Regulation of the endothelial plasminogen activator system by fluvastatin. Role of Rho family proteins, actin polymerisation and p38 MAP kinase

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Sylvie Dunoyer-Geindre[1*]; Richard J. Fish[1,2*]; Egbert K. O. Kruithof[1]

[1]Division of Angiology and Hemostasis, Department of Internal Medicine, Geneva University Hospital; [2]Current address: Department of Genetic Medicine and Development, Geneva University Medical School, Geneva, Switzerland

Summary

Statins are cholesterol-lowering drugs that exert pleiotropic effects which include changes in the plasminogen activation (PA) system of endothelial cells (EC). It was the objective of this study to investigate the signal transduction pathways by which statins increase the expression of tissue-type PA (t-PA) and decrease PA inhibitor type 1 (PAI-1) in human umbilical vein EC. Fluvastatin treatment increased t-PA expression more than 10-fold and reduced PAI-1 expression up to five-fold. This effect was mimicked by geranylgeranyl transferase inhibition. The role of geranylgeranylated small G-proteins of the Rho family was assessed by adenovirus-mediated expression of dominant negative (DN) RhoA, Cdc42 and Rac1 and by siRNA-mediated suppression of these proteins. DN-Cdc42 and DN-Rac1, as well as siRNA for Cdc42, increased t-PA expression, while DN-RhoA and DN-Rac1 decreased PAI-1 expression. Latrunculin B, an inhibitor of actin polymerisation, increased t-PA mRNA and reduced PAI-1 mRNA to the same extent as fluvastatin. Inhibition of p38, as well as p38α or p38β siRNA, reversed the effects of fluvastatin on t-PA expression. Treatment with p38β siRNA partially reversed the effect of fluvastatin on PAI-1, whereas p38α siRNA had no significant effect. Inhibition of jun kinase reduced basal and fluvastatin-induced t-PA expression to the same extent and increased PAI-1. MEK/ERK inhibition had no effect. In human EC, the fluvastatin-induced increase in t-PA is mediated by Cdc42 and, as with t-PA induced by inhibition of actin polymerisation, requires activation of p38MAP kinase. The mechanisms by which fluvastatin treatment reduces PAI-1 are different from those that increase t-PA.

Keywords

Endothelial cells, statin, t-PA, PAI-1, p38, Rho family proteins, actin polymerization

Correspondence to:
Egbert K. O. Kruithof
Division of Angiology and Hemostasis
University Medical Center, Laboratory 9094
Rue Michel Servet 1, CH-1211 Genève 4, Switzerland
Tel.: +41 22 3795493, Fax: +41 22 37 29299
E-mail: Egbert.Kruithof@hcuge.ch

*These authors contributed equally to this article.

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Introduction

3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, or statins, lower cholesterol levels in patients with cardiovascular disease. They exhibit pleiotropic effects that are related to their inhibition of protein prenylation (1). Thus, proteins, which require prenylation for their activity, are at least partially inhibited by statin treatment. Such proteins include the small G-protein superfamily, which are involved in cytoskeletal organisation, membrane trafficking and gene regulation.

Several studies reported an increased expression of tissue-type plasminogen activator (t-PA) and reduced expression of its major inhibitor, plasminogen activator inhibitor-1 (PAI-1), in statin-treated endothelial cells or smooth muscle cells (2–5). As endothelium-derived t-PA is the principal factor for thrombus dissolution at the intact vascular wall, this implies a modified balance between t-PA and PAI-1 leading to a beneficial more pro-fibrinolytic vessel wall in statin-treated patients.

The increased t-PA activity, and reduced PAI-1 activity, in statin-treated rat aortic endothelial cells has been linked to inhibition of RhoA, a small G-protein (2). There are a number of well-defined links between small G-protein activity and gene expression, notably in the activation of signalling to MAP kinase pathways which in turn control gene expression (6). While small prenylated G-proteins are therefore good candidate target proteins for mediating the activity of statins on gene regulation, how inhibition of small G-protein prenylation translates into changes in t-PA and PAI-1 expression is largely unclear.

In this study we probed the mechanism by which fluvastatin alters the balance of the endothelial cell plasminogen activator system. We demonstrate that the mechanism of statin-induced changes in t-PA and PAI-1 expression are distinct and can be partially mimicked by inhibition of several Rho family GTPases and fully by inhibition of actin polymerisation. In addition, the statin-mediated increase in t-PA production requires p38 MAP kinase activity.

Materials and methods

Reagents

Fluvastatin sodium, IPA3, SB202190, SP600125, latrunculin B and cytochalasin D were from Calbiochem (San Diego, CA, USA); BIRB796 from Axon (Groningen, The Netherlands); Y-27632 dihydrochloride from Tocris (Ellsville, MI, USA) and U0126 from Biomol (Plymouth Meeting, PA, USA). Other fine chemicals were from Sigma-Aldrich (Schnelldorf, Germany).

Cells

Human umbilical cord derived endothelial cells (HUVEC) were isolated as described (7) and cultured in EGM-2 medium (Cambrex Walkersville Inc, Baltimore, MD, USA). All treatments with fluvastatin or other reagents were made in EGM-2 medium with 2% FCS but without the addition of growth factors. Umbilical cords were obtained with informed consent from parents and with local hospital ethics committee approval. Cells were used at passage 1 to 4.

Quantitative reverse transcriptase real time PCR (qPCR)

Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using the Improm-II reverse transcriptase system from Promega (Madison, WI, USA). qPCR was performed as described previously (8) using the ΔΔCT method and GAPDH as the control housekeeping gene. The oligonucleotide sequences used for qPCR are given in ▶Table 1.

Measurement of t-PA and PAI-1 antigen concentrations

t-PA antigen concentrations were measured by ELISA, as described previously (9). PAI-1 antigen concentrations were measured by ELISA (DuoSet SerpinE1/PAI-1, R&D systems, Abingdon, UK) according to the manufacturer's instructions.

Immunoblotting

Cells were lysed with lysis buffer (Cell Signaling Technology, Danvers, MA, USA) supplemented with complete mini protease inhibitors (Roche Diagnostics, Rotkreuz, Switzerland) and 1 mM NaF. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed using antibodies for RhoA, Cdc42 and Rac1 (Cell Signaling Technology) and β-actin (Sigma-Aldrich).

Adenoviral vectors

Adenoviral vectors for expression of Myc-tagged N19RhoA, N17Rac1 and N17Cdc42 (referred to as DN [dominant negative]) were a gift from Dr A. J. Ridley (Ludwig Institute, London, UK). The vectors were amplified in human 293 embryonic kidney cells and used as described previously (10).

Short interfering RNA transfection

Short interfering RNA (siRNA) specific for RhoA, Rac1, Cdc42, PAK1, PAK2, PAK3, p38α, p38β and control siRNA were obtained from Qiagen (Germantown, MD, USA). The following HP validated siRNA sets were used: RhoA (Hs_RHOA_6 and Hs_RHOA_7), Rac1 (Hs_RAC1_6 and Hs_RAC1_10), Cdc42 (Hs_Cdc42_4, Hs_Cdc42_12 and Hs_Cdc42_17), PAK1 (Hs_PAK1_6, Hs_PAK1_7 and Hs_PAK1_9), PAK2 (Hs_PAK2_6 and Hs_PAK2_12), PAK3 (Hs_PAK3_5 and Hs_PAK3_11), p38α (Hs_MAPK14_5, Hs_MAPK14_6 and Hs_MAPK14_7) and p38β (Hs_MAPK11_5, Hs_MAPK11_6 and Hs_MAPK11_7).

Cells were plated in a six-well plate to obtain about 60% confluency and maintained in medium without antibiotics for 3 hours (h). Then, the medium was replaced by OptiMEM® medium (Invitrogen). Three µl RNAiMax transfection reagent (Invitrogen) was incubated for 5 minutes (min) in 50 µl OptiMEM at room temperature, combined with 50 pmol of siRNA in 50 µl OptiMEM, incubated for 20 min and added to cells. Transfection was performed for 4 h at 37°C, thereafter the medium was replaced by EGM2 medium without antibiotics. A second transfection was made 24 h later. Forty-eight hours after the second siRNA treat-

Table 1: Primers used for quantitative PCR analysis.

Protein	Forward primer	Reverse primer
GAPDH	GGTGAAGGTCGGAGTCAAC	CCATGGGTGGAATCATATTG
t-PA	CCGGCTACGGCAAGCA	AGCGGCTGGATGGGTACA
PAI-1	AAGGGTCTGCTGTGCACCAT	AAACACCCTCACCCCGAAGT
RhoA	TGGAAAGACATGCTTGCTCAT	GCCTCAGGCGATCATAATCTTC
Rac1	ATCCGCAAACAGATGTGTTCT	CGCACCTCAGGATACCACT
Cdc42iso 1	GGAGTGTTCTGCACTTACACAG	CGGCTCTTCTTCGGTTCTG
PAK1	CAGCACTATGATTGGAGTCGG	TGGATCGGTAAAATCGGTCCT
PAK2	CACACCACCCTCTGCTGAAAA	GGGTCCCCACACTCACAATG
PAK3	AAATTGGTCAAGGGGCATCAG	ACCCATAGTTCATCACCCACC
p38α	CAGCTTCAGCAGATTATGCGT	CTTGGGCCGCTGTAATTCTC
p38β	AAGCACGAGAACGTCATCGG	TCACCAAGTACACTTCGCTGA

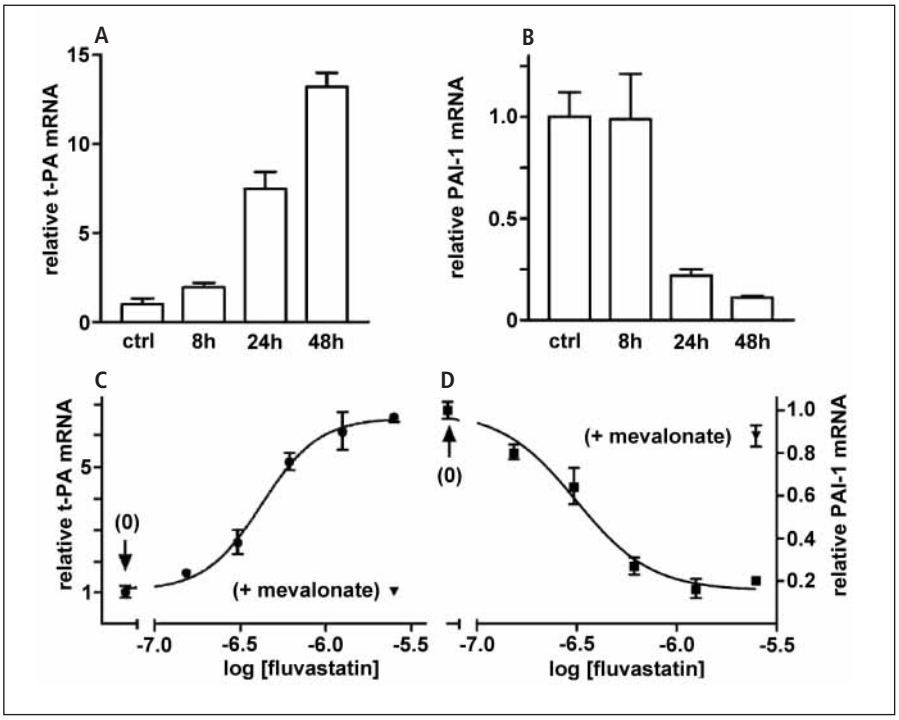


Figure 1: Effect of fluvastatin on t-PA and PAI-1 expression in HUVEC. HUVEC were treated with 2.5 μM fluvastatin for 8, 24 or 48 h. Levels of t-PA mRNA (A) and PAI-1 mRNA (B) were compared to untreated cells (ctrl). C and D) Dose-response curve for the effect at 24 h of fluvastatin on t-PA and PAI-1 mRNA. Note the similar fluvastatin concentrations that gave a half maximal effect (in C EC50 = 0.43 μM and in D EC50 = 0.32 μM) and the complete reversal of the effect of 2.5 μM fluvastatin by 0.5 mM mevalonate (▼). Data are from a single representative experiment made with triplicate RNAs for each condition. Error bars represent the SD of technical replicates.

ment, the cells were treated with agonist or control medium (without growth factors), and 24 h later mRNA was extracted and analysed by qPCR to determine target mRNA reduction and t-PA and PAI-1 mRNA expression. For each target two or three different siRNAs were used. Results were only used when target reduction was over 70%. Two negative controls were used for siRNA, in one we added the RNAiMax transfection reagent alone to cells, in another we added a control siRNA (All star, Qiagen). No difference in expression of t-PA and PAI-1 was observed with either control.

Statistics

Unpaired Student t-tests were performed on data as described in the figure legends, but only on data where multiple biological replicates (independent experiments) are shown. Statistical tests were not made on technical replicates from single representative experiments.

Results

Effects of fluvastatin on t-PA and PAI-1 expression in HUVEC

As effects of statins on the PA system of endothelial cells have been described before, mainly in rat aortic EC, we first set-out to find appropriate cell culture conditions in which to study the effects of fluvastatin on t-PA and PAI-1 expression in HUVEC. We performed a time course using a previously optimised fluvastatin concentration of 2.5 μM (10), which is close to peak concentrations in patients (11). We observed a time-dependent increase in t-PA and a decrease in PAI-1 mRNA (▶Fig. 1A and B). While increased t-PA mRNA could already be measured at 8 h, decreased PAI-1 mRNA was evident only after 24 h. Changes over time in expression of t-PA and PAI-1 in control cells were limited. At the 24 h time point, t-PA mRNA as compared to the 0 h control point was 0.9 ± 0.2, whereas for PAI-1 this was 1.2 ± 0.2.

As a 48 h incubation period in medium lacking growth factors is not ideal for HUVEC cultures, we used 24 h incubations for further experiments. At 24 h, the increase in t-PA mRNA was accompanied by an increase in t-PA antigen in cell conditioned media (data not shown). Dose-response curves showed that the fluvastatin concentration required for a half-maximal t-PA increase or the reduction in PAI-1, were the same (▶Fig. 1C and D). Mevalonate (0.5 mM) completely reversed the effects of 2.5 μM fluvastatin (▶Fig. 1C and D).

As statins may also affect protein geranylgeranylation we studied the effect of geranylgeranyl pyrophosphate (GGPP). We observed that GGPP reversed (▶Fig. 2A-D) the effect of fluvastatin on t-PA and PAI-1 mRNA and protein secretion. The geranylgeranyl transferase inhibitor GGTI-298 mimicked the effect of fluvastatin by increasing t-PA mRNA and decreasing PAI-1 mRNA (▶Fig. 2E and F). This confirms that the effects of fluvastatin on t-PA/PAI-1 expression involve inhibition of protein geranylgeranylation.

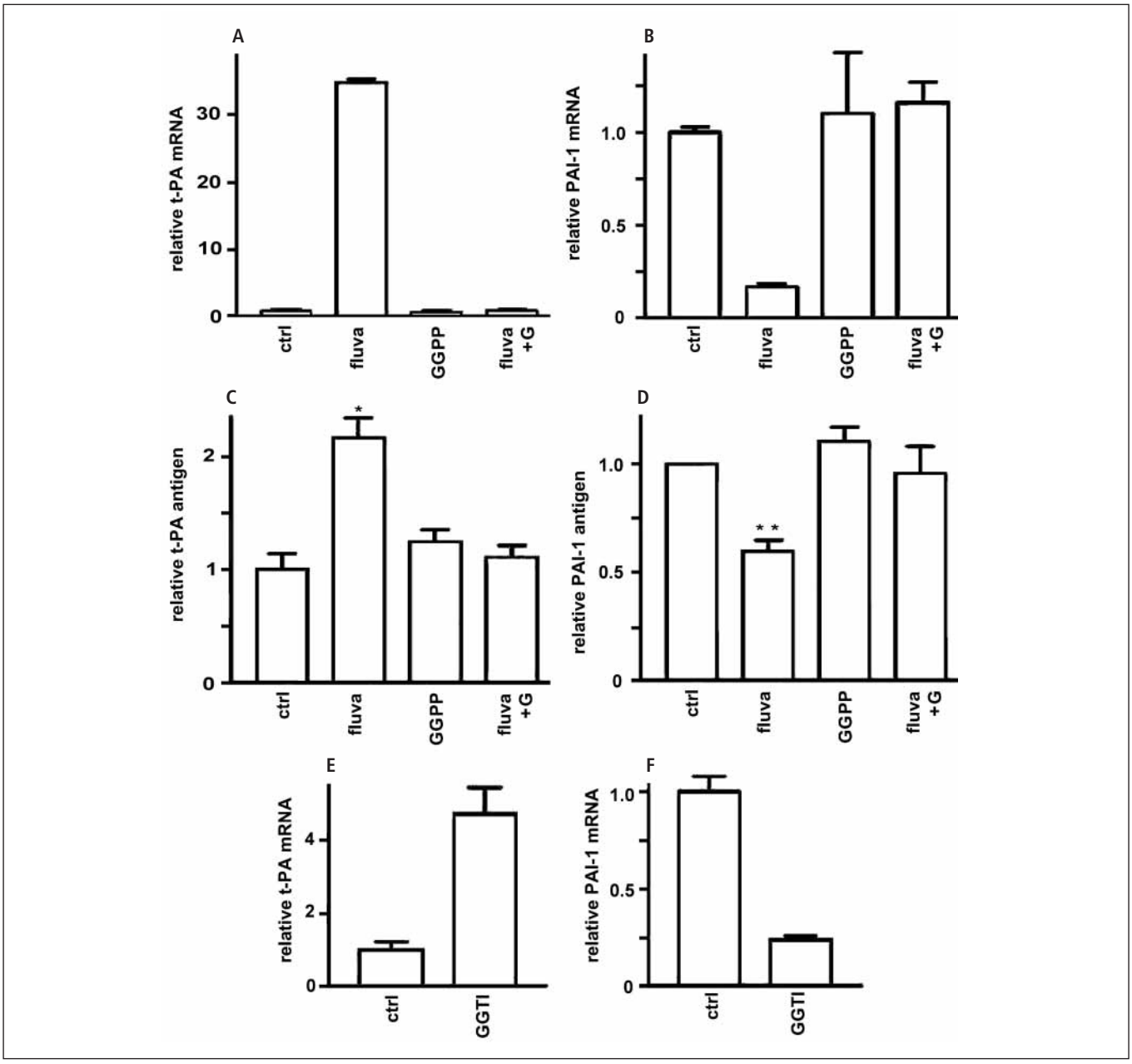


Figure 2: Role of protein geranylgeranylation in the fluvastatin-induced changes in t-PA and PAI-1. HUVEC were incubated for 24 h with 2.5 μM fluvastatin (fluva), 10 μM GGPP, or both (fluva + G) and levels of t-PA mRNA (A), of PAI-1 mRNA (B) or t-PA (C) and PAI-1 (D) antigen in cell-conditioned medium compared to untreated cells (ctrl). E) and F) Effect of the geranylgeranyl transferase inhibitor GGTI-298 (10 μM) on t-PA and PAI-1 mRNA levels. Graphics showing mRNA expression are from a single representative experiment made with triplicate RNA samples for each condition. Error bars represent the SD of technical replicates (n=3). Data for t-PA or PAI-1 antigen show the mean ± SEM of four independent experiments. Results were assessed by an unpaired Student's t-test (* $p<0.05$, ** $p<0.01$).

Role of Rho family proteins

As the major cellular target for protein geranylgeranylation are small G-proteins, and inhibition of RhoA was previously shown to increase t-PA activity in rat aortic EC (2), we sought to determine whether RhoA and two further Rho family proteins, Rac1 and Cdc42, undergo modifications in HUVEC treated with fluvastatin. Inhibition of prenylation can be assessed by a change in electrophoretic mobility in SDS-PAGE (12). We demonstrate that under our experimental conditions an important fraction of cellular RhoA, Rac1 and Cdc42 had a slower migration in SDS-PAGE gels following fluvastatin treatment (▶Fig. 3A). The fluvastatin-induced changes in migration of these small GTPases were reversed by GGPP. We also noted a clear increase in RhoA expression in sta-

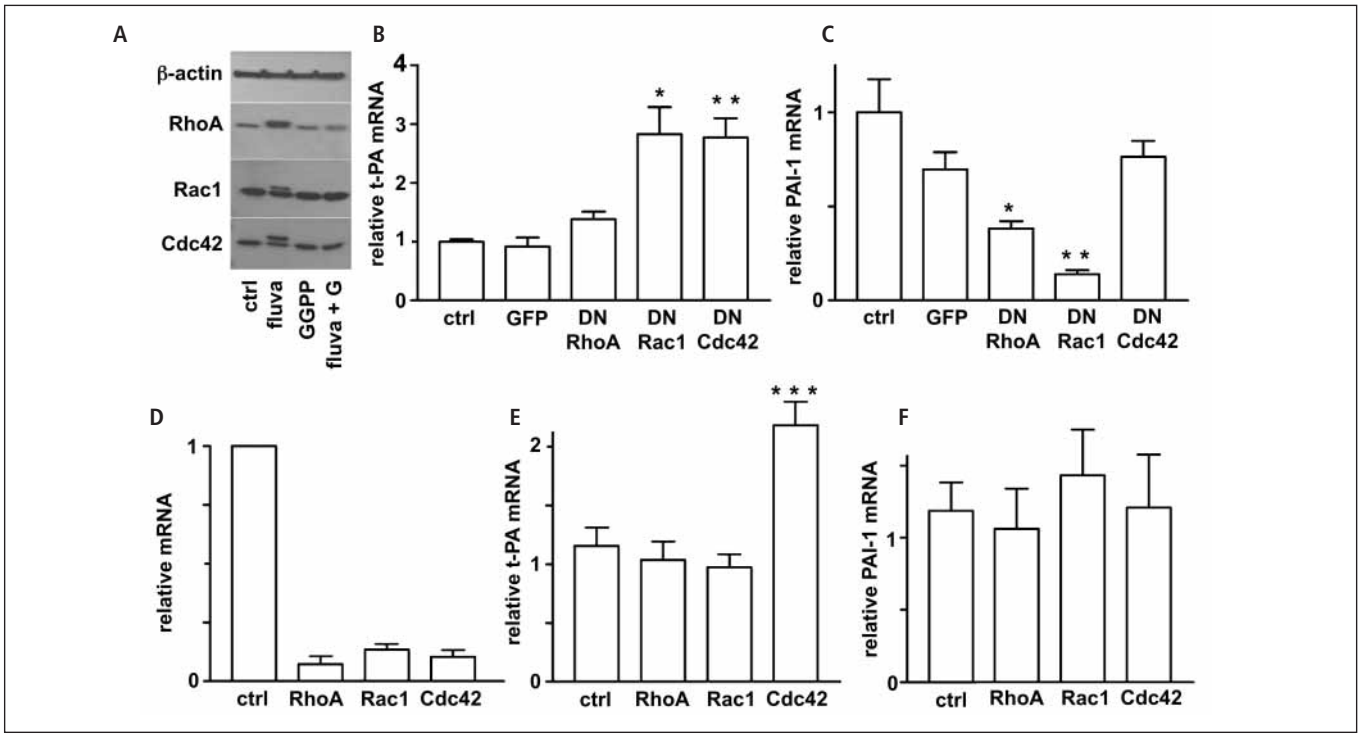


Figure 3: Rho family GTPases and the PA system. A) HUVEC were treated for 24 h with medium (ctrl), 2.5 μM fluvastatin (fluva), GGPP (10 μM) or fluvastatin and GGPP (fluva + G), and cell lysates used for immunoblotting with antibodies to RhoA, Rac1, Cdc42 and β-actin as a loading control. Note the change in migration of Rho family proteins extracted from fluvastatin-treated cells. B and C) HUVEC were transduced with adenoviral vectors for overexpression of GFP, DN-RhoA, DN-Rac1 or DN-Cdc42. Relative mRNA levels of t-PA (B) and PAI-1 (C) were compared to non-transduced cells (ctrl). Data are from three independent experiments, each made with duplicate or triplicate RNA samples of each condition, error bars show SEM (n=3). Unpaired Student's t-tests were used to compare the values for each treatment to control cells. For * $p < 0.05$, ** $p < 0.01$. D) Downregulation of RhoA, Rac1 or Cdc42 mRNA after siRNA-mediated silencing. E and F) HUVEC were twice transfected at a 24 h interval with siRNA for RhoA, Rac1 and Cdc42 or control siRNA. Seventy-two hours later t-PA mRNA (E) and PAI-1 mRNA (F) levels were assessed. Data are expressed as ratio with respect to untreated cells and are from three independent experiments, each made with two or three different siRNA preparations, error bars show SEM (n=8 to 10 data points). *** $p < 0.001$.

tin-treated cells, which confirms results described previously (13). This increase in RhoA was reversed by GGPP, suggesting it requires inhibition of protein geranylgeranylation.

To assess the role of these small G-proteins in the fluvastatin-induced changes in t-PA or PAI-1 expression, we overexpressed DN versions of each in HUVEC cultures, using adenoviral vectors, and measured t-PA and PAI-1 mRNA. A green fluorescent protein (GFP) vector was used as a transduction control, and non-transduced cells were included in the analysis. A significant three-fold increase in t-PA expression was measured upon expression of DN-Rac1 and DN-Cdc42 and a 50% increase with DN-RhoA, compared to non-transfected and GFP-transfected cells (▶Fig. 3B). DN-Cdc42 did not alter PAI-1 expression whereas DN-RhoA and DN-Rac1 reduced PAI-1 expression by about 55% and about 80%, respectively (▶Fig 3C). DN Rho family proteins not only affect their active homologs, but by depletion of guanine exchange factors, modify the activity of other Rho family proteins (14). Therefore, the effect of a DN-Rho family protein does not allow us to unequivocally identify the protein involved. To further investigate the role of RhoA, Rac1 and Cdc42 we used siRNA-mediated suppression of these proteins using at least two different siRNAs for each protein. The siRNA approach chosen resulted in greater than 80% reduction of RhoA mRNA, Rac1 mRNA and Cdc42 mRNA, respectively (▶Fig. 3D). Control siRNA had no effect on mRNA levels of these Rho family proteins. Knockdown of RhoA or Rac1 had no effect on t-PA, whereas knockdown of Cdc42 resulted in a significant ($p<0.001$) two-fold increase of t-PA mRNA (▶Fig. 3E). No significant changes were observed for PAI-1 mRNA by siRNA-mediated downregulation of RhoA, Rac1 or of Cdc42 (▶Fig. 3F). Results with control siRNA were comparable those obtained with untreated cells.

Inhibition of Rho kinase by 10 μM of Y27632 (i.e. more than 10-fold higher than the IC_{50}) for 6 h and 24 h had no effect on t-PA mRNA expression (0.75-fold and 1.03-fold control values, respectively), or on PAI-1 mRNA (0.63-fold and 1.55-fold control values, respectively).

Taken together, these results show that the effect of fluvastatin on t-PA and PAI-1 expression is mediated by a different combination of Rho family proteins.

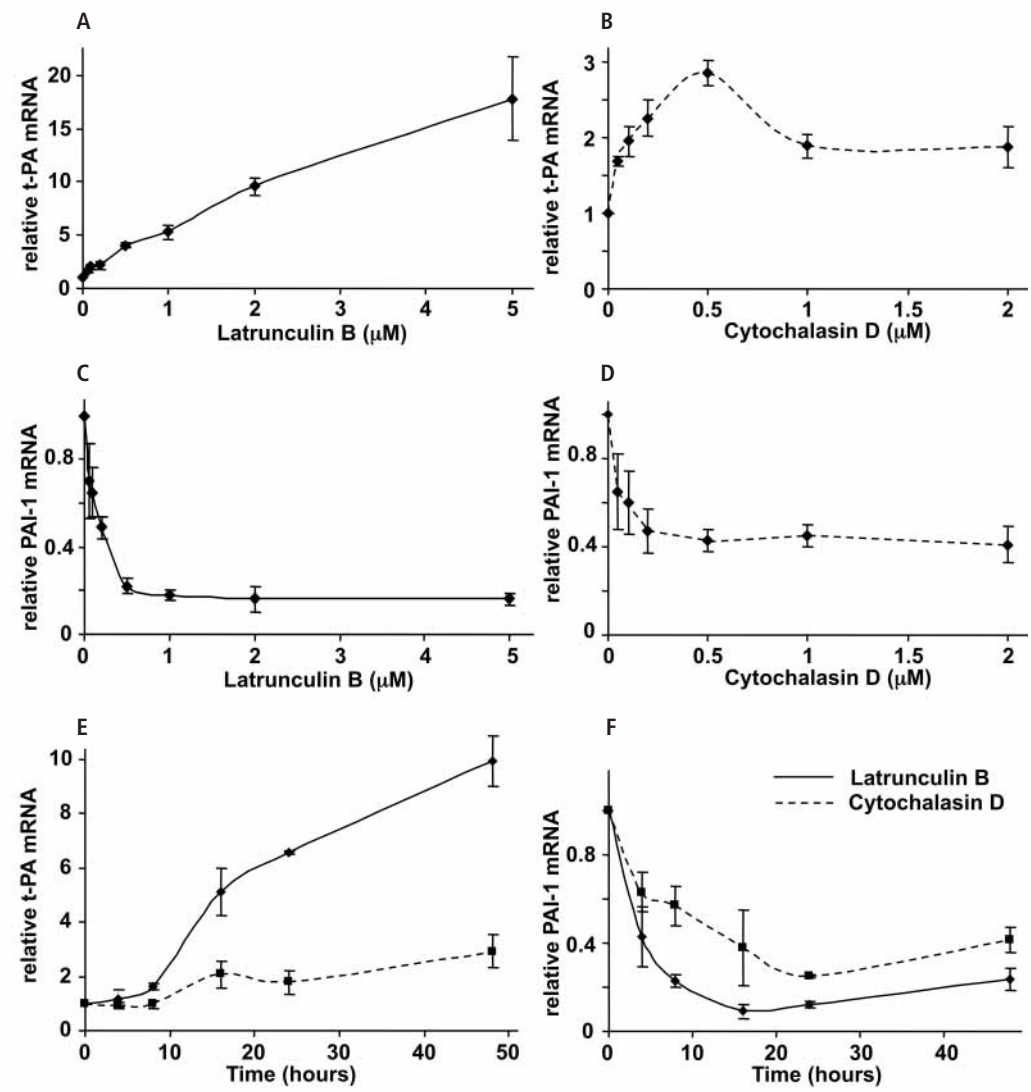


Figure 4: Effect of latrunculin B or cytochalasin D on t-PA and PAI-1 expression by HUVEC. HUVEC were incubated with the indicated concentrations of the actin-polymerisation inhibitors latrunculin B or cytochalasin D, and 24 h later mRNA levels of t-PA (A and B) and PAI-1 (C and D) mRNA analysed. Results are given as mean ± SEM of 3–6 independent experiments. E and F) Time course experiments were done with 2 μM of latrunculin B or 2 μM of cytochalasin D and mRNA levels of t-PA (E) and PAI-1 (F) analysed. Results are given as mean ± SEM of three independent experiments.

Effect of PAK family inhibition on t-PA and PAI-1 expression

Common intermediates downstream of Rac1 and Cdc42 are the p21-activated kinases (PAK). To investigate the role of PAKs we used IPA3, a selective inhibitor of PAK1, PAK2 and PAK3. At 10 μM of IPA-3, t-PA mRNA and PAI-1 mRNA levels were unchanged (t-PA: 1.09-fold ± 0.32, SEM, n=3; and PAI-1: 1.17-fold ± 0.10 over control cells). Downregulation of individual PAKs, using siRNA for PAK1, PAK2 or PAK3 had no effect on t-PA expression (ratio of siRNA treated versus control untreated cells for PAK1 siRNA 1.01 ± 0.29, SEM, n=6 ; for PAK2 siRNA : 0.76 ± 0.08; for PAK3 siRNA: 1.13 ± 0.07). PAK3 siRNA or control siRNA induced a small increase in PAI-1 mRNA (PAK3 siRNA: 1.43 ± 0.20 and control siRNA 1.48 ± 0.24, as compared to control untreated cells), while PAK1 and PAK2 siRNA induced a further increase in PAI-1 mRNA (PAK1 siRNA: 2.26 ± 0.31, SEM, n=6; and PAK2 siRNA : 3.49 ± 0.69, n=6).

Effect of actin polymerisation inhibitors

Previously, an increase in t-PA activity was observed in supernatants of rat aortic endothelial cells treated with the actin polymerisation inhibitor cytochalasin D (2). To determine whether the increase in t-PA activity was due to an increase in t-PA expression, a reduction in PAI-1 expression or both, HUVEC were treated with

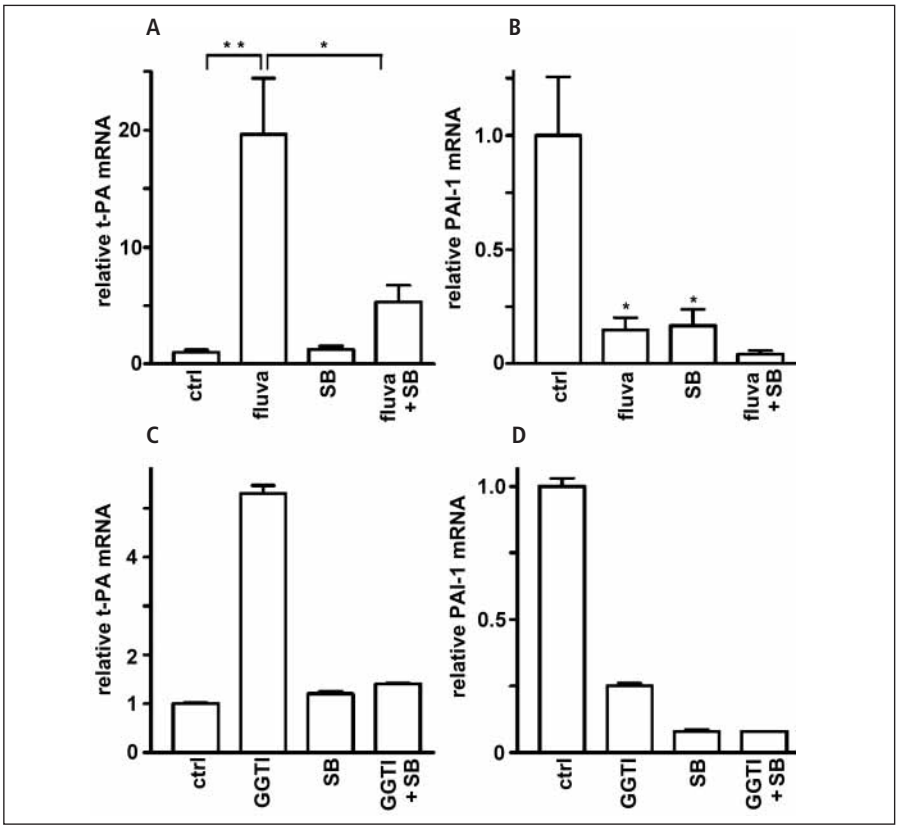


Figure 5: Fluvastatin- or GGTI-induced t-PA expression is reversed by inhibition of p38 MAP kinase. To assess the dependence of fluvastatin-induced changes in t-PA and PAI-1 on p38 MAP kinase activity, HUVEC were treated with 2.5 μM fluvastatin (fluva), 10 μM SB202190 (SB), a p38 MAPK inhibitor, or both (fluva + SB) and t-PA (A) and PAI-1 (B) mRNA levels compared to untreated cells (ctrl). Data are from four independent experiments. Error bars show SEM (n=4). * $p < 0.05$, ** $p < 0.01$. Dependence of GGTI-298-induced changes of t-PA (C) and PAI-1 (D) expression on p38 MAP kinase activity was measured by treating HUVEC with medium (ctrl), GGTI-298, SB202190 or GGTI and SB202190. Data are from a single representative experiment performed with triplicate RNAs for each condition. Error bars represent the SD of technical replicates.

various concentrations of the actin polymerisation inhibitors latrunculin B or cytochalasin D. After 24 h, we observed a dose-dependent increase in t-PA mRNA and decrease in PAI-1 mRNA in HUVEC treated with latrunculin B (▶Fig. 4A and C). Maximal increase in t-PA mRNA was 18-fold and the maximal reduction in PAI-1 mRNA was over 80%. With 2 μM cytochalasin D, we observed a more modest two-fold increase in t-PA mRNA and two-fold reduction in PAI-1 mRNA (▶Fig. 4B and D). The marked difference in effect of the two actin polymerisation inhibitors is unlikely to be due to the use of suboptimal cytochalasin D concentrations, because a plateau was already reached at a concentration of 0.5 μM, whereas the effect of latrunculin B had not yet reached its maximum at 5 μM. The effect of 2 μM latrunculin B and 2 μM cytochalasin D on PAI-1 mRNA is already detectable after 4 h and reached a maximum after 24 h, whereas the effect on t-PA expression is only significant after 16 h and did not reach a maximum even after 48 h (▶Fig. 4E and F).

Role of p38 MAP kinase

MAP kinases are known signalling intermediates between Rho family proteins and actin structure on one hand and gene expression on the other. Here, we investigated the role of MAP kinases in the statin-mediated increase in t-PA mRNA and reduction in PAI-1 mRNA. First, we investigated the role of p38 MAP kinase in the effects seen with fluvastatin. HUVEC were incubated for 24 h with fluvastatin or GGTI-298, with SB202190 (an inhibitor of p38α and p38β) or with both. SB202190 had a slight, non-significant, stimulating effect on basal t-PA expression, but reversed the fluvastatin- or GGTI-298-mediated increase in t-PA mRNA (▶Fig. 5A and C). SB202190 reduced steady-state PAI-1 expression and accentuated the fluvastatin- or GGTI-298-mediated decrease in PAI-1 (▶Fig. 5B and D). SB202190 is not only an efficient inhibitor of p38α and p38β, but also of several other kinases (15). Therefore we used BIRB796, another inhibitor of p38α and p38β, which has an inhibitory profile distinct from that of SB202190 (15). BIRB796 increased basal t-PA mRNA up to two-fold, but inhibited the fluvastatin-mediated increase in t-PA mRNA (▶Fig. 6A). It had no effect on basal PAI-1 mRNA or on the fluvastatin-mediated reduction of PAI-1 expression (▶Fig. 6B). The difference in effect of SB202190 and BIRB796 on basal PAI-1 expression suggests that the effect of SB202190 is not due to inhibition of p38, but rather to its effect on other kinases. The experiments with the p38 inhibitors were supplemented with studies on the effect of siRNA-mediated down-regulation of p38α (MAPK14) and p38β (MAPK11). We used three different siRNA preparations for each; using these siRNAs p38α mRNA was reduced by 79 ± 7% (mean ± SD) and p38β mRNA by 81 ± 10%. In agreement with the results obtained with the two p38 inhibitors, we observed that both MAPK14 (p38α) siRNA and MAPK11 (p38β) siRNA treatment counteracted the effect of fluvastatin on t-PA mRNA (▶Fig. 6C and E). MAPK14 (p38α) siRNA but not

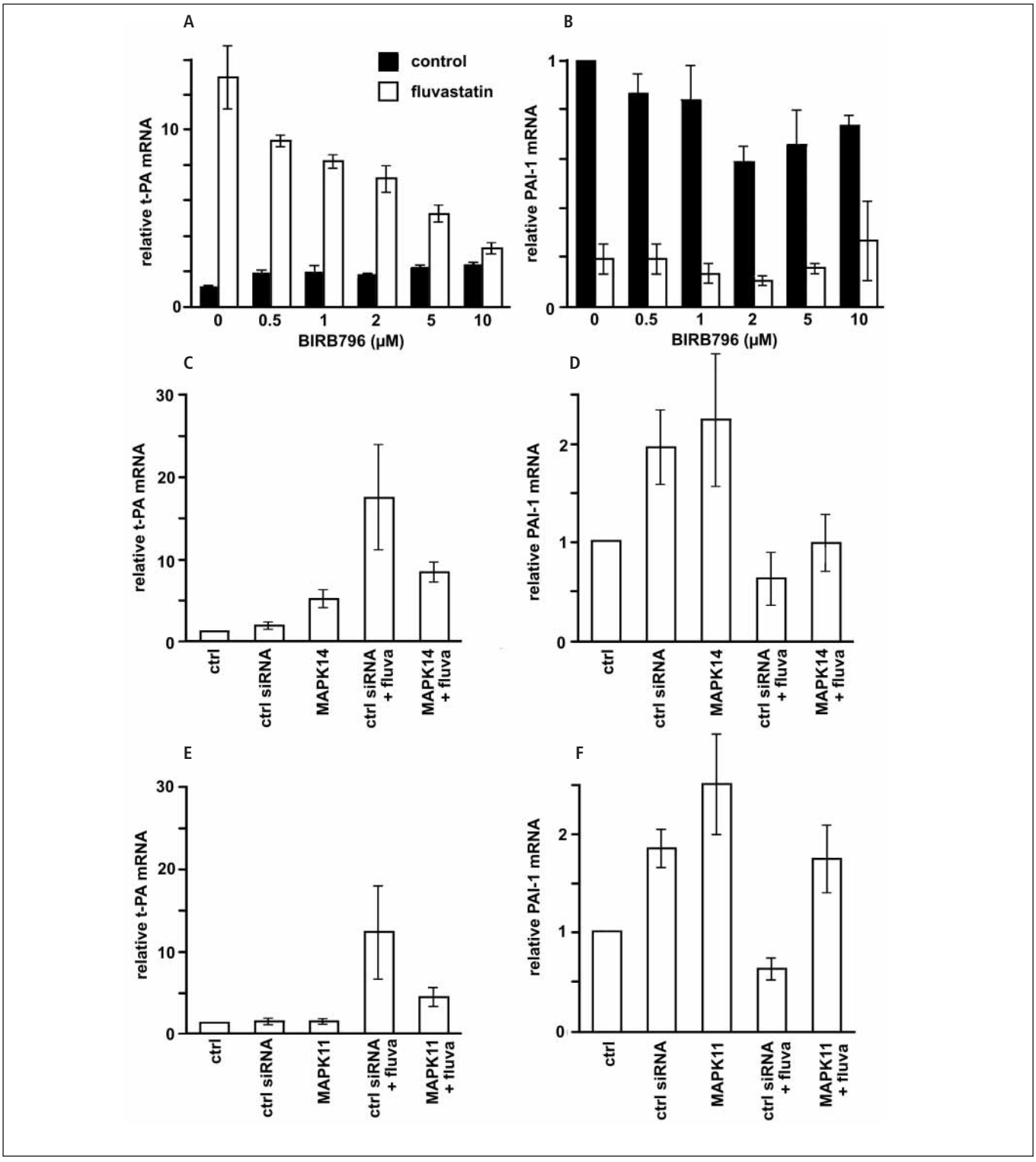


Figure 6: Effect of BIRB796 and siRNA for p38α and p38β on t-PA and PAI-1 expression by HUVEC. HUVEC were incubated for 24 h with BIRB796, an inhibitor of p38α and p38β, and the effect on t-PA mRNA (A) and PAI-1 mRNA (B) was assessed. The results show the mean ± SEM of three independent experiments. HUVEC were twice transfected at a 24 h interval with siRNA for p38α (MAPK14) (C and D), p38β (MAPK11) (E and F) or control siRNA, and 72 h later t-PA mRNA (C and E) and PAI-1 mRNA (D and F) levels were assessed. Fluvastatin was added during the last 24 h (+F). The results show mean ± SEM of three independent experiments, with three different siRNA sets used in each experiment.

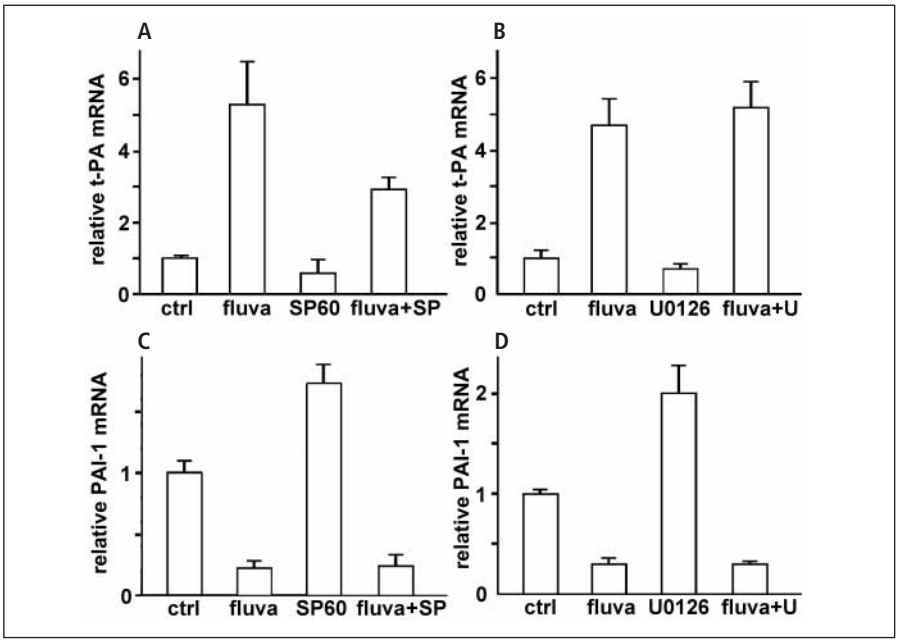


Figure 7: Fluvastatin-induced t-PA expression is not sensitive to JNK or MEK1/MEK2 inhibitors. A and C) HUVEC were treated with medium (ctrl), fluvastatin (fluva, at 2.5 μM), SP600125 (SP60, a JNK inhibitor, at 10 μM) or fluvastatin and SP600125, and relative t-PA mRNA (A) or PAI-1 mRNA (C) levels measured. B and D) U0126 (an inhibitor of MEK1 and MEK2, at 2 μM) was used and samples analysed as in A and C. Data are from a single representative experiment made with three RNA samples per condition. Error bars represent SEM (n=3).

MAPK11 (p38β) siRNA increased basal t-PA mRNA three-fold compared to control siRNA-transfected cells. The effect of MAPK14 (p38α) siRNA and MAPK11 (p38β) on basal PAI-1 mRNA was comparable to that of control siRNA, which induced a two-fold increase in PAI-1 (▶Fig. 6D and F). MAPK11 (p38β) siRNA partially reversed the effect of fluvastatin on PAI-1 mRNA, whereas MAPK14 (p38α) siRNA had no significant effect. To determine the role of p38 in the increased expression of t-PA after treatment with latrunculin B, we incubated HUVEC with 5 μM latrunculin B, with 5 μM BIRB796 or a combination of both agents. Treatment with BIRB796 reduced the effect of latrunculin B on t-PA mRNA by 39.4 ± 5.0% (SEM, n=3 independent experiments) whereas BIRB796 alone increased t-PA mRNA (ratio: 2.02 ± 0.24, n=3). BIRB796 did not counteract the effect of latrunculin B on PAI-1 (data not shown).

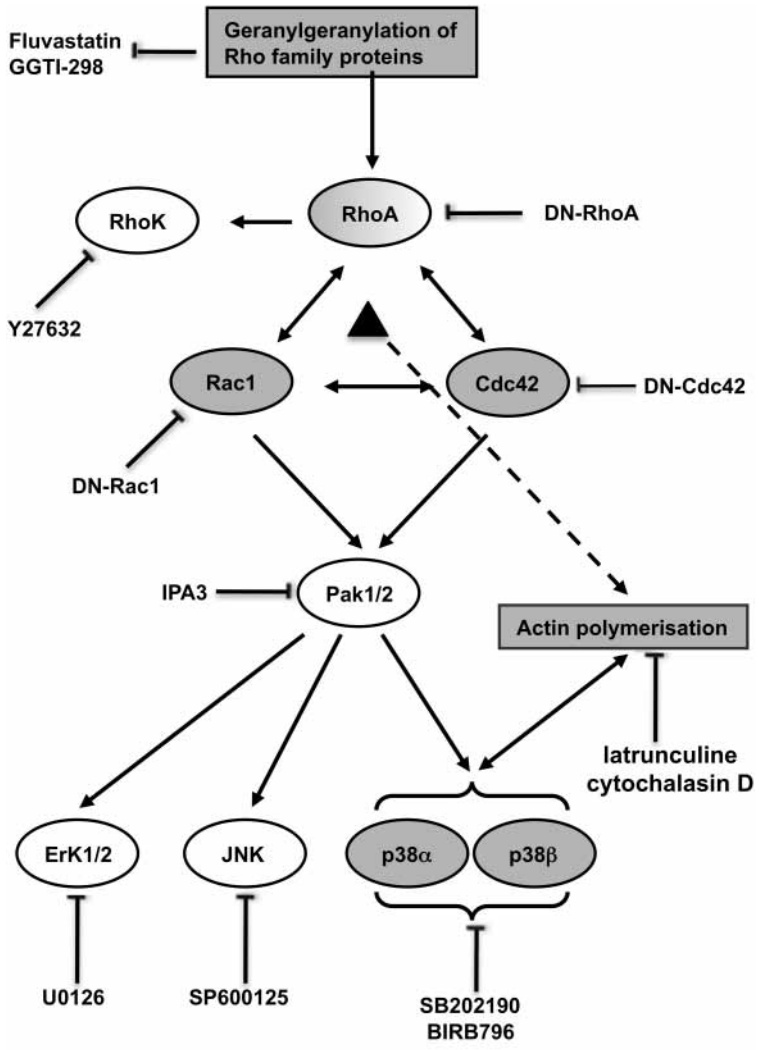
Role of JNK and ERK MAP kinases

Since statins are known to inhibit JNK activation, we also tested the effects of SP600125 (10 μM), an inhibitor of JNK1, JNK2 and JNK3 and not of p38 or ERK, on the fluvastatin-increased t-PA expression in HUVEC. While this inhibitor reduced statin-induced t-PA levels, it also lowered basal t-PA expression by approximately the same magnitude, suggesting that t-PA expression may require JNK/SAPK activity in a mechanism that is not exclusive to statin-induced t-PA (▶Fig. 7A). The MEK inhibitor U0126 (10 μM), which efficiently blocks ERK1/2 signalling, had no effect on basal or fluvastatin-induced t-PA expression in HUVEC (▶Fig. 7B). SP600125 and U0126 increased basal PAI-1 mRNA 1.8– and two-fold, respectively, and had no effect on PAI-1 mRNA in fluvastatin-treated cells (▶Fig. 7C and D).

Discussion

By increasing t-PA and reducing PAI-1, statins modify the balance between activator and inhibitor, leading to an increase in endothelial fibrinolytic potential (2). In agreement with previous reports, we found that the statin-induced changes in expression of t-PA and PAI-1 were independent of the cholesterol lowering activities of statins, were mimicked by inhibition of protein geranylgeranylation and involved members of the Rho family of small G-proteins. In addition, the statin-mediated increase in t-PA, but not the decrease in PAI-1, was dependent on p38 MAP kinase. The changes in t-PA and PAI-1 expression were detectable only 8 h (t-PA) or 24 h (PAI-1) after initiation of statin treatment. This implies that the effect of fluvastatin is indirect and requires a lag period, most likely involving a "wash-out" of the geranylgeranylated forms of the small G-proteins involved in modifying t-PA or PAI-1 expression.

In a previous study, treatment of rat EC or HUVEC with C3 exoenzyme, an inhibitor of RhoA, RhoB and RhoC, resulted in increased t-PA activity (2). We confirmed that RhoA inhibition, using DN-RhoA, increased t-PA expression. However, the increase was a modest 50%, which is of the same magnitude as previously reported (2), whereas DN-Rac1 and DN-Cdc42 increased t-PA expression three-fold. As fluvastatin increased t-PA expression more than 10-fold, these results suggest a primary role for Rac/Cdc42 subfamily proteins and a less important role for RhoA. The pattern of inhibition of PAI-1 expression was different, with DN-Rac1 and DN-RhoA reducing PAI-1 mRNA about 80% and 50%, respectively, while DN-Cdc42 had no significant effect. We also used siRNA-mediated knockdown of RhoA, Rac1 or Cdc42 to probe their contribution to the effect of fluvastatin on t-PA or PAI-1. Transfection with Cdc42 siRNA increased t-PA expression two-fold which is comparable to that observed with DN-Cdc42. Cdc42 siRNA had no effect on PAI-1 expression. Transfection with siRNA



Direct effect of inhibitor on t-PA and PAI-1 mRNA in HUVEC

Inhibitor	Effect on t-PA	Effect on PAI-1
Fluvastatin	+ + +	- - -
GGTI-296	+ + +	- - -
Y27632	0	0
DN-RhoA	+	-
DN-Rac1	+ +	- -
DN-Dcd42	+ +	0
IPA3	0	0
Latrunculin B	+ + +	- - -
Cytochalasin D	+ +	-
SB202190	0	-
BIRB796	+	0
siRNA p38α	+	0
SP600125	-	+
U0126	0	+

Figure 8: Effect of inhibitors on t-PA and PAI-1 expression in normal and fluvastatin treated HUVEC. Upper panel: Schematic representation of the signalling pathways that mediate the effect of fluvastatin on t-PA and PAI-1 expression in HUVEC. Inhibition of proteins marked in grey had an effect on expression of t-PA and/or PAI-1, whereas inhibition of the proteins marked in white had no effect. Lower panel: direct effect of inhibitors of signalling intermediates on t-PA and PAI-1 expression. The effect of fluvastatin on t-PA expression, but not PAI-1 expression, was inhibited by the p38 inhibitors BIRB796 and SB202190, by siRNA for p38α and p38β. The MEK/ERK inhibitor U0126 or the JNK inhibitor SP600125 did not modify the responses to fluvastatin. The effect of latrunculin B on t-PA expression, but not PAI-1 expression, was inhibited by BIRB796.

for RhoA or Rac1 had no effect on t-PA or PAI-1 expression, even though RhoA or Rac1 mRNAs were reduced by more than 80%. The lack of an effect of these siRNAs may reflect a contribution of inhibition of other Rho family proteins to the fluvastatin-mediated increase in t-PA and reduction in PAI-1. Indeed, down regulation of one Rho family protein, may modify expression of another Rho family protein (i.e. siRNA for RhoA leads to upregulation of RhoB) (16). Furthermore, dominant-negative Rho family proteins contain a mutation that inhibits their downstream effects but allows binding of guanine exchange factors (GEF). Overexpression of one particular DN-Rho protein titrates out the GEFs that bind to this protein and thereby reduces its activity. Because GEFs can function on several Rho GTPases, overexpression of particular DN-Rho family protein could therefore prevent their target GEFs from activating other Rho GTPases (14). Under our experimental conditions DN-RhoA reduced PAI-1 expression by two-fold. Potential downstream effectors of RhoA are the Rho kinases. As the Rho kinase inhibitor Y27632 had no effect on PAI-1 expression, the effect of DN-RhoA in HUVEC is unlikely to be mediated by Rho kinase. Our results are in contrast with previous findings on the role of Rho kinase in the angiotensin II-induced increase in PAI-1 in rat aortic EC (17, 18) and the hyperglycaemia-induced increase of PAI-1 in human saphenous vein or bovine aortic EC (19). Taken together, our results imply a role for Cdc42 in the statin-mediated upregulation of t-PA, whereas downregulation of PAI-1 appears to depend on a different combination of Rho family proteins.

PAK family proteins are a common signalling intermediate downstream of Cdc42 and Rac1. Upregulation of t-PA by statins was not mediated by PAK family proteins, as shown by the lack of effect of IPA3, a PAK inhibitor, and of siRNA for PAK1, PAK2 or PAK3. Also, the downregulation of PAI-1 appears to be independent of PAK proteins: IPA3 and siRNA for PAK3 had no effect and siRNA for PAK1 and PAK2 led to a small increase of PAI-1 instead of the decrease seen with fluvastatin. In any case, our results imply that the mechanisms downstream of Rac1 and Cdc42 modulating t-PA and PAI-1 expression do not depend on PAK family proteins.

Statins are known to modify the polymerisation state of actin with a loss of stress fiber formation (20). Inhibition of actin polymerisation by cytochalasin D resulted in increased t-PA activity in conditioned medium of rat aortic EC (2). The change in t-PA activity could have been due to an increase in t-PA or a decrease in PAI-1, or both. Here we show that inhibition of actin polymerisation not only increases t-PA expression but also decreases PAI-1 expression. This was shown using two compounds, latrunculin B and cytochalasin D, each having a different mode of action. Latrunculin induces actin depolymerisation, whereas cytochalasin D prevents actin polymerisation (21, 22). The extent of their effects was strikingly different: while cytochalasin D increased t-PA mRNA two-fold and decreased PAI-1 expression two-fold, latrunculin B increased t-PA mRNA 18-fold and reduced PAI-1 mRNA by more than 80%. The magnitude of effect of latrunculin B, but not of cytochalasin D, on t-PA or PAI-1 mRNA was comparable to that achieved with fluvastatin. The two-fold increase in t-PA mRNA and two-fold decrease in PAI-1 after treatment with cytochalasin D explains the increase in t-PA activity in rat aortic EC observed previously (2) and, together with the even stronger effect of latrunculin B, suggests that the effect of fluvastatin in HUVEC is, to a large extent, mediated by its effect on actin polymerisation. To what extent the quantitative differences in the effect of cytochalasin D and latrunculin B on t-PA and PAI-1 expression are related to their different mechanisms of action remains to be established. The effect of actin polymerisation inhibitors on PAI-1 expression appears to be cell type-specific. In endothelial cells (this report) and in mesangial cells (21) both latrunculin B and cytochalasin D reduce basal PAI-1 expression, whereas in epithelial cells (23) and smooth muscle cells (24) cytochalasin D increases PAI-1 expression.

Several studies using actin-targeting agents demonstrated that the cytoskeleton plays an important role in activation of p38, jun kinase or ERK MAP kinases (25). We investigated whether one of these MAP kinase families was involved in the statin-induced changes in t-PA or PAI-1 expression by endothelial cells. Inhibition of p38 MAP kinase with two small molecule inhibitors of p38 (SB202190 and BIRB796), as well as by siRNA for p38α and p38β, partially reversed the effect of fluvastatin on t-PA. Interestingly, BIRB796, as well as p38α siRNA, consistently induced a small, up to two-fold, increase in basal t-PA mRNA. The latter is in agreement with previous observations showing that inhibition of p38 increased basal t-PA mRNA in EC (26). Activation of p38 was also observed in atorvastatin-treated EC, and inhibition of p38 by SB203580 reduced the atorvastatin-mediated increase of vascular endothelial growth factor (VEGF) (27), which is known to increase t-PA expression in EC (28). It remains to be established whether a p38/VEGF pathway may contribute to the statin-mediated increase in t-PA. We observed that SB202190 strongly reduced PAI-1 expression under basal conditions and after fluvastatin or GGTI-286 treatment. As BIRB796 and siRNA for p38α or for p38β had no down-regulating effect on basal PAI-1 it is likely that inhibition of a SB202190 target other than p38, such as glycogen synthase kinase 3β, casein kinase1δ, receptor-interacting protein 2, or cyclin G-associated kinase (14), underlies the decrease in PAI-1 mRNA.

Inhibition of JNK reduced basal t-PA expression, as well as fluvastatin-induced t-PA expression to a similar extent. This suggests a role for JNK in maintaining basal t-PA expression, but not as a mediator of the effect of fluvastatin. Our results are in agreement with a previous study showing that inhibition of JNK resulted in a decrease in t-PA mRNA (26). Inhibition of ERK had no effect on basal or fluvastatin-induced t-PA mRNA. For PAI-1 we observed a two-fold increase in expression after inhibition of JNK or ERK, whereas combined treatment with fluvastatin and JNK or ERK inhibitors resulted in a similar PAI-1 expression as after treatment with fluvastatin alone. Taken together these results suggest that JNK and ERK are not involved in the statin-mediated changes in t-PA or PAI-1 expression.

►Figure 8 gives an overview of the relationship between the factors that are modified by statin treatment and the inhibitors used to determine the role of each factor.

In conclusion, statins profoundly modify the fibrinolytic system of human endothelial cells by strongly increasing t-PA and reducing

What is known about this topic?

- Statins increase expression of t-PA and decrease expression of PAI in human endothelial cells.
- The statin effect was reversed by geranylgeranyl pyrophosphate, and not farnesyl-pyrophosphate, suggesting that it acts via inhibition of protein geranylgeranylation rather than inhibition of cholesterol biosynthesis or of protein farnesylation.
- A role for RhoA was observed in some studies.
- Inhibition of actin polymerisation by cytochalasin D led to an increase in t-PA activity.

What does this paper add?

- We provide a quantitative analysis of the effect of statin on both t-PA and PAI-1 antigen release and mRNA expression and direct evidence for a role of inhibition for protein geranylgeranylation by using a geranylgeranyl transferase inhibitor.
- By using dominant negative protein expression and siRNA down-regulation, we show that changes in t-PA and PAI-1 are mediated by distinct Rho family proteins; Cdc42/Rac1 subfamily proteins play a major role, whereas RhoA plays a minor role. PAK1, PAK2 and PAK3, common signaling intermediates downstream of Rac1 and Cdc42, are not involved.
- We show that inhibition of actin polymerization affects expression of both t-PA and PAI-1; latrunculin B fully mimics the effect of fluvastatin, whereas cytochalasin D is 10 times less efficient, even at optimal dosages.
- We show an essential role for p38, but not JNK and ERK, in mediating the statin effect.

PAI-1 expression. Inhibition of actin polymerisation quantitatively reproduces the modifications induced by statins, suggesting that statins act via their known effect on the integrity of the actin cytoskeleton. Up-regulation of t-PA and down-regulation of PAI-1 are mediated by distinct signal transduction pathways, involving different combinations of Rho family proteins. Moreover, p38 MAP kinase mediates the changes in t-PA, but not in PAI-1.

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