Labeled PAI-1 was detectable in a band of approximately 50 kD that was revealed in extracts obtained from conditioned medium, cells and extracellular matrix. Fluvastatin (2.5 μM) reduced the amount of [^{35}S]-PAI in the three compartments and all-*trans*-geranylgeraniol (5 μM) reversed these effects (Fig. 4). Overall protein synthesis evaluated by measuring the incorporation of TCA-precipitable [^{35}S]-methionine into cells and CM was not modified. In contrast it was reduced by 50% in the extracellular matrix, in which the PAI-1 is the most abundant protein (28).

Effect of Fluvastatin on PAI-1 Release Induced by PMA, LPS and TNFα

PMA, LPS and TNFα drastically enhanced PAI-1 release in conditioned medium of stimulated HUVEC (Figs. 5, 6) in accordance with previous data (29). Fluvastatin (2.5 μM) inhibited the release of PAI-1 induced by 0.1 μM PMA and this inhibition was fully prevented by 100 μM mevalonate or 5 μM all-*trans*-geranylgeraniol, but not by 10 μM farnesol (Fig. 5). Fluvastatin (2.5 μM) inhibited PAI-1 secretion in cells treated with LPS (10 μg/ml) or TNFα (100 U/ml) and 10 μM all-*trans*-geranylgeraniol restored it (Fig. 6).

Effect of Fluvastatin on t-PA Synthesis by Unstimulated HUVEC

In our experimental conditions HUVEC released relatively low amounts of t-PA antigen within 16 h incubation (0.081 ± 0.006 ng/μg cell protein). HUVEC, however, incubated with media containing increasing amounts of fluvastatin (0.1-1-2.5 μM) released greater amounts of t-PA antigen with a 70% increase at the 2.5 μM concentration. This effect was reverted by mevalonate (Table 1). The fluvastatin-induced t-PA increases reflected the accumulation of steady state levels of mRNA for t-PA, which was prevented by 100 μM mevalonate (Fig. 7).



	C	F	F+M
t-PA	110	274	107
GAPDH	99	98	70
Ratio t-PA/GAPDH	1.11	2.8	1.5

Fig. 7 Effect of fluvastatin and mevalonate on t-PA mRNA levels. Confluent HUVEC were preincubated and then incubated with vehicle or fluvastatin (2.5 μM) and fluvastatin plus mevalonate (100 μM) as described in Fig. 1. Total RNA was extracted and analyzed by Northern blotting with radiolabeled human-t-PA and human-GAPDH cDNA probes. A) Autoradiographic results: lane 1, vehicle-treated cells; lane 2, fluvastatin-treated cells; lane 3, cells treated with fluvastatin plus mevalonate. B) Quantitative data obtained from the imaging screen expressed in arbitrary units

t-PA activity in unstimulated HUVEC was below the sensitivity of the assay (< 0.125 UI/ml) and was not influenced by fluvastatin (not shown). In fact the increase in t-PA secretion induced by 2.5 μM fluvastatin did not exceed 0.055 ng/μg cell protein, whereas the residual PAI-1 antigen, after 2.5 μM fluvastatin treatment, averaged 0.29 ng/μg cell protein, an amount grater than that of t-PA.

Table 1 Effect of fluvastatin and mevalonate on t-PA antigen secretion by HUVEC

Effect of fluvastatin and mevalonate on t-PA antigen secretion by HUVEC	t-PA (ng /μg cell protein)
Vehicle	0.081 ± 0.006
Fluvastatin (0.1 μM)	0.084 ± 0.008
Fluvastatin (1 μM)	0.114 ± 0.003 *
Fluvastatin (2.5 μM)	0.136 ± 0.003 *
Mevalonate (100 μM)	0.081 ± 0.005
Fluvastatin (2.5 μM) + Mevalonate (100 μM)	0.088 ± 0.007

Confluent HUVEC were preincubated and then cultured for further 16h with vehicle or fluvastatin (2.5 μM) and fluvastatin plus mevalonate (100 μM) as described in *Fig. 1*. t-PA antigen levels were measured in the conditioned medium as indicated in methods section. Data are the mean ± SEM of 4 individual experiments performed in duplicate.
* $p < 0.01$ *vs* vehicle-treated cells by ANOVA followed by Dunnett's test.

Discussion

The plasminogen system plays an integral role in the regulation of vascular physiology by regulating fibrinolysis and proteolysis of extracellular matrix (30). Endothelial cells are central regulators of both systems determining the availability of both t-PA and PAI-1 (31). Increases in t-PA over PAI-1 thus favour fibrinolysis and proteolysis, the opposite resulting in an impairment of both systems. The synthesis and secretion of t-PA and PAI-1 by endothelial cells is highly regulated, few agonists stimulate t-PA synthesis without affecting PAI-1 synthesis (29, 32). Only a few drugs, however, have been shown to affect the biosynthesis of these proteins. Fibric acid derivatives have been reported to reduce, with various degrees of potency, PAI-1 transcription and protein synthesis in HepG2 cells, with gemfibrozil as the most potent and bezafibrate with minimal effects (33, 34). Gemfibrozil has also been shown to impair PAI-1 biosynthesis in endothelial cells (35). These effects occur, however, only at very high concentrations (0.3-1 mM). As for t-PA biosynthesis, only a few compounds have been shown to influence it (32). Recently, lovastatin and simvastatin have been reported to increase fibrinolytic activity in the rat aortic endothelial cell as the result of reduction in PAI-1 and increase in t-PA (21).

Data presented in this study show that fluvastatin, a synthetic HMG-CoA reductase inhibitor, improves endothelial cell function by significantly reducing basal and stimulated PAI-1 biosynthesis and increasing t-PA secretion. Fluvastatin reduced PAI-1 mRNA steady state levels, with an effect on both 2.2 and 3.2 kb transcripts, as well as *de novo* synthesis of PAI-1 protein. In cultured cells > 97 % of PAI-1 is secreted into the medium, 0.3% accumulates in the extracellular matrix and 2.5–3% remains within the cells (data not shown). Fluvastatin reduced the amount of newly synthesized PAI-1 in the supernatant, cell extracts and extracellular matrix, which indicates that the drug affects the plasminogen system in both its fibrinolytic and proteolytic functions.

Fluvastatin not only reduced basal PAI-1 biosynthesis but also its increase as induced by a variety of agonists, the most pronounced effects being with PMA. The signalling pathways involved in PAI-1 biosynthesis were not addressed in this study. In this context, however, it is worth mentioning that PAI-1 biosynthesis in endothelial cells induced by PMA or TNFα recognizes a complex network of second messengers ultimately involving protein kinase C activation and translocation (36).

Fluvastatin influenced also t-PA levels in conditioned medium of unstimulated HUVEC, with a 70% increase at 2.5 μM, and this effect reflected an increase in t-PA steady-state mRNA levels. Simvastatin and lovastatin, at similar concentrations, have been reported to increase t-PA biosynthesis in Simian virus 40-transformed aortic endothelial cell line (21), with an effect more marked than that observed in the present paper in fluvastatin-treated HUVEC. In contrast, no effect of fluvastatin has been recently reported on t-PA antigen release by HUVEC incubated with medium containing 20% FCS (37).

The increase in t-PA antigen exerted by fluvastatin here described did not result in appreciable changes in t-PA activity. This observation can be explained by the fact that PAI-1 secretion by HUVEC far exceeds that of t-PA not only in basal condition but also after treatment with fluvastatin and therefore the t-PA/PAI-1 molar ratio remains in favour of PAI-1 protein.

All the effects of fluvastatin were prevented by mevalonate, the primary product of the HMG-CoA reductase enzyme, thus suggesting that fluvastatin influence PAI-1 and t-PA through inhibition of the isoprenoid pathway. The effects of fluvastatin were not explained by depletion of intracellular cholesterol, because experiments were performed in the presence of 2.5% FCS, which provides an extracellular source of cholesterol. Besides cholesterol, mevalonate is the precursor of intermediates, which are substrates for prenylation of several proteins. FPP is the precursor for farnesylation of proteins such as laminin B and ras, whereas all-trans-GGPP is a substrate for geranylation of other proteins, including many low molecular weight GTP-binding proteins (38, 39), that are involved in intracellular cell signaling. Interestingly, all-*trans*-geranygeraniol mimicked the effects of mevalonate in preventing the effects of fluvastatin on PAI-1. Thus, an effect of fluvastatin upstream of PAI-1 or t-PA gene transcription, via intermediates of isoprenoids, can be hypothesized.

Lovastatin was shown to reduce PAI-1 biosynthesis as a result of the inhibition of geranylgeranylated Rho proteins and of disruption of the actin microfilaments in endothelial cells (21). A similar mechanism may be involved with fluvastatin. On the basis of this study and on that of Essig (21) it appears that the reduced availability of geranylgeraniol influences the biosynthesis of t-PA and PAI-1 by HUVEC in opposite directions. Thus, our data indicate that fluvastatin influences PAI-1 and t-PA biosynthesis via inhibition of the HMG-CoA reductase enzyme and that an intermediate of isoprenoid biosynthesis, e. g. geranylgeraniol mediates this effect.

In conclusion, this study demonstrates that fluvastatin, at concentrations readily achieved *in vivo* (22) influences endothelial cell function positively by effectively inhibiting PAI-1 and increasing t-PA secretion.

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