

## Theme Issue Article

# Regulation of plasminogen activator inhibitor type I gene expression by inflammatory mediators and statins

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### Summary

Elevated plasma concentrations of plasminogen activator inhibitor type I (PAI-I), also named serpin E1, are encountered in patients with thrombophilia, atherosclerosis, septicemia and the metabolic syndrome and may be associated with an increased risk of complications. Expression of PAI-I is increased by inflammatory stimuli and decreased by statins, drugs widely used in patients with cardiovascular disease. Increased expression of PAI-I

by inflammatory stimuli is mediated by a large variety of signal transduction pathways, which include the NF- $\kappa$ B and MAP kinase pathways. The downregulating effect of statins on PAI-I expression is dependent on the inhibition of Rho family proteins and may involve an activation of PI-3 kinase/Akt signaling pathways. In this review we summarize the findings on the effect of inflammation and statins on PAI-I expression.

### Keywords

Plasminogen activator inhibitor type I, gene regulation, inflammation, statins

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## Introduction

The plasminogen activator (PA) system plays a key role in many important physiological and pathological processes, such as coagulation/fibrinolysis, inflammation, wound healing and malignancy. The trypsin-like enzyme plasmin is generated from its precursor plasminogen by the plasminogen activators t-PA or u-PA. Plasmin degrades fibrin deposits and contributes to the degradation of extracellular matrix proteins. The activity of the PA system is under tight control at different levels, such as regulation of gene expression of the PA system components, receptor-mediated accumulation of PAs at the cell surface, receptor-mediated degradation of PAs and inhibition by  $\alpha_2$ -antiplasmin and PA-inhibitors (PAIs). PAI-I, also named serpin E1, is the principal inhibitor of both t-PA and u-PA. It is produced by endothelial cells, smooth muscle cells, fibroblasts, monocytes/macrophages, adipocytes, liver cells and cardiac myocytes. The relative contribution of each of these cell types to plasma PAI-I concentrations in healthy and pathologic conditions is not well established. Elevated plasma concentrations of PAI-I are encountered in patients with thrombophilia, atherosclerosis, septi-

cemia and the metabolic syndrome and may be associated with an increased risk of complications. Among the factors that are known to influence plasma PAI-I concentrations are sex hormones, inflammation, obesity, metabolic factors, physical activity, and the renin-angiotensin system. Common polymorphisms in the PAI-I gene promoter appear to modify PAI-I gene responses to environmental stimuli. In addition PAI-I appears to play an important role in malignancy and inflammatory diseases. The various basal and clinical aspects of PAI-I have been subjects of a number of recent reviews (1–9). This review deals primarily with the effect of inflammatory stimuli and of statins on the expression of PAI-I by endothelial cells or other cell types and the signaling pathways involved.

## Signaling pathways mediating PAI-I induction by inflammatory cytokines

An important aspect of PAI-I is its relation to inflammation. This is most strikingly illustrated in septicemia, where elevated PAI-I is associated with a poor prognosis (10). The increase of

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Cell type	Inflammatory stimulus	Signaling intermediates	References
HUVEC	TNF	NF- $\kappa$ B, ERK	28, 29
HSEC, BAEC	TNF, AngII	PPAR- $\alpha$ , ERK, p38	30
Adipocyte	IL-6, oncostatin	JAK/STAT	17
Adipocyte	TNF	ERK, PKC	31
Mesangial cells	TNF	JNK	32
HepG2 hepatoma	IL-1	p38, JNK, ERK	24
	IL-6	JAK/STAT	24

AngII, angiotensin II; HUVEC: human umbilical vein endothelial cells; HSEC, human saphenous vein EC; BAEC, bovine aortic EC.

**Table 1: Signalling pathways mediating the induction of increased PAI-1 expression by inflammatory cytokines in cultured cells.**

PAI-1 as well as the other changes in haemostatic parameters during septicemia are most likely caused by a characteristic pattern of inflammatory cytokine release, as illustrated by studies of experimental endotoxemia in man or primates (11). Thus, injection of endotoxin is followed by transient increases in tumor necrosis factor (TNF) and interleukin 6 (IL-6), reaching maximal levels at 1 hour (h) and 2 h, respectively. Within 2 h after injection of endotoxin, activation of the coagulation system is apparent as well as endothelial cell activation, as deduced from increases in plasma concentrations of von Willebrand factor and t-PA. Increased plasma concentrations of PAI-1 were observed at a later time period. Experimental in-vivo studies in man and baboons make it likely that both TNF and IL-6 contribute to the increases of PAI-1 (12, 13). The cell types responsible for maintaining plasma PAI-1 levels in man or in primates under basal conditions or after endotoxin stimulation are not known. In healthy human arterial tissue PAI-1 immunostaining was found to be associated both with the arterial endothelial cells and with the medial smooth muscle cells (14, 15), whereas in aortas of normal rats or endotoxin-treated rats PAI-1 was detected only in association with smooth muscle cells of the tunica media and not with endothelial cells (16). Other cell types that may contribute to PAI-1 expression in vivo are monocytes that respond to inflammatory stimuli with an increased expression of PAI-1 (see below) and adipocytes, which may provide a link between PAI-1 and the metabolic syndrome (4). Treatment of human explant adipose tissue with IL-6 or oncostatin, another member of the glycoprotein 130 ligand family, resulted in a ten-fold increase of PAI-1 mRNA and antigen release, which was dependent on JAK/STAT signal transduction (17). This result illustrates the link between PAI-1, obesity and inflammation.

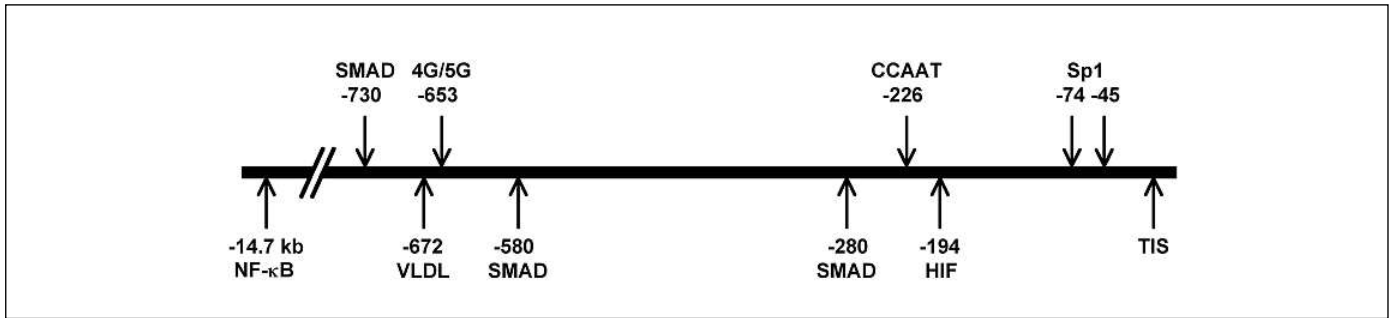
The effect of inflammatory stimuli such as TNF, IL-1 or lipopolysaccharide (LPS) on PAI-1 gene expression has been investigated in different cell types. In most of the cell types studied, including endothelial cells, smooth muscle cells, adipocytes or hepatocytes, these inflammatory stimuli strongly upregulated PAI-1 expression at the protein and mRNA level (18–24), which shows that these cell types have maintained their responsiveness to inflammatory stimuli when put into culture.

In human monocyte-like U937 cells, TNF or IL-1 treatment induced an increase in PAI-1 antigen release; PAI-1 antigen release and PAI-1 mRNA was potentiated further by treatment

with the calcium ionophore A23187 or the endoplasmic Ca<sup>2+</sup>-ATPase inhibitor thapsigargin (25).

Different signal transduction pathways contribute to inflammatory responses. Of these, mitogen activated protein (MAP) kinase-dependent and nuclear factor kappa-B (NF- $\kappa$ B)-dependent signaling are well characterized. Extracellular stimuli such as inflammatory cytokines, growth factors, TLR ligands or ligands of G-protein-coupled receptors activate a phosphorylation cascade where MAP3K activate MAP2K which then activate MAP kinases (26). There are three MAP kinase families, p38MAP kinases, extracellular signal-regulated kinases (ERK) and c-jun-N-terminal kinases (JNK), which either directly activate transcription factors or act via so-called MAP kinase activated protein kinases. The NF- $\kappa$ B transcription factors are activated by a large number of stimuli, including TNF, IL-1 and LPS (27). In one pathway of NF- $\kappa$ B activation, activated I $\kappa$ B-kinases phosphorylate I $\kappa$ B, which is then ubiquitinated and degraded by the proteasome. Degradation of I $\kappa$ B leads to release of NF- $\kappa$ B from its complex with I $\kappa$ B and migration of the transcription factor to the nucleus, where it binds to NF- $\kappa$ B promoter elements leading to transcription of NF- $\kappa$ B target genes. The MAP kinase and NF- $\kappa$ B pathways interact at several points: e.g. MAP3K2, MAP3K3 and MAP3K7 (TAK1) are able to phosphorylate I $\kappa$ B and thereby induce NF- $\kappa$ B release. Other signal transduction pathways like protein kinase C-, protein tyrosine kinase- or JAK/STAT-dependent pathways also influence inflammatory cell activation Table 1.

The contribution of MAPKs, NF- $\kappa$ B and other signaling molecules to the upregulation of PAI-1 protein and mRNA by inflammatory stimuli has been investigated using specific signaling pathway inhibitors. The upregulation of PAI-1 expression by TNF in human umbilical vein endothelial cells was partly inhibited by the ERK inhibitor PD98059 and by the NF- $\kappa$ B inhibitors, BAY11-7082 or emodin; in addition a role for protein tyrosine kinases and PPAR $\alpha$  could be inferred by the effect of the inhibitors genistein and troglitazone, respectively (28, 29). In these cells no inhibition was observed for the JNK inhibitor SP600125, the p38 MAP kinase inhibitors SB202190 or SB203580 or the protein kinase C inhibitor calphostin C. PAI-1 expression in bovine aortic and human saphenous vein endothelial cells was increased by treatment with TNF, angiotensin II or insulin. This effect was mediated, in part, by ERK and p38 MAPK. Inhibition of PI3-kinase with wortmannin or LY-294002



**Figure 1: PAI-1 promoter elements mediating PAI-1 induction by inflammatory cytokines.** All promoter positions are given with respect to the transcription initiation site (TIS). The SP1 elements at -42 and -73 mediate glucose responsiveness. A hypoxia responsive element (HIF) is located at -194 and a very low density lipoprotein-responsive element (VLDL) at -672. A CCAAT-enhancer binding protein  $\delta$  element

at -226 bp mediates upregulation of PAI-1 by IL-1 or IL-6 and three SMAD 3 and 4 protein-binding sites at -280, -580 and -730 mediate responsiveness to TGF- $\beta$ . A distant enhancer element at -14.7 kb contributes to TNF responsiveness. A common 4G/5G polymorphism at -653 modifies cellular responses to inflammatory stimuli and is of clinical importance.

or further enhanced PAI-1 mRNA expression induced by these extracellular stimuli. Dominant-negative mutants of PI3-kinase or Akt1 had the same effect (30).

In 3T3 L1 mouse adipocytes, TNF induced an acute (after 3 h) increase of PAI-1 mRNA, and of PAI-1 mRNA and protein after chronic treatment (24 h). Pretreatment with the MEK/ERK inhibitor PD98059 or the PKC inhibitor GF109203X abolished both the acute and chronic increase in PAI-1 mRNA in TNF-treated adipocytes, whereas treatment with the p38 inhibitor SB203580, the NF- $\kappa$ B inhibitor NG50, the PI3 kinase inhibitor wortmannin or the tyrosine kinase inhibitor genestein inhibited PAI-1 expression only in response to chronic TNF treatment (31). Inhibition of JNK had no effect on PAI-1 expression in these cells. In human mesangial cells, the TNF induced expression of PAI-1 mRNA was inhibited by DMAP, an inhibitor of JNK (32). In contrast, in these cells, the MEK/ERK inhibitor PD98059, the p38 MAPK inhibitor SB203580 and the PI3 kinase inhibitor LY294002 had no effect. Preincubation of the mesangial cells with prostaglandin E1 reduced the PAI-1 response to TNF in these cells via a protein kinase A-dependent pathway (32). In early osteoarthritic tissue cultures, with or without IL-1 or LPS treatment, the secretion PAI-1 was significantly decreased by the ERK 1/2 inhibitor U0126, by the p38 MAP kinase inhibitor SB203580, and by the PI3K inhibitor LY294002 (33). In human HepG2 hepatoma cells, treatment with IL-1 or IL-6 increased PAI-1 mRNA expression and the combination of both had an additive effect (24). Induction of PAI-1 mRNA by IL-1 was increased by the PI3-kinase inhibitor LY-294002 and decreased by the p38 inhibitor SB-203580, the JNK inhibitor SP-600125, and the ERK inhibitor U-0126. Induction of PAI-1 by IL-6 was inhibited by JAK inhibitor 1. The NF- $\kappa$ B inhibitor SN-50 and the Akt inhibitor API-2 had no effects on PAI-1 (24).

Taken together these studies highlight the large variety of signaling pathways that impact on PAI-1 expression and interact with each other in a complex fashion. It is interesting to note that different combinations of signaling pathways may be used by different cell types and at different stages (acute or chronic) of inflammation.

### PAI-1 promoter elements mediating PAI-1 induction by inflammatory cytokines

A number of regulatory elements have been identified in the PAI-1 promoter, including two Sp1 elements (at -42 bp and -73 bp with respect to the transcription initiation site) that mediate glucose responsiveness (34), a hypoxia responsive element (at -194) (35), a very-low-density lipoprotein-responsive element (at -672/-657) (36), and SMAD 3 and 4 protein-binding sites that mediate TGF- $\beta$  responsiveness (at -280, -580 and -730) (37). Upregulation of PAI-1 by IL-1 or IL-6 in human HepG2 hepatoma cells was found to be mediated by a CCAAT-enhancer binding protein  $\delta$  element at -226 (24). The elements mediating the upregulation of PAI-1 by TNF in endothelial cells proved to be more elusive. Expression of reporter genes downstream of a 2.5 kbp or a 280 bp PAI-1 promoter construct induced only modest increases in reporter gene activity (38-40). In contrast, experiments using a gene construct containing 2.9 kb of the human PAI-1 promoter fused to a GFP reporter gene, and stably integrated into the genome of transgenic mice or transient transfection experiments with reporter constructs containing up to 6.4 kb from the transcription start site failed to show responsiveness to TNF (29). These experiments imply that TNF-mediated PAI-1 upregulation requires distant enhancer elements. By using cross-species homology analysis and DNase-1 hypersensitivity analysis a conserved NF- $\kappa$ B-binding site was identified at -14.7 kbp that may contribute to TNF responsiveness (29) Fig. 1.

A common 4G/5G polymorphism in the PAI-1 promoter, with an allele frequency of 0.47/0.53 at 653 bp upstream of the transcription initiation site may be important for PAI-1 expression. This polymorphism may be of particular importance for responses to inflammatory stimuli, because PAI-1 promoter reporter gene constructs containing the 4G allele and transfected into human HepG2 hepatoma cells showed a six-fold higher reporter gene activity after IL-1 treatment than promoter reporter constructs containing the 5G allele (41). In human endothelial cells, PAI-1 induction after treatment with TNF or reactive oxygen species was dependent on NF- $\kappa$ B nuclear translocation and a potential NF- $\kappa$ B binding site was identified adjacent to the

Cell type	Inhibitor	Effect	Reference
RAEC	C3-exotoxin	Decrease in basal PAI-I	47
HUVEC	DN-RhoA	Decrease in basal PAI-I	own work
Monocytes	Y27632, fasudil	Reduced PAI-I response to LPS	58
Monocytes	Y27632, fasudil	Reduced PAI-I response to hyperglycemia	68
Vascular SMC	C3- exotoxin	Reduced PAI-I response to TGF- $\beta$	70
	Y27632, fasudil		

RAEC, rat aortic EC; HUVEC, human umbilical cord EC; SMC, smooth muscle cells.

**Table 2: Effect of inhibition of RhoA by C3 exotoxin or dominant negative (DN) RhoA or of Rho-kinase by Y27632 or fasudil on PAI-I expression in cultured cells.**

4G/5G polymorphic site (38). The clinical relevance of the 4G/5G polymorphism with respect to its effect on plasma PAI-I concentrations and its relation with cardiovascular risk or in septicemia is still being debated (1, 3).

### Effect of statins on PAI-I expression by vascular cells

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and used by millions of patients to prevent cardiovascular complications. They inhibit the conversion of HMG-CoA into mevalonate, an essential intermediate in the biosynthesis not only of cholesterol, but also of the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Originally, the clinical benefits of statins were thought to be exclusively due to their cholesterol lowering activity. However, statins also give protection from cardiovascular events to patients with normal LDL-cholesterol levels (42), which implies that they have beneficial cholesterol-independent effects. These may be due to an inhibitory effect of statins on farnesylation or geranylgeranylation of small GTPases (43) that have fundamental roles in cell biology. Small GTPase superfamily proteins require prenylation, at C-terminal cysteine-bearing motifs, for association with specific membrane compartments and for interactions with protein binding partners (44). Small GTPases cycle between a GTP-bound active state and a GDP-bound inactive state. This cycling is modulated by three activities: guanine nucleotide exchange factors that catalyze GDP release and promote GTP-loading and GTPase activation; GTPase activating proteins that enhance GTP hydrolysis; and guanine nucleotide dissociation inhibitors that prolong the inactive state of GDP-bound GTPases. About 170 small GTPases been identified and are classified into several families. In general, Ras family proteins are involved in gene expression; Rho proteins in cytoskeletal organization and gene expression; and Rab, Ran and Arf/Sara proteins in intracellular vesicle trafficking (45).

Statins have profound effects on the plasminogen activator system. As an example, in rabbits an atherogenic diet led to increased plasma antigen concentrations of PAI-1 and an increased PAI-1 mRNA in isolated glomerular mesangial cells; a four week treatment with simvastatin significantly reduced plasma PAI-1 and PAI-1 mRNA in the glomerular mesangial cells (46). These in-vivo results are in agreement with studies on the effect of statins in cultured cells, which mainly lead to an increased t-PA expression and

a decreased PAI-1 expression. These effects on t-PA and PAI-1 mRNA and protein levels were observed in human endothelial cells (23, 47–52), in human smooth muscle cells (23, 51, 52), in human cardiac myocytes (53), in human mesothelial cells (54), in human adipocytes (55), in adipose tissue from atherosclerotic rabbits (56) and in rat renal proximal tubular cells (57). In basal and LPS-stimulated cultured monocytes, statins reduced PAI-1 antigen and mRNA (58). In contrast to the latter study, atorvastatin increased PAI-1 protein synthesis in human monocytes during the early stages of differentiation into macrophages, but had no effect on PAI-1 synthesis in mature human monocyte-derived macrophages (59). In a mouse model of focal ischemia, statin treatment increased t-PA and eNOS mRNA, but had no effect on PAI-1 mRNA; ischemic lesion volumes and neurologic deficits were reduced and the protective mechanisms involved both eNOS and t-PA (60).

In human endothelial cells and vascular smooth muscle cells, statins reduced the activation of the inflammatory transcription factors NF- $\kappa$ B and c-jun and reduced PAI-1 mRNA levels; this may explain the reported anti-inflammatory effect of statins (61). In accordance with such an inhibitory effect of statins on inflammation, statins were found to counteract the enhancing effect of TNF on PAI-1 antigen release and the suppressive effect on t-PA antigen release in human mesothelial cells; promoter studies revealed that this effect was due to an inhibition of NF- $\kappa$ B- and AP-1-dependent gene transcription (62). A similar inhibition of TNF-mediated upregulation of PAI-1 mRNA and antigen release was observed for human endothelial and smooth muscle cells and the hybrid endothelial-like cell line EA.hy 926 (23, 41). The downregulating effects of statins on PAI-1 expression may involve promoter elements situated within 800 bp upstream of the PAI-1 transcription initiation site (41, 52, 55).

Treatment of human renal mesangial cells with oxidized low-density lipoprotein induced an increase in PAI-1 antigen and mRNA. The increase was mediated by transforming growth factor-beta and required ERK activation. Treatment with lovastatin reduced transforming growth factor-beta release, which then resulted in a reduction in PAI-1 expression (63).

### Role of Rho family proteins in statin-mediated downregulation of PAI-I expression

Several studies addressed the mechanisms by which statins increase t-PA expression and suppress PAI-1 expression. The results of these studies imply that these effects are unrelated to the

inhibition by statins of cholesterol biosynthesis. Consistently, the effects of statins on t-PA and PAI-1 expression were mimicked by geranylgeranyl transferase inhibitors and counteracted by geranylgeranyl pyrophosphate, whereas farnesyl transferase inhibitors and farnesyl pyrophosphate had no effect (47, 49, 50, 52, 57, 58). Further investigations pointed to a role for Rho family proteins. Essig et al. (47) observed that the increase in t-PA and decrease in PAI-1 could be partially mimicked by C3 exotoxin, an inhibitor of Rho. In own unpublished studies, we observed that adenovirus-mediated gene transfer of dominant negative (DN)-RhoA indeed leads to an increase of t-PA mRNA and a reduction of PAI-1 mRNA. However, DN-Rac1 or DN-Cdc42 also increased t-PA mRNA, and to a greater extent than DN-RhoA, whereas DN-Rac1, but not DN-Cdc42, had a stronger down regulating effect on PAI-1 mRNA than DN-RhoA (Fish et al., unpublished data). These findings imply that the effects of Rho family proteins on t-PA and PAI-1 expression are more complex than thus far imagined Table 2.

RhoA is activated by G-protein coupled receptors, growth factor receptors, integrins and cytokine receptors. Important downstream, effectors of activated RhoA are the Rho kinases ROCK-1 and ROCK-2 (64, 65). Expression of ROCK-1 and ROCK-2 is upregulated in human smooth muscle cells by the inflammatory stimuli IL-1 and angiotensin II, via protein kinase C and NF- $\kappa$ B-dependent pathways (66). This implies a link between inflammation and ROCK function. Several reports mentioned that activation of ROCK increases PAI-1 expression. In rat aortic endothelial cells and vascular smooth muscle cells, angiotensin II induced a marked increase in PAI-1 expression. Inhibition of ROCK by Y27632 blocked the effect of angiotensin II on PAI-1 mRNA and antigen release (50, 67). Exposure of human saphenous vein or bovine aortic endothelial cells to hyperglycemia led to an increased expression of ROCK activity and of PAI-1 mRNA and antigen release. The hyperglycemia-mediated increase of PAI-1 was inhibited by the ROCK inhibitors fasudil and Y27632 and by a dominant-negative mutant of Rho-kinase (68). In addition, in bovine aortic endothelial cells, inhibition of NF- $\kappa$ B action by parthenolide, BAY 11-7082 and SN50 blocked the hyperglycemia-induced increase in PAI-1 antigen release, which suggests that the increase in PAI-1 is mediated by NF- $\kappa$ B activation through the Rho/Rho kinase pathway (69). In basal and LPS-stimulated human peripheral blood monocytes the Rho-kinase inhibitors, Y-27632 and fasudil, decreased PAI-1 synthesis (58). In a recent study, the upregulation of PAI-1 mRNA and antigen release by tumor growth factor

(TGF)- $\beta$  in vascular smooth muscle cells was reduced by the RhoA inhibitor C3-exotoxin, the ROCK inhibitors Y27632 and by the MEK-inhibitors PD98059 and U0126; this implies that both pathways are required. (70). BN hypertensive rats were shown to have an increased expression in the aorta of PAI-1 mRNA, which normalized after treatment with the ROCK inhibitor fasudil (71). Taken together these studies suggest a major role for ROCK in regulating PAI-1 expression.

### Role of Akt proteins in statin-mediated downregulation of PAI-1 expression

Among the substrates of ROCK's are not only a large number of proteins involved in cytoskeletal function, but also PTEN. Phosphorylation of PTEN by ROCK enhances its phosphatase activity, which inhibits the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway. This pathway is known to be activated by statins (72) and is involved in the regulation of cell growth, protein synthesis, transcriptional regulation, and cell survival (64). Conversely, inhibition of ROCK increases Akt signaling. The importance of Akt activation as a mediator of the down modulating effect of statins on PAI-1 expression was demonstrated by the finding that treatment with PI3K inhibitors reversed the inhibitory effects of simvastatin on TNF- and insulin-induced PAI-1 mRNA (30). Additional support for a role of Akt in suppressing PAI-1 expression is given by findings that treatment of ovarian SKOV3 carcinoma cells with the PI3-kinase inhibitors LY294002 and wortmannin as well as with a siRNA for Akt, resulted in an increased expression of PAI-1 antigen (73).

Taken together these studies suggest a mechanism by which statins downregulate PAI-1 expression. In this mechanism statins prevent the addition of a geranylgeranyl anchor on RhoA, which interferes with its activity. This then leads to a reduced expression of ROCK activity and a decrease of PTEN activity, an inhibitor of the PI3 kinase-Akt pathway. The resulting increase in Akt activity then downregulates PAI-1 expression. Which of the more than 20 different pathways downstream of Akt1 are involved remains to be established. Also the role of Rac1, which appears to have a stronger effect on PAI-1 expression than RhoA, remains to be established. A question that needs to be addressed is whether the action of Rac1 on PAI-1 expression is upstream of the PI3-kinase/Akt pathway, possibly indirectly by activating RhoA (74, 75), or downstream, as PI3-kinases can activate Rac indirectly via PtdIns(3,4,5)P3-sensitive Rac-guanine nucleotide exchange factors (76).

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