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HMG CoA reductase inhibitors affect the fibrinolytic system of human vascular cells *in vitro*: a comparative study using different statins

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1 The results of several clinical studies investigating the effect of statin therapy on the fibrinolytic system *in vivo* are inconclusive. We compared the effect of six different statins (atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, simvastatin) on components of the fibrinolytic system expressed by human vascular endothelial cells and smooth muscle cells and by the human hepatoma cell line HepG2.

2 All statins used except pravastatin significantly decreased PAI-1 production in human endothelial and smooth muscle cells. This effect was also seen in the presence of IL-1α and TNF-α. All statins except pravastatin increased t-PA production in human smooth muscle cells. On a molar basis cerivastatin was the most effective HMG CoA reductase inhibitor used. Only simvastatin and lovastatin increased t-PA production in endothelial cells. The effects on the fibrinolytic system were reversed by mevalonate. Statins decreased mRNA levels for PAI-1 in endothelial and smooth muscle cells and increased mRNA levels for t-PA in smooth muscle cells. Statins did not affect PAI-1 expression in HepG2 cells. Cell viability was not influenced by statins in endothelial cells and HepG2 cells whereas in smooth muscle cells a cytotoxic effect was seen at high concentrations.

3 If the effects on the fibrinolytic system of vascular cells *in vitro* shown in this study are also operative *in vivo* one could speculate that by increasing t-PA and decreasing PAI-1 at sites of vascular lesions statins might reduce fibrin formation and thrombus development. Such an effect might contribute to the clinically proven benefits of statin therapy.
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Abbreviations: BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phopshate dehydrogenase; HCASMC, human coronary artery smooth muscle cells; HMG CoA, 3-hydroxy-3methylglutaryl-coenzyme A; HSMEC, human skin microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; HUVSMC, human umbilical vein smooth muscle cells; LDH, lactate dehydrogenase; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloprotease; PAI-1, plasminogen activator inhibitor-1; SCS, supplemented calf serum; TF, tissue factor; t-PA, tissue type-plasminogen activator; u-PA, urokinase type-plasminogen activator

Introduction

The discovery of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors or statins has led to significant improvements in the management of hypercholesteremia. Several studies have shown that statin therapy reduces not only the incidence of cardiovascular events but also decreases mortality in patients with high or 'average' cholesterol levels (Sacks *et al*., 1996; Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al*., 1995). However recently a significant number of investigations have given evidence that beyond their lipid lowering capacity statins exert 'pleiotropic' actions resulting in vasoprotection. Such vasoprotective effects include the inhibition of smooth muscle cell growth and the inhibition of neointima formation, the induction of apoptosis in smooth muscle cells, the reduction of leukocyte adhesion to and transmigration through endothelial cells, the induction of endothelial nitric oxide synthase and the inhibition of endothelin and monocyte chemotactic protein-1 (MCP-1) expression in endothelial cells and the inhibition of MCP-1, tissue factor (TF) and matrix metalloprotease-9 (MMP-9) expression in macrophages (Sindermann *et al*., 2000; Stark *et al*., 1998; Indolfi *et al*., 2000; Kimura *et al*., 1997; Dunzendorfer *et al*., 1997; Laufs *et al*., 1997; Hernandez-Perera *et al*., 1998; Romano *et al*., 2000; Bellosta *et al*., 1998a; Ferro *et al*., 2000; for recent reviews see also Rosenson & Tangney, 1998; Vaughan *et al*., 2000).

Recently, two studies have shown effects of lovastatin and simvastatin, respectively, on the fibrinolytic system of cultured smooth muscle cells and endothelial cells. In these studies a decrease in plasminogen activator inhibitor type-1 (PAI-1) in both cell types was observed whereas tissue type PA (t-PA) was increased in endothelial cells (Essig *et al*.,

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1998; Bourcier & Libby, 2000). PAI-1 is the major physiological inhibitor of t-PA and urokinase type PA (u-PA) and therefore plays a key role in the regulation of fibrinolysis (Loskutoff *et al*., 1993). High PAI-1 plasma levels and decreased levels of t-PA activity have been shown to be associated with coronary heart disease and PAI-1 mRNA has been found in human atherosclerotic lesions underlining its role in the development of these disorders (Aznar & Estelles, 1994; Schneiderman *et al*., 1992). Several *in vivo* studies have also investigated the effect of statin therapy on this key regulator of the fibrinolytic system. The results, however, are inconclusive. Pravastatin decreased PAI-1 plasma levels in several studies whereas atorvastatin was reported to increase PAI-1 in plasma (Avellone *et al*., 1994; Wada *et al*., 1992; Dangas *et al*., 2000; Davidson *et al*., 1997). For both fluvastatin and lovastatin an increase and a decrease in PAI-1 levels in plasma was reported in different clinical investigations (Davidson *et al*., 1997; Bevilacqua *et al*., 1997; Leren *et al*., 1988; Kostner *et al*., 1989; Tan *et al*., 1999; Isaacsohn *et al*., 1994). No data are currently available on the effect of cerivastatin on PAI-1 *in vivo*. As pointed out in a recent review experimental/clinical disparities such as these raise questions about the clinical applicability of *in vitro* studies performed with isolated statins (Vaughan *et al*., 2000). In this study we have for the first time compared the effect of six different statins on the fibrinolytic system of human vascular smooth muscle cells, endothelial cells and hepatocytes *in vitro*.

Methods

Materials

Stock solutions of atorvastatin (kindly provided by Pfizer, Sandwich, U.K.), cerivastatin (kindly provided by Bayer, Wuppertal, Germany) and fluvastatin (kindly provided by Novartis, Basel, Switzerland) were prepared at a concentration of 10 mM in ethanol. A stock solution of pravastatin (kindly provided by Bristol-Myers-Squibb, Paris, France) at the same concentration was prepared in distilled water. Stock solutions (10 mM) of lovastatin and simvastatin (kindly provided by Merck Sharpe and Dome, Ballydine, Ireland) were prepared after activation of the molecules as described (Cutts & Melnykovych, 1988). Such stocks were aliquoted and stored at -70°C. Mevalonate was purchased from Sigma (St. Louis, MO, U.S.A.). Human recombinant interleukin 1-α (IL-1α) and human recombinant tumor necrosis factor-α (TNF-α) were purchased from R&D Systems (Minneapolis, MN, U.S.A.).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords by mild collagenase treatment and cultured and characterized as described (Wojta *et al*., 1992). Human skin microvascular endothelial cells (HSMEC) were isolated from skin biopsies by trypsin digestion and the use of Dynabeads® (Dynal, Norway) coated with *Ulex europaeus* agglutinin as described (Wojta *et al*., 1992). Human umbilical vein smooth muscle cells (HUVSMC) were isolated by the explant technique from pieces of human umbilical cords obtained after normal vaginal delivery (Wojta *et al*., 1993). Human coronary artery smooth muscle cells (HCASMC) were isolated by the same technique from pieces of human coronary arteries obtained from explanted recipients' hearts after heart transplantation. Such smooth muscle cells were cultured and characterized as described (Wojta *et al*., 1993). All cells used in this study were between passage 2 and 4. The human hepatoma cell line HepG2 obtained from American Type Culture Collection (ATCC; Rockville, MD, U.S.A.) was cultivated as described (Wojta *et al*., 1994). All human material was obtained and processed according to the recommendations of the hospital's Ethics Committee and Security Board.

Treatment of cells with statins

Endothelial cells were incubated in Medium 199 (M199; Sigma) containing 1.25% supplemented calf serum (SCS; Hyclone, Logan, UT, U.S.A.) with different concentrations of the respective statin for the indicated time periods. Smooth muscle cells and HepG2 cells were incubated in M199 containing 0.1% bovine serum albumin (BSA; Sigma) for 24 h prior to treatment with statins. Thereafter the medium was replaced with fresh M199 containing 0.1% BSA, statins were added at the concentrations indicated and the cells were incubated for various time periods. After incubation, the culture supernatants were collected following removal of cell debris by centrifugation and stored at -70°C until used. The total cell number of the respective cultures after trypsinisation was counted with a haemocytometer.

Tissue type-plasminogen activator (t-PA) antigen and plasminogen activator inhibitor-1 (PAI-1) antigen assays

t-PA and PAI-1 antigen in conditioned media were determined by specific enzyme-linked immunosorbent assays (ELISAs) using monoclonal antibodies (Technoclone, Vienna, Austria). The PAI-1 ELISA measures active, complexed and latent PAI-1. The t-PA ELISA measures free and complexed t-PA.

Northern blots

Total cellular RNA was prepared by the guanidinium thiocyanate-phenol-chloroform extraction from confluent cells treated as indicated. RNA samples were electrophoresed in 1.2% agarose gels, transferred to a Duralon-UV™ membrane (Stratagene, U.S.A.). Hybridizations were performed overnight in 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, 5% SDS, containing 10^6 c.p.m./ml of the ^{32}P labelled cDNA probes for human PAI-1, t-PA or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). After hybridization the membranes were washed in 5% SDS, 1 × SSC at room temperature for 10 min, then washed in the same buffer at 57°C for 20 min three times. Autoradiography was performed with XAR-5 X-ray films (Eastman Kodak, U.S.A.) at -70°C (Zhang *et al*., 1997).

Determination of cell viability

In order to determine possible cytotoxic effects of the statins used, lactate dehydrogenase (LDH) leakage was measured in

cultures treated with statins as described above using a commercially available assay for photometric determination of LDH activity (Sigma).

Statistical analysis

Data were compared statistically by performing a Student's *t*-test for paired observations unless otherwise stated in the legend to the respective figure. Values of $P \leqslant 0.05$ were considered significant.

Results

Effect of statins on cell viability

As can be seen from Table 1, cell viability as determined by leakage of lactate dehydrogenase was not significantly altered by statins in HUVEC and HepG2 whereas a cytotoxic effect was observed at a concentration of 5 μM of the respective statins in HUVSMC. At lower concentrations cell viability of HUVSMC was not affected by the statins used.

Statins affect the expression of PAI-1 and t-PA in smooth muscle cells and endothelial cells

When HUVSMC were treated for 40 h with statins at concentrations between 0.1 and 2.5 μM, all statins tested except pravastatin decreased PAI-1 production and increased t-PA production in HUVSMC concentration dependently (Figure 1). Whereas atorvastatin, fluvastatin, lovastatin and simvastatin showed similar concentration response curves, the concentration response curve for cerivastatin was shifted to the left indicating that cerivastatin could be used at significantly lower concentrations to obtain effects comparable to the other statins. A time course for the effect of fluvastatin is shown in the respective insets. A similar reduction in PAI-1 production after statin treatment was seen in HCASMC (Table 2). At concentrations between 0.1 and 5.0 μM all statins with the exception of pravastatin decreased PAI-1 production concentration dependently in HUVEC after 40 h (Figure 2). Again cerivastatin was the most effective when compared to the other statins on a molar basis. A time course for the effect of fluvastatin is shown in the inset of Figure 2. Similar effects were seen when HSMEC were treated with statins (Table 2). In contrast to the results obtained with smooth muscle cells, upon incubation with statins only a slight increase in t-PA production in endothelial cells was observed which reached significance only in the case of simvastatin and lovastatin (Table 2). No effect of statins on u-PA production by either smooth muscle cells or endothelial cells was seen (data not shown). As can be seen from Figure 3 the effect of the respective statins was also evident on the level of specific mRNA expression. All statins tested reduced PAI-1 mRNA in HUVSMC and HUVEC and increased t-PA mRNA levels in HUVSMC.

Table 1 Effect of statins on viablity of human umbilical vein smooth muscle cells (HUVSMC), human umbilical vein endothelial cells (HUVEC)and HepG2 cells

	HUVSMC		*HUVEC*	*HepG2*	
	(5.0 μM)	(2.5 μM)	(5.0 μM)	(5.0 μM)	(100.0 μM)
Atorvastatin	231.3±10.5**	102.2±5.7	102.7±12.5	94.1±3.5	n.d.
Cerivastatin	257.9±17.3**	107.6±8.1	103.9±9.7	97.1±5.6	n.d.
Fluvastatin	218.5±20.2**	105.9±3.7	108.5±8.2	97.9±7.3	95.2±6.8
Lovastatin	290.7±24.6**	97.7±8.7	103.5±4.5	99.4±4.3	101.2±7.9
Pravastatin	n.d.	n.d.	97.9±7.8	97.5±4.6	100.5±3.4
Simvastatin	282.9±7.4**	101.5±11.2	107.8±9.6	99.3±4.3	98.7±5.1

Confluent monolayers of the respective cell type were incubated for 48 h in the presence or absence of statins at the indicated concentration. Thereafter lactate dehydrogenase leakage as an indicator for cell damage was measured photometrically using a commercially available assay as described in the Methods section. Absorbance values obtained with untreated control cells were set as 100%. Values of statin treated cells are expressed as per cent of control and represent mean values±s.d. of three independent determinations. $^{**}P<0.01$; n.d.: not determined.

Table 2 Effect of statins on PAI-1 production in human coronary artery smooth muscle cells (HCASMC), in human skin microvascular endothelial cells (HSMEC) and in HepG2 cells and on t-PA production in human umbilical vein endothelial cells (HUVEC)

	PAI-1 antigen				*t-PA antigen*
	HCASMC	*HSMEC*	*HepG2 #1*	*HepG2 #2*	*HUVEC*
Control	856.2±90.4	275.5±20.8	98.5±9.3	103.3±12.3	1.03±0.12
Atorvastatin	n.d.	148.4±12.3*	102.9±11.3	n.d.	1.15±0.20
Cerivastatin	254.9±22.1**	125.6±14.7**	97.1±8.8	n.d.	1.17±0.07
Fluvastatin	387.1±49.3*	117.8±11.3**	112.9±11.2	103.8±8.7	1.13±0.05
Lovastatin	n.d.	150.3±17.5*	109.9±12.8	101.2±10.2	1.57±0.05**
Pravastatin	825.4±75.8	277.7±20.9	96.3±17.6	104.9±7.8	1.09±0.07
Simvastatin	426.1±56.8*	134.3±16.1**	105.3±17.8	102.2±13.5	1.65±0.13**

Confluent monolayers of the respective cell type were incubated for 40 h in the absence or presence of the respective statin at a concentration of 1.0 μM (HCASMC), 5.0 μM (HSEMC, HepG2 #1 and HUVEC) or 100 μM HepG2 #2). Conditioned media of such treated cells were collected and PAI-1 and t-PA antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values±s.d. of three independent determinations. n.d.: Not determined, $^{**}P<0.01$, $^{*}P<0.05$.

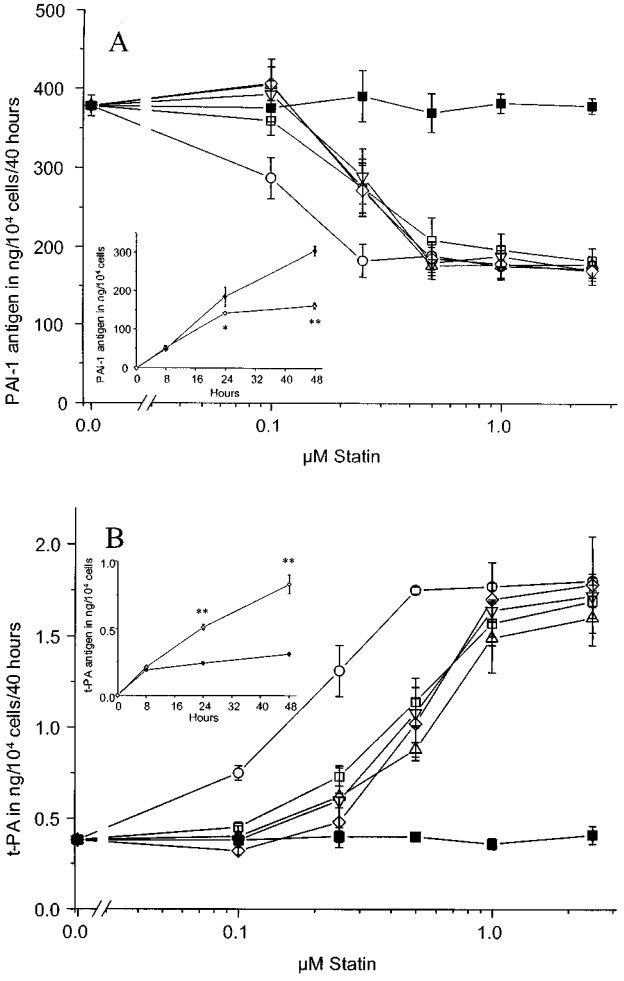

Figure 1 (A) Effect of statins on PAI-1 production in human umbilical vein smooth muscle cells (HUVSMC). Confluent monolayers of HUVSMC were incubated for 40 h in the absence or presence of atorvastatin (open squares), cerivastatin (open circles), fluvastatin (open upright triangles), lovastatin (open inverted triangles), pravastatin (filled squares) or simvastatin (open diamonds) at a concentration of 0.1, 0.25, 0.5, 1.0 and 2.5 μM, respectively. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values$\pm$s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. PAI-1 antigen was significantly decreased by all statins used except pravastatin at concentrations $\geqslant 0.25$ μM ($P<0.01$); as determined by ANOVA the concentration response curve for cerivastatin was significantly different from the concentration response curves obtained for atorvastatin, fluvastatin, lovastatin and simvastatin respectively ($P<0.01$). Inset (A) Timecourse of the effect of fluvastatin on PAI-1 production in human umbilical vein smooth muscle cells (HUVSMC): Confluent monolayers of HUVSMC were incubated for 8, 24 or 48 h in the absence (full diamonds) or presence of 2.5 μM fluvastatin (open diamonds). Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells and represent mean values$\pm$s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. PAI-1 antigen was significantly decreased in the presence of fluvastatin after 24 h (*$P<0.05$) and 48 h (**$P<0.01$) of incubation. (B) Effect of statins on t-PA production in human umbilical vein smooth muscle cells (HUVSMC). Confluent monolayers of HUVSMC were incubated for 40 h in the absence or presence of atorvastatin (open squares), cerivastatin (open circles), fluvastatin (open upright triangles), lovastatin (open inverted triangles), pravastatin (filled squares) or simvastatin (open diamonds) at a concentration of 0.1, 0.25, 0.5, 1.0 and 2.5 μM, respectively. Conditioned media of such treated cells were collected and t-PA antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values$\pm$s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. t-PA antigen was significantly increased by all statins used except pravastatin at concentrations $\geqslant 0.5$ μM ($P<0.01$); as determined by ANOVA, the concentration response curve for cerivastatin was significantly different from the concentration response curves obtained for atorvastatin, fluvastatin, lovastatin and simvastatin respectively ($P<0.01$). Inset (B) Timecourse of the effect of fluvastatin on t-PA production in human umbilical vein smooth muscle cells (HUVSMC). Confluent monolayers of HUVSMC were incubated for 8, 24 or 48 h in the absence (full diamonds) or presence of 2.5 μM fluvastatin (open diamonds). Conditioned fresh media of such treated cells were collected and t-PA antigen was determined as described in the Methods section. Values are given in ng/10^4 cells and represent mean values$\pm$s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. t-PA antigen was significantly increased in the presence of fluvastatin after 24 h (**$P<0.01$) and 48 hours (**$P<0.01$) of incubation.

Statins affect the production of PAI-1 in smooth muscle cells and endothelial cells activated by cytokines

When HUVSMC and HUVEC were treated with cytokines such as interleukin-1α (IL-1α) or tumor necrosis factor-α (TNF-α) an increase in PAI-1 production was seen. When such cells were treated with these cytokines in the presence of statins PAI-1 levels were reduced almost to levels seen in cells incubated with statins alone (Figure 4).

Mevalonate reverses the effects of statins on PAI-1 and t-PA in smooth muscle cells and endothelial cells

As can be seen from Table 3 the effects of the statins were reversed by addition of 100 μM mevalonate. When mevalonate was present during the incubation period of 40 h together with the respective statin, PAI-1 as well as t-PA levels were not different from levels seen in untreated cells.

Statins do not affect PAI-1 in HepG2 cells

Neither of the statins used at 5.0 or 100.0 μM did affect PAI-1 production in the human hepatoma cell line HepG2 after 40 h of incubation (Table 2).

Discussion

HMG CoA reductase inhibitors or statins have been shown to exert various 'vasoprotective' effects besides their lipid lowering properties (for recent reviews see references Rosenson & Tangney, 1998; Vaughan *et al*., 2000). In this study we have investigated the effect of different statins on the fibrinolytic system of human vascular endothelial cells, human vascular smooth muscle cells and the human hepatoma line HepG2. There is growing evidence for a role of components of the fibrinolytic system not only in the lysis of blood clots but also in the development and in the progression of atherosclerosis. High PAI-1 plasma levels and decreased levels of t-PA are associated with coronary heart disease and PAI-1 mRNA has been found

Table 3 Mevalonate reverses the effects of statins on PAI-1 and t-PA production in human umbilical vein smooth muscle cells (HUVSMC) and in human umbilical vein endothelial cells (HUVEC)

	HUVSMC				*HUVEC*			
	PAI-1 antigen		*t-PA antigen*		*PAI-1 antigen*		*t-PA antigen*	
	No mevalonate	*Mevalonate*	*No mevalonate*	*Mevalonate*	*No mevalonate*	*Mevalonate*	*No mevalonate*	*Mevalonate*
Control	310.0±20.0	311.2±43.3	0.40±0.04	0.41±0.06	851.7±87.0	824.3±24.6	0.85±0.03	0.88±0.09
Atorvastatin	194.5±13.3*	322.5±12.2	1.05±0.08**	0.38±0.02	331.7±16.5**	808.7±81.6	1.11±0.23	0.91±0.09
Cerivastatin	170.0±21.1*	302.3±41.2	1.35±0.12**	0.47±0.05	331.9±56.1**	852.3±97.8	0.73±0.02	0.77±0.03
Fluvastatin	190.1±12.2*	366.7±41.2	1.16±0.12**	0.40±0.03	379.9±16.5**	912.0±65.4	1.05±0.15	0.78±0.09
Lovastatin	176.6±21.2*	320.0±35.6	1.54±0.15**	0.42±0.06	371.7±73.6**	836.2±81.2	1.80±0.12**	0.77±0.04
Pravastatin	322.2±13.4	322.5±8.9	0.42±0.02	0.45±0.05	863.5±88.9	838.7±40.2	0.75±0.09	0.72±0.03
Simvastatin	180.3±21.2*	294.4±28.6	1.57±0.13**	0.49±0.05	363.1±65.1**	831.2±88.5	1.62±0.12**	0.77±0.04

Confluent monolayers of HUVSMC and HUVEC were incubated for 40 h in the absence or presence of the respective statin at a concentration of 2.5 μM (HUVSMC) or 5.0 μM (HUVEC) alone or together with mevalonate at a concentration of 100 μM. Conditioned media of such treated cells were collected and PAI-1 and t-PA antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values±s.d. of three independent determinations. $^{**}P<0.01$, $^{*}P<0.05$.

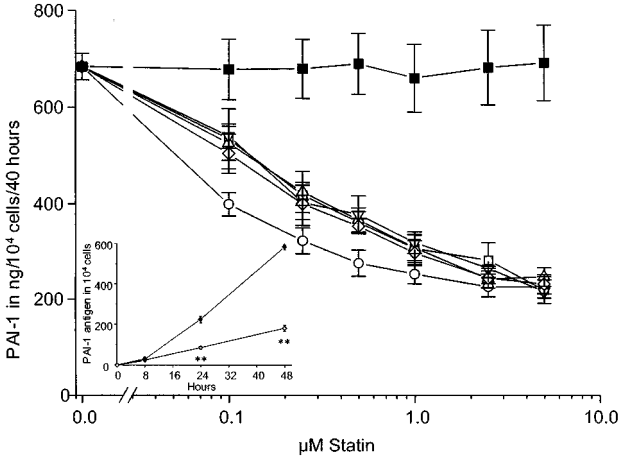

Figure 2 Effect of statins on PAI-1 production in human umbilical vein endothelial cells (HUVEC). Confluent monolayers of HUVEC were incubated for 40 h in the absence or presence of atorvastatin (open squares), cerivastatin (open circles), fluvastatin (open upright triangles), lovastatin (open inverted triangles), pravastatin (filled squares) or simvastatin (open diamonds) at a concentration of 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 μM, respectively. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values±s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. PAI-1 antigen was significantly decreased by all statins used except pravastatin at concentrations $\geqslant 0.25$ μM ($P<0.01$); as determined by ANOVA, the concentration response curve for cerivastatin was significantly different from the concentration response curves obtained for atorvastatin, fluvastatin, lovastatin and simvastatin respectively ($P<0.01$). Inset to (2) Timecourse of the effect of fluvastatin on PAI-1 production in human umbilical vein endothelial cells (HUVEC). Confluent monolayers of HUVEC were incubated for 8, 24 or 48 h in the absence (full diamonds) or presence of 5.0 μM fluvastatin (open diamonds). Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells and represent mean values±s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. PAI-1 antigen was significantly decreased in the presence of fluvastatin after 24 h ($^{**}P<0.01$) and 48 h ($^{**}P<0.01$) of incubation.

in human atherosclerotic lesions (Loskutoff *et al*., 1993; Aznar & Estelles, 1994; Schneiderman *et al*., 1992). Various *in vivo* studies have investigated the effects of statins on plasma levels of PAI-1 (Avellone *et al*., 1994; Wada *et al*., 1992; Dangas *et al*., 2000; Davidson *et al*., 1997; Bevilacqua *et al*., 1997; Leren *et al*., 1988; Kostner *et al*., 1989; Tan *et al*., 1999; Isaacsohn *et al*., 1994). The results of these clinical studies, however, are inconsistent. E.g. for both fluvastatin and lovastatin an increase and a decrease in PAI-1 levels in plasma was reported in different clinical investigations (Davidson *et al*., 1997; Bevilacqua *et al*., 1997; Leren *et al*., 1988; Kostner *et al*., 1989; Tan *et al*., 1999; Isaacsohn *et al*., 1994).

Here we present evidence that all statins tested except pravastatin concentration dependently decreased PAI-1 production in cultured endothelial cells and smooth muscle cells isolated from human umbilical vein. Similar results were obtained when human microvascular endothelial cells and human coronary artery smooth muscle cells were treated with statins indicating that the observed effect was not specific for a particular type of endothelial cell or smooth muscle cell. Furthermore t-PA production in smooth muscle cells was also concentration dependently increased by all statins, with the exception of pravastatin which showed no effect. In that respect it is of interest that in other *in vitro* studies pravastatin did not inhibit smooth muscle cell growth and migration and did not induce apoptosis whereas cerivastatin, fluvastatin, simvastatin and lovastatin inhibited proliferation and migration of these cells and the latter two induced apoptosis (Sindermann *et al*., 2000; Stark *et al*., 1998; Indolfi *et al*., 2000; Bellosta *et al*., 1998b). This lack of efficacy of pravastatin in cell culture studies might be brought about by its lipophobic properties hindering pravastatin's penetration of the cell membrane. In addition it should be noted that – when comparing the effects of the different statins on PAI-1 and t-PA production in the cells used in our study – atorvastatin, fluvastatin, lovastatin and simvastatin showed similar concentration response curves. The concentration response curves for cerivastatin in these experiments were shifted to the left, indicating that significantly lower concentrations of cerivastatin were necessary to elicit effects on the fibrinolytic system in endothelial cells and smooth muscle cells comparable to the effects induced by the other statins used. Interestingly the recommended *in vivo* dosages for cerivastatin are significantly lower than for the other HMG CoA reductase inhibitors (Hunninghake, 1998; Desager & Horsmans, 1996; Marais *et al*., 1997). In endothelial cells only simvastatin and lovastatin increased t-PA production whereas the other statins had no effect. This is in agreement with two recent studies that investigated the

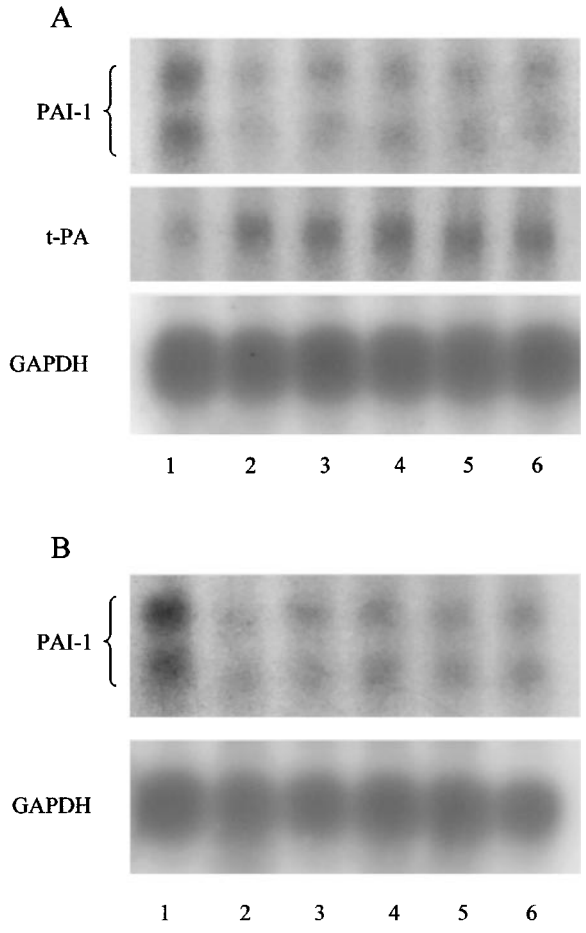

Figure 3 (A) Effect of statins on PAI-1 and t-PA mRNA expression in human umbilical vein smooth muscle cells (HUVSMC). Confluent monolayers of HUVSMC were incubated for 16 hours in the absence or presence of 2.5 μM of the respective statin (lane 1: control, lane 2: atorvastatin, lane 3: cerivastatin, lane 4: fluvastatin, lane 5: lovastatin, lane 6: simvastatin). mRNA of such treated cells was prepared and PAI-1, t-PA and GAPDH mRNA was visualized by Northern blotting as described in the Methods section. Experiments were performed twice with a representative experiment shown. (B) Effect of statins on PAI-1 mRNA expression in human umbilical vein endothelial cells (HUVEC). Confluent monolayers of HUVEC were incubated for 16 h in the absence or presence of 5.0 μM of the respective statin (lane 1: control, lane 2: atorvastatin, lane 3: cerivastatin, lane 4: fluvastatin, lane 5: lovastatin, lane 6: simvastatin). mRNA of such treated cells was prepared and PAI-1 and GAPDH mRNA was visualized by Northern blotting as described in the Methods section. Experiments were performed twice with a representative experiment shown.

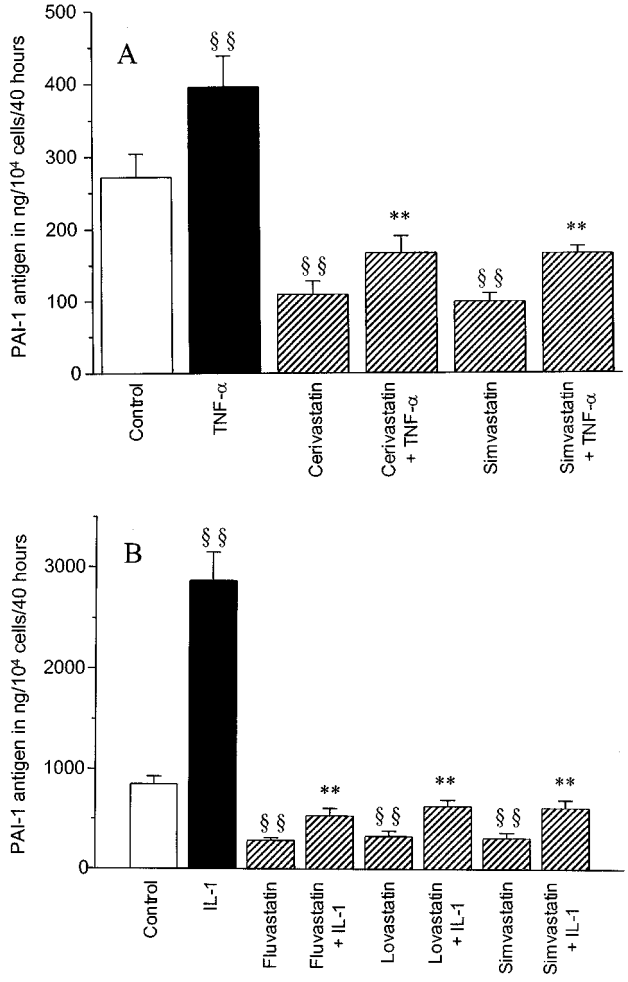

Figure 4 (A) Effect of statins on tumor necrosis factor-α (TNF-α) induced PAI-1 production in human umbilical vein smooth muscle cells (HUVSMC). Confluent monolayers of HUVSMC were incubated for 40 h in the absence (open bar) or presence of TNF-α (10.0 nM; full bar), cerivastatin (2.5 μM; hatched bar), or simvastatin (2.5 μM; hatched bar), respectively, or with the respective statin together with TNF-α (hatched bars) at the concentrations indicated above. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values $\pm$ s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. §§ Significantly different from control, $P<0.01$; **significantly different from TNF-α treated cells, $P<0.01$. (B) Effect of statins on interleukin-1α (IL-1α) induced PAI-1 production in human umbilical vein endothelial cells (HUVEC). Confluent monolayers of HUVEC were incubated for 40 h in the absence (open bar) or presence of IL-1α (200 u ml^{-1}; full bar), fluvastatin (5.0 μM; hatched bar), lovastatin (5.0 μM; hatched bar) or simvastatin (5.0 μM; hatched bar), respectively, or with the respective statin together with IL-1α (hatched bars) at the concentrations indicated above. Conditioned media of such treated cells were collected and PAI-1 antigen was determined as described in the Methods section. Values are given in ng/10^4 cells/40 h and represent mean values $\pm$ s.d. of three independent determinations. Experiments were performed three times with a representative experiment shown. §§ Significantly different from control, $P<0.01$; **significantly different from IL-1α treated cells, $P<0.01$.

effects of these two statins on the fibrinolytic system of endothelial cells and smooth muscle cells (Essig *et al*., 1998; Bourcier & Libby, 2000).

As indicated by the results of a cell viability assay using lactate dehydrogenase leakage as a marker of cell damage, the effects of the HMG CoA reductase inhibitors on the fibrinolytic system of vascular cells were not due to cytotoxicity. Lactate dehydrogenase leakage was not affected in endothelial cells by statins at concentrations of $\leqslant 5$ μM whereas in smooth muscle cells a cytotoxic effect was observed at a concentration of 5 μM. No difference in lactate dehydrogenase leakage to control cells was seen in smooth muscle cells treated with statins at concentrations of $\leqslant 2.5$ μM. The effects of statins on PAI-1 and t-PA production could be completely reversed by mevalonate indicating that these effects were brought about by inhibition

of the mevalonic acid pathway. This is in agreement with several other cell culture studies (Sindermann *et al*., 2000; Laufs *et al*., 1997; Bellosta *et al*., 1998a; Essig *et al*., 1998; Bourcier & Libby, 2000).

The effects of statins on components of the fibrinolytic system on the level of proteins were also reflected on the level of specific mRNA expression. As evidenced by Northern blotting, statin treatment decreased the steady state level of PAI-1 specific mRNA in endothelial cells and smooth muscle cells whereas levels of t-PA specific mRNA were increased in smooth muscle cells after incubation with HMG CoA reductase inhibitors.

Furthermore we could show that statins significantly reduced the IL-1α and TNF-α induced increase in PAI-1 in vascular cells. Thus we present evidence that HMG CoA reductase inhibitors might ameliorate the response of vascular cells towards activation by inflammatory cytokines. Recently it was shown that fluvastatin and atorvastatin modestly inhibited TNF-α induced PAI-1 synthesis in endothelial cells, whereas simvastatin was shown to inhibit the platelet derived growth factor (PDGF)- and transforming growth factor-β (TGF-β) stimulated expression of PAI-1 in vascular cells (Lopez *et al*., 2000; Bourcier & Libby, 2000).

In contrast to the effects seen in vascular cells, statins at 5.0 μM did not regulate PAI-1 production in the human hepatoma cell line HepG2 which has been extensively used as a model to study the regulation of PAI-1 in human hepatocytes (Wojta *et al*., 1994; Healy & Gelehrter, 1994; Arts *et al*., 1999). To account for possible metabolization of the statins by HepG2 cells, the cells were incubated with the respective HMG CoA reductase inhibitor at a concentration of 100.0 μM. Even at this high concentration no effect on PAI-1 production in these cells was seen. Cell viability of HepG2 cells was not affected by statins used at concentrations of $\leqslant$100.0 μM. Thus one could speculate that the effects of statins on the fibrinolytic system might be tissue specific.

In general, caution should be used in extrapolating results of experimental studies to the clinical setting. The concentrations of statins used in this *in vitro* study are higher than observed plasma concentrations in patients treated with these HMG CoA reductase inhibitors (Desager & Horsmans, 1996). They are, however, in the same range as concentrations of statins used in numerous other tissue culture studies (Sindermann *et al*., 2000; Indolfi *et al*., 2000; Laufs *et al*., 1997; Hernandez-Perera *et al*., 1998; Romano *et al*., 2000; Bellosta *et al*., 1998a; Ferro *et al*., 2000; Essig *et al*., 1998; Bourcier & Libby, 2000). Furthermore one could speculate that the local concentration in tissue in the *in vivo* situation might depend on the exposure time of the tissue to the HMG CoA reductase inhibitors. Considering the fact that in most clinical studies patients are treated with statins over a period of days or even weeks, these exposure times are much longer as compared to the *in vitro* setting where cells are treated with these drugs only for hours. Given the different response of vascular cells and HepG2 cells towards statin treatment, further studies are needed to investigate a possible tissue specificity of the described effect of statins on components of the fibrinolytic system.

We present evidence that in vascular cells all statins tested except pravastatin reduced PAI-1 and increased t-PA expression. It should be noted, however, that under culture conditions human endothelial cells and smooth muscle cells produce PAI-1 in an up to 100-fold excess over t-PA leading to complex formation between t-PA and PAI-1 and total inhibition of the plasminogen activator by its inhibitor. Thus no t-PA activity is detectable in supernatants from cultured human endothelial cells and smooth muscle cells (Bartha *et al*., 1988; Wojta *et al*., 1991; 1993). In contrast, in freshly isolated human endothelial cells and in resting murine endothelial cells *in vivo* PAI-1 is hardly detectable whereas t-PA is present throughout the vasculature (van den Berg *et al*., 1988; Fearns *et al*., 1995; Kristensen *et al*., 1984). If the effects of statins on PAI-1 and t-PA expression in vascular cells *in vitro* are also operative *in vivo,* one could therefore hypothesize that at sites of vascular lesions a reduced expression of PAI-1 and an increased expression of t-PA by endothelial cells and smooth muscle cells would increase the fibrinolytic capacity locally thereby favouring thrombolysis and reducing fibrin formation after plaque rupture. It should be emphasised that we have shown in this study that statins exert their downregulating effect on PAI-1 production also in the presence of inflammatory cytokines, which are thought to be present at such sites of plaque rupture and which in the *in vivo* setting result in strong induction of PAI-1 expression in the endothelium (Loskutoff *et al*., 1993). Again, if statins downregulate PAI-1 production in vascular cells activated by inflammatory mediators to basal levels also *in vivo*, one could speculate that the fibrinolytic potential of these cells would be increased and local fibrinolysis would be favoured. On the other hand a decrease in PAI-1 might also favour extracellular matrix degradation by proteases thereby leading to plaque instability. Different studies investigating the effect of statins on PAI-1 plasma levels in patients have produced inconsistent results (Avellone *et al*., 1994; Wada *et al*., 1992; Dangas *et al*., 2000; Davidson *et al*., 1997; Bevilacqua *et al*., 1997; Leren *et al*., 1988; Kostner *et al*., 1989; Tan *et al*., 1999; Isaacsohn *et al*., 1994). The contribution of endothelial cells under normal conditions to the amount of PAI-1 present in plasma remains to be defined. Freshly isolated human endothelial cells produce only little PAI-1 and no PAI-1 mRNA could be detected in normal murine endothelium *in vivo* (van den Berg *et al*., 1988; Fearns *et al*., 1995). PAI-1 has been detected in human endothelium *in vivo* but these findings should be interpreted with some caution since most of these tissues have been obtained under stressed conditions (e.g. after trauma or major surgery) and PAI-1 is an acute phase protein (Healy & Gelehrter, 1994; Lupu *et al*., 1993; Chomiki *et al*., 1994). Other possible sources of plasma PAI-1 are platelets, liver cells and adipocytes (for a review see Loskutoff & Samad, 1998). Thus – given the doubtful contribution of endothelial cells to plasma PAI-1 – we hypothesize that statins might not influence systemic plasma PAI-1 levels by decreasing its expression in vascular endothelial cells, but rather exert their clinically proven beneficial effects by shifting the balance locally at the site of vascular lesions towards increased fibrinolysis thereby reducing the risk of thrombus formation at such sites.

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