

Theme Issue Article

Metabolic, hormonal and environmental regulation of plasminogen activator inhibitor-1 (PAI-1) expression: Lessons from the liver

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Summary

Plasminogen activator inhibitor-1 (PAI-1) controls the regulation of the fibrinolytic system in blood by inhibiting both urokinase-type and tissue-type plasminogen activators. Enhanced levels of PAI-1 are found in patients with type 2 diabetes mellitus which is associated with a dysbalance in glucose and lipid homeostasis. Especially a defective insulin response in the liver contributes to the development of hyperglycemia, dyslipidemia and peripheral insulin resistance and may contribute to hepatic overexpression of PAI-1 in diabetes type 2. Furthermore, a substantial upregulation of PAI-1 expression has also been shown in a

variety of liver injury models. Thus, the liver appears to be not only a major site of PAI-1 synthesis in response to hormonal changes, but also in response to a variety of other pathological events. PAI-1 expression in liver largely depends on activation of signalling pathways and transcriptional regulators which may be the basis for a new level of cross-talk between different signalling pathways and thus may represent attractive therapeutic candidates. This article will primarily focus on the regulation of PAI-1 expression in liver cells and discuss potential cross-talks between metabolic, hormonal and environmental signals.

Keywords

Fibrinolysis inhibitors, gene expression, plasminogen activator inhibitors, transcription factors, hypoxia

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Introduction

A number of physiological and pathological processes among them fibrinolysis and matrix degradation are regulated by the tissue-type (tPA) and urokinase-type (uPA) plasminogen activators. Both PAs are serine proteases which catalyze the conversion of the inactive proenzyme plasminogen into active plasmin (1). In contrast to tPA and uPA, plasmin has a wide range of substrate specificity and degrades fibrin, basement membrane components such as laminin, collagen type IV and fibronectin as well as activates matrix metalloproteases and growth factors like HGF (hepatocyte growth factor) and transforming growth factor (TGF)- β (2). The action of tPA and uPA is primarily antagonized by plasminogen activator inhibitors (PAIs). First evidence for the existence of these inhibitors was gained from experiments with conditioned medium from the rat hepatoma cell line HTC which showed the presence of a glucocorticoid-inducible fibrinolytic inhibitor (3, 4). This inhibitor could then also be purified from various biological sources and based on immunological properties at least two types of inhibitors were distinguished which became known as endothelial-type and placental-type PAI, respect-

ively (for review see [5]). With the cloning of cDNAs for each type (6–10) and immunological expression studies it became clear that both types are present in placenta (11) and the names PAI-1 and PAI-2 were adopted (12). In addition to PAI-1 and PAI-2 which belong to the serine protease inhibitor (serpin) superfamily, protein C inhibitor (PAI-3) and the protease nexin-1 also possess plasminogen activator inhibitor activity. From the PAIs known so far, PAI-1 appears to be the most important. It is a single-chain glycoprotein with an apparent molecular mass of 48 kDa and can be secreted from a variety of cells but the major producers are hepatocytes, vascular endothelial and smooth muscle cells, adipocytes and platelets (13–17). Although circulating and primarily measured in blood, PAI-1 is also a component of the extracellular matrix where it is found to interfere with the interaction between vitronectin and its cell surface ligand $\alpha v \beta 3$ integrin (18). Further, PAI-1 was found to induce uPA-uPAR internalization and appears to be cleared from the circulation with the help of scavenger receptors from the low-density lipoprotein receptor related family (LRP) (19) and thus PAI-1 may affect intracellular signaling processes by itself (20, 21).

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The importance of PAI-1 is emphasized by several clinical studies: decreased PAI-1 levels cause bleeding diathesis, whereas increased PAI-1 levels are associated with conditions associated with hypoxia like atherosclerosis, coronary heart disease, deep-vein thrombosis, acute and chronic inflammatory lung disorders as well as cancer (for review see [22]).

Additionally, enhanced PAI-1 levels are found in patients with type 2 diabetes mellitus (23, 24) which is associated with a dysbalance in glucose and lipid homeostasis (25). Under normal conditions glucose and lipid homeostasis is mainly achieved by the adequate regulation of hepatic metabolism. This occurs primarily by the action of the pancreatic hormones insulin and glucagon. By contrast, it was shown that especially a defective insulin response in liver contributes to the development of hyperglycemia, dyslipidemia and peripheral insulin resistance (26–28). In addition, hepatic overexpression of PAI-1 in diabetes

type 2 was considered to contribute to the decreased basal membrane and extracellular matrix degradation and the resulting angiopathies (for review see [29]). Further, a substantial upregulation of PAI-1 expression has also been shown in a variety of liver injury models including hemorrhagic shock (30), bile duct ligation (31, 32), acetaminophen hepatotoxicity (33, 34) and alcohol-induced liver injury (35). Thus, the liver appears to be not only a major site of PAI-1 synthesis in response to hormonal changes, but also in response to a variety of other pathological events.

The liver is an organ composed of different cell types functioning in cooperation as a center of metabolism, a center of defence, a control station of the hormonal system and a blood reservoir as well as in all processes required for the formation and maintenance of its own cellular and extracellular structures (37, 38).

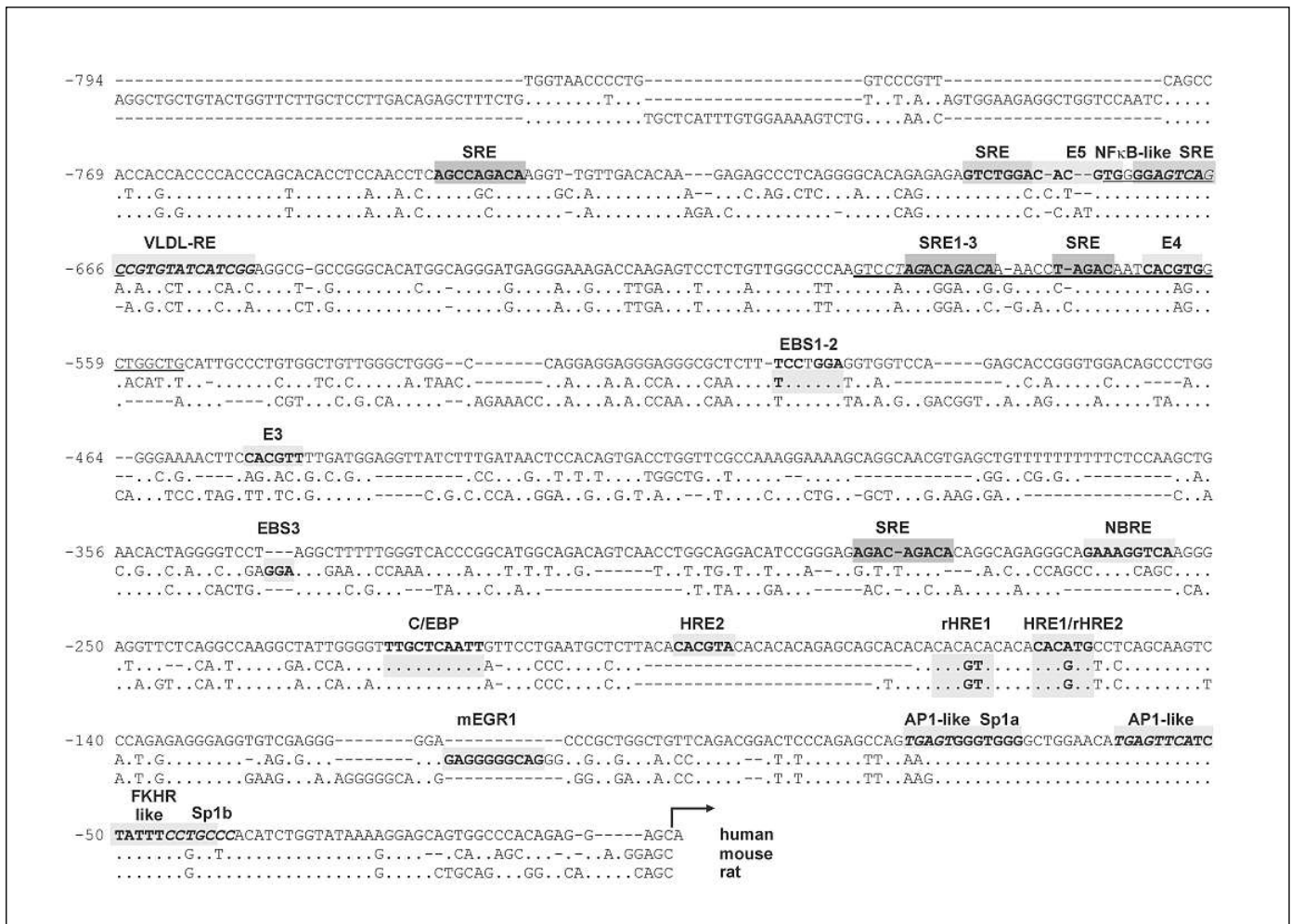


Figure 1: Transcription factor binding sites in the human, mouse and rat PAI-1 promoters. The mouse and rat promoters were aligned to the human -796 bp promoter using the Clustal Alignment algorithm. Dots indicate the positions of sequence identity, and dashes reflect gaps introduced into the sequence to obtain optimal sequence homology. The transcription factor binding sites identified in the respective promoters are boxed and in bold letters. AP1-like, Activator protein I-like binding element; C/EBP, CAAT enhancer binding protein response element; EBS, Ets/Net binding sites; mEGR I, mouse EGR I

binding element; E, E-box; FKHR-like, FoxO/forkhead like binding element; HRE, hypoxia response element; rHRE, rat HRE; NBRE, NGFI-B responsive element/Nurr77 response element; NF-κB-like, nuclear factor kappa B-like element (underlined); SP1a/Sp1b, stimulatory protein-I response element a and b, respectively; SRE, Smad 3–4 binding element/TGF-β response element; VLDL-RE, very-low-density lipoprotein response element. The P2.I (-592/-552) element encompassing SRE1–3, another SRE and E4 is underlined.

A major role of the liver in metabolism is determined by its glucostat function which keeps blood glucose levels constant. Thereby the parenchymal cells, the hepatocytes proper, rather than the non-parenchymal cells operate as glucose storing cells. They remove excess glucose after a meal and release glucose in-between meals for the use of the glucose-dependent erythrocytes and the central nervous system (CNS). In addition, they use nutrient- or muscle-derived amino acids to produce glucose and remove the ammonia by ureagenesis. Further, the liver is involved in cholesterol synthesis and processing of nutrient fat reaching the liver as chylomicrons and fatty acids. Moreover, ketone bodies can be produced as intermediates to economize the use of glucose in fasting periods (37, 39).

This glucostat function of the liver is controlled by a complex nervous-humoral network (40, 41). Glucose uptake is mainly stimulated by insulin and the parasympathetic liver nerves while utilization of the glucose stores and thus glucose output is stimulated by glucagon, glucocorticoids and the catecholamines adrenaline and noradrenaline. Moreover, glucose output and uptake are also controlled by the circulating concentrations of glucose, lactate and last but not least oxygen (37, 38).

Thus, disturbances in the metabolic function of the liver will have profound effects on the other functions of the liver such as the synthesis of PAI-1 and other plasma proteins, the xenobiotic metabolism as well as the maintenance of the biomatrix components.

It is commonly accepted that the perturbations in PAI-1 expression in liver largely depends on the aberrant activation of signaling pathways and transcriptional regulators for which several binding sites have been identified within the promoters of the human, mouse and rat PAI-1 promoter (Fig. 1). Consequently,

these transcriptional regulators may be the basis for a new level of cross-talk between different signaling pathways and thus may represent attractive therapeutic candidates (36). Therefore, this article will primarily focus on the regulation of PAI-1 expression in liver cells and discuss potential cross-talks between metabolic, hormonal and environmental signals.

Glucagon and cAMP

The 29 amino acid peptide glucagon acts via its receptor which belongs to the superfamily of G-protein-coupled receptors (42, 43). Although enhancement of Ca^{2+} and activation of protein kinase C have been described upon binding of glucagon to its receptor, the major signaling pathway in liver results in activation of the adenylate cyclase and subsequent enhancement of cAMP levels which in turn activate protein kinase A (PKA). This signaling pathway leads to the activation of hepatic glucose production by glycogenolysis and gluconeogenesis as well as to changes in the gene expression pattern mainly due to the PKA-dependent phosphorylation of the transcription factor cAMP responsive element binding protein (CREB) (44–46).

Indeed, first studies with primary rat hepatocytes showed that treatment with cAMP increased PAI-1 mRNA in these cells (47). In addition, increases of PAI-1 were also observed in the livers of rats upon injection of cAMP alone or in combination with dexamethasone (47). In addition, stimulation of the cAMP/PKA/CREB signaling cascade by starvation *in vivo* or by treatment of primary hepatocytes with glucagon *in vitro* induced PAI-1 gene expression. This response was associated with enhanced phosphorylation of CREB. Interestingly, binding of CREB did not occur at a cAMP responsive element (CRE). Instead, CREB was

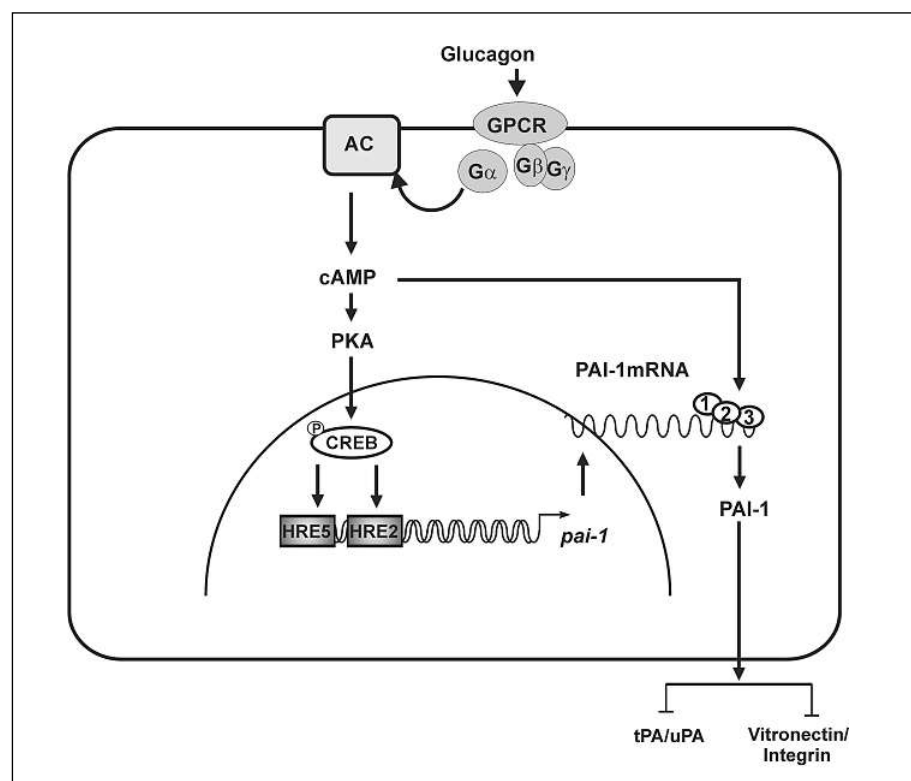


Figure 2: Regulation of PAI-1 expression in response to glucagon. Glucagon binding to its receptor in liver results in activation of the adenylate cyclase and subsequent enhancement of cAMP levels and activation of protein kinase A (PKA). Upon PKA-dependent phosphorylation the transcription factor cAMP responsive element binding protein (CREB) can bind HRE2 and E5 to induce PAI-1 expression. In addition, the 3'-UTR from the PAI-1 mRNA can be destabilized in the presence of three cAMP regulatable 3'-UTR PAI-1 mRNA-binding proteins.

able to bind to three elements, hypoxia-responsive element 2 (HRE2) (-194/-187) and two classical E-boxes (E), E4 (-566/-559) and E5 (-681/-674), within the human PAI-1 promoter (Fig. 2) but only HRE2 and E5 appeared to be functionally active (48); In the rat PAI-1 promoter the HRE2 (-164/-157) is completely conserved and also contributed to the cAMP-dependent induction (48) but, the involvement of E5 could not be demonstrated due to the lack of E5 in the rat promoter.

The effect of cAMP or agonists that increase intracellular cAMP levels on PAI-1 expression appears to be cell type-specific, since the rat HTC hepatoma cell line responded with a decrease in PAI-1 levels upon stimulation with cAMP (49).

In addition, the different effects of cAMP on PAI-1 mRNA levels in the various cell types may in part be explained by an additional mode of post-transcriptional regulation. Interestingly, the 3'-UTR from the PAI-1 mRNA appears to be destabilized in the presence of cAMP in HTC rat hepatoma cells. At least two regions within the 3'-UTR could be identified from which the 3'-most 134 nucleotides were sufficient to mediate this effect also in a heterologous system (50). It was then found by ultraviolet cross-linking analyses that three cytosolic proteins of about 38–76 kDa could bind to that region (51). Although one of these mRNA-binding proteins was cloned (52), the exact identity of the other PAI-1 mRNA-binding proteins remains unknown. Thus, PAI-1 regulation by cAMP appears to be controlled at the transcriptional and post-transcriptional level.

Interestingly, the cAMP concentration is not only increased upon starvation but also during liver regeneration to stimulate DNA synthesis and the cell cycle (53, 54). In addition to its role in matrix remodelling and fibrinolysis, PAI-1 has been found to be an early response gene in the liver (55). In addition, PAI-1 can be induced in regenerating liver after partial hepatectomy which suggests that it is necessary for the modulation of the hepatocytes growth and differentiation. Indeed, tPA and uPA are known to be involved in the activation of HGF (56) and TGF- β (57). Thus, the induction of PAI-1 by cAMP possibly represents a negative feedback for the regeneration process which may inhibit hepatocyte proliferation. Moreover, the induction by cAMP in the liver may have consequences for patients suffering from diabetes. In those patients, glucagon appears to be the dominant metabolic hormone compared to healthy individuals (58). This would indicate that the glucagon-mediated PAI-1 induction in the liver may contribute to the angiopathies occurring during diabetes. Indeed, PAI-1 was found to be overexpressed in patients suffering from non-proliferative diabetic retinopathy (59). Thus, the decreased matrix degradation due to PAI-1-induced inhibition of plasmin formation may contribute to these vessel abnormalities during diabetes.

Catecholamines and angiotensin II

The receptors for catecholamines in liver are the α 1-adrenergic receptors (α 1AR) which like the glucagon receptor belong to the seven transmembrane domain G protein-coupled receptor (GPCR) family. Three α 1AR subtypes are known (1A, 1B, and 1D) which couple to Gq and regulate phospholipase C (PLC) (60–62). Thus, hormone binding to the α 1AR leads via Gq to ac-

tivation of phospholipase C and generation of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) which release intracellular Ca^{2+} and mediate activation of protein kinase C, respectively. Additionally, α 1-AR has also been linked to other intracellular cascades in several extrahepatic cell types, including activation of phospholipase A2 (63), phospholipase D (64), and MAP kinases (65) and production of reactive oxygen species (66). These responses appear to be tissue- and cell type-specific and it remains obscure whether alternative signaling mechanisms are involved in hepatic α 1-AR actions.

Interestingly, it was found in mice *in vivo* that noradrenaline increased PAI-1 expression in liver under special circumstances. While noradrenalin induced PAI-1 expression in the heart and aorta but not the kidney or liver of wild-type mice, it induced PAI-1 in liver and kidney when the angiotensin II receptor subtype AT(1a) was deficient. Although this study did not specifically address the mechanism through which noradrenaline increases PAI-1 expression, it implicated that the angiotensin II receptor subtype AT(1a) mediated signaling suppresses PAI-1 expression in liver and also in kidney. Interestingly, at the same time the noradrenaline effects on blood pressure were diminished upon AT(1a) deficiency (67).

This appears to be an interesting crosstalk, since the octapeptide angiotensin II which is best known for its action in the cardiovascular system also elicits a variety of responses including stimulation of cell proliferation on various organs among them the liver (68–71). Two major types of receptors, i.e. angiotensin AT1 (with the a and b subtypes) and AT2 receptors have been characterized and like the α 1AR the AT1 receptors in hepatocytes are primarily coupled to the IP3/ Ca^{2+} /PKC signal transduction pathway (72).

Since it was found *in vivo* that Ang II stimulated PAI-1 expression (73) in part through the AT(1b) receptor in kidney and liver (67), it can be speculated that the transcriptional mechanisms may be similar between kidney and liver. Although the transcription factors and PAI-1 promoter elements mediating Ang II-dependent induction in hepatocytes were not yet determined, it was found in kidney mesangial cells that the transcription factor Sp1 binding to two Sp1 binding sites (Sp1a, -76/-71 and Sp1b, -46/-41) (Fig. 3) in the human PAI-1 promoter was involved in the upregulation by Ang II (74). Similarly, it was reported that both Sp1a and AP-like sequences (-59/-52), respectively, mediated angiotensin II-stimulated PAI-1 promoter activation in a cooperative manner in vascular smooth muscle cells (75).

The catecholamine and angiotensin II modulated PAI-1 regulation may be important for regulation during liver fibrosis. It appeared also that the renin-angiotensin system mediates key actions involved in hepatic tissue repair and fibrosis, including myofibroblast proliferation, infiltration of inflammatory cells, and collagen synthesis. Thus enhanced PAI-1 expression may contribute to inhibition of collagenases and important growth factors like TGF- β and HGF (76–78). However, it has also been proposed that inhibition of the renin-angiotensin system could prevent fibrosis progression in chronic liver diseases (79), but so far these antifibrotic effects in patients were not yet shown.

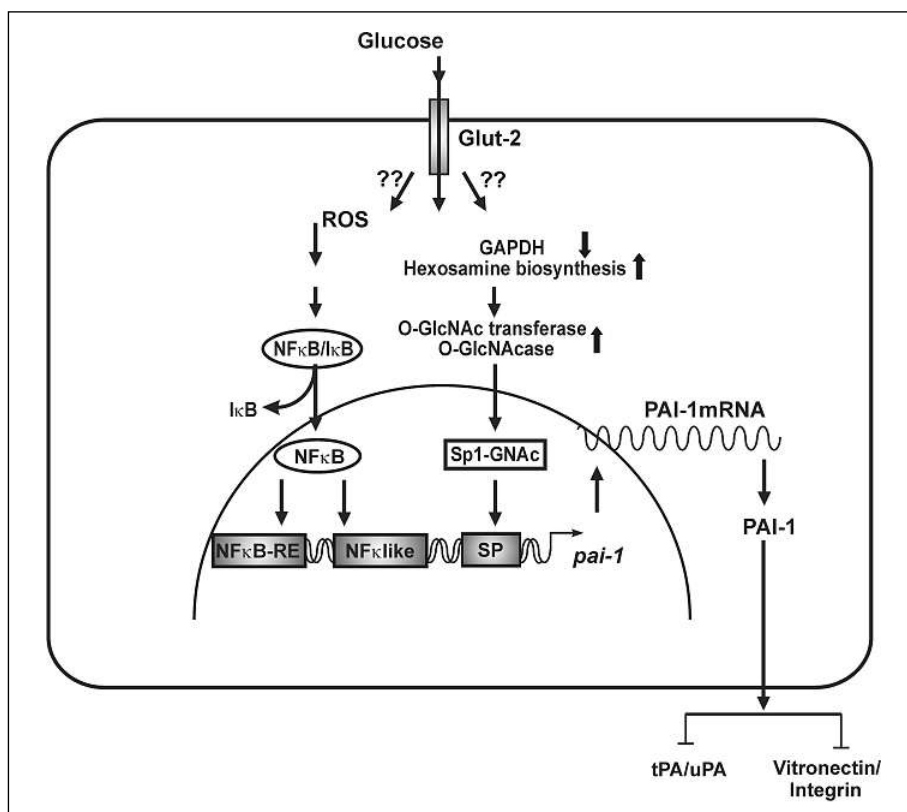


Figure 3: Regulation of PAI-I expression in response to glucose. Glucose is taken up by hepatocytes via the glucose transporter Glut-2 and an excessive intake of carbohydrates may result in the formation of reactive oxygen species (ROS) via a so far unknown mechanism. ROS in turn appear to activate NF- κ B which then can induce PAI-I transcription via binding the NF- κ B response element (NF- κ B-RE) in the far upstream (-15kB) located enhancer or eventually via the NF- κ B-like sequence at -678/-665 of the human PAI-I promoter. Glucose may also elicit an increased serine/threonine O-linked N-acetylglucosamination of Sp1. Then O-linked N-acetylglucosamination of Sp1 activates expression of PAI-I via binding of Sp1 to the Sp1 or Sp1c site in the promoter.

Glucose

An excessive intake of carbohydrates as often seen with Western diets can contribute to fat accumulation via glucose- and insulin-regulated *de novo* lipogenesis in the liver.

In humans it was shown that combined hyperinsulinemia, hypertriglyceridemia and hyperglycemia increased the blood levels of PAI-1 and may thus contribute to hypofibrinolysis in type 2 diabetic patients, which underlines the importance of achieving glycemic and lipidemic control in those patients (80). The contributions of liver cells were then further investigated in the human HuH7 hepatocyte cell line which was treated with high glucose from 3 to 24 mM. Upon treatment with glucose in the presence of insulin, these cells displayed enhanced nuclear factor kappa-B (NF- κ B) activity. Further, proinflammatory cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF α) showed an additive effect with high glucose. Similar effects were obtained with the human PAI-1 promoter which appeared to be regulated by NF- κ B (81). Finally, pretreatment of the cells with pyrrolidine dithiocarbamate (PDTC), which is thought to act as antioxidant, completely abolished the effect of high glucose and markedly attenuated that of TNF α (81). These data implicate that the responsiveness of the PAI-1 gene to glucose as well as to TNF α and IL-1 can be mediated via a common mechanism involving reactive oxygen species triggering NF- κ B activation.

The role of reactive oxygen species as universal NF- κ B activators was challenged by a number of studies and it became evident that this response occurs in a cell type-dependent manner (reviewed in [82, 83]). In addition, the experiments in which

PDTC antagonized the glucose effect do not specifically indicate involvement of reactive oxygen species because a recent study demonstrated that compounds considered being antioxidants such as PDTC and N-acetylcysteine (NAC) can inhibit NF- κ B activity independent of their antioxidative properties. PDTC inhibited I κ B-ubiquitin ligase activity while NAC blocked TNF receptor-induced signaling by lowering the receptor affinity (84). Thus, NF- κ B activation does not seem to be a universal response to oxidative stress induced by high glucose but might partially contribute to this response in a cell type-specific manner.

So far, binding of NF- κ B subunits to the PAI-1 promoter was found with a conserved NF- κ B site in an about 15 kb upstream located enhancer mediating the TNF α response (see below) (85). In addition, another sequence at -678/-665 of the human PAI-1 promoter was shown to mediate the IL-1-dependent stimulation of an 805 bp PAI-1 promoter-driven reporter construct in HepG2 cells (86). Although this sequence was named NF- κ B-like binding site due to sequence similarity, binding of NF- κ B subunits was not directly determined (86). Thus, it remains entirely open whether binding of NF- κ B to any of these sites in the PAI-1 promoter contributes to the glucose effect.

In addition to hepatocytes, the glucose-dependent induction occurs also in smooth muscle cells (87). Like in hepatocytes glucose activated the hexosamine pathway. However and in contrast to hepatocytes, high glucose elicited an increased serine/threonine O-linked N-acetylglucosamination of Sp1 (88). Furthermore, hyperglycemia increased expression of a 740 bp and 85 bp PAI-1 promoter Luc reporter containing two Sp1 sites (see above). When the two Sp1 sites were mutated, hyperglycemia did not increase expression of an 85-bp truncated PAI-1 promoter

Luc reporter (87). Thus, hyperglycemia induced hexosamine synthesis and then O-linked N-acetylglycosamination of Sp1, which activates expression of PAI-1 in vascular smooth muscle cells (88). In addition to Sp1, two other reports suggested that the glucose-increased PAI-1 gene transcription was dependent on activation of AP-1 in human (89) and rat vascular smooth muscle cells (90). Thus, to what extent NF- κ B, Sp1, and AP-1 contribute to PAI-1 gene expression in response to glucose requires further investigations.

Insulin and insulin-like growth factor-I (IGF-I)

Insulin signaling involves second messengers including members of the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascades (91). Thereby the PI3K, which generates phosphatidylinositol-3,4,5-phosphate (PI(3,4,5)P₃), has a key role in the metabolic actions of insulin while the MAPK pathway is more involved in the growth promoting actions of insulin. PI(3,4,5)P₃ regulates the activity or subcellular localization of a variety of signaling molecules such as phosphatidylinositol-dependent kinase (PDK) and protein ki-

nase B (PKB) known as Akt, which are also involved in the transmission of the insulin signal (92, 93).

It has long been suggested that the effects of insulin are mediated through a common insulin-responsive element (IRE) and a transcription factor that binds to an IRE (94–96). However, up to now at least eight distinct IREs have been characterized (97), suggesting that there is no single consensus IRE.

Insulin has been shown to increase the endogenous PAI-1 gene expression in HepG2 cells (98), primary rat hepatocytes (99), and the transcription of a human PAI-1 promoter Luc construct in human umbilical vein endothelial cells (100, 101).

Insulin acted as an inducer of PAI-1 gene expression in all different model systems studied, but the search for the insulin responsive elements displayed some differences. The insulin response of the human PAI-1 promoter was first suggested to involve the regions -93/-62, -157/-128, and -777/-741 and the PKC/MAPK pathway when HepG2 cells were used (102). In another study with HepG2 cells, the involvement of the MAPK pathway was confirmed, and it was additionally shown that the insulin effect was associated with activation of the transcription factor hypoxia-inducible factor-1 (HIF-1) (103). At first glance this may

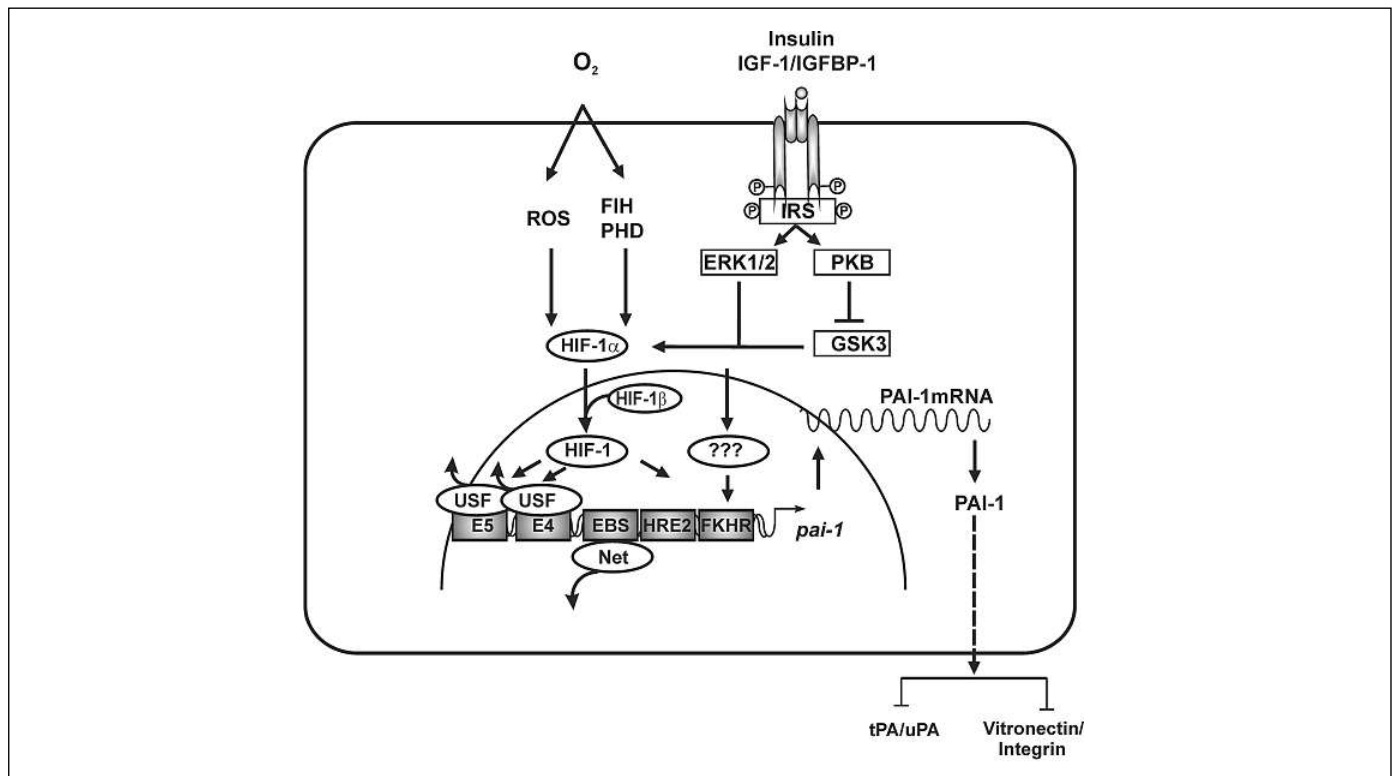


Figure 4: Regulation of PAI-1 expression in response to insulin, IGF-I and hypoxia. Insulin signaling involves activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/PKB) and mitogen-activated protein kinase (MAPK) cascades via insulin receptor substrates (IRS). The activation of the MAPK pathway and the PI3K/PKB pathway, via inhibition of glycogen synthase-3 (GSK3), stabilizes the transcription factor hypoxia-inducible factor-1 α (HIF-1 α). HIF-1 α then becomes transported to the nucleus where it then recruits its partner HIF-1 β , thus forming HIF-1. HIF-1 acts primarily via its high-affinity binding site HRE2 in the human PAI-1 promoter, while the low-affinity HIF-1 but high affinity upstream stimulatory factor (USF) binding E-boxes, E4 and E5 as well

as a putative FoxO1/FKHR-like binding site affect the insulin-dependent induction. The nature of the protein binding to the FoxO1/FKHR-like binding site has not been identified yet. Under hypoxia, HIF-1 α becomes stabilized due to the inhibition of HIF-prolylhydroxylases (PHD) and the factor-inhibiting HIF (FIH) as well as due to the modulated formation of reactive oxygen species. Once stabilized HIF-1 α gets into the nucleus, forms HIF-1 and acts via the HRE2 and E4 and E5. Thereby it outcompetes USF transcription factors. In addition, hypoxia downregulates Net levels; Thereby Net binding to the Ets-binding sites (EBS) is lost, however, the role of Net in liver and for hypoxia-mediated PAI-1 gene regulation in human cells has not been determined yet.

be surprising since this factor normally induces expression of genes under hypoxic conditions by binding hypoxia responsive elements (HREs), but this activation can also occur in the presence of insulin and other growth factors (104–109). HIF-1 appears to act primarily via the HRE2 in the human PAI-1 promoter which constitutes a high-affinity HIF-1 binding site. In addition, the low-affinity HIF-1 but high affinity upstream stimulatory factor (USF) binding E-boxes, E4 and E5 (110) as well as a putative FoxO1/FKHR-like (111) binding site (-52/-45) (Fig. 4), affected the insulin-dependent induction but only under normoxia (103). This indicates that the insulin effects on PAI-1 may be partially mediated via USFs which would be in line with findings from the fatty acid synthase promoter (112, 113). Although the E4- and E5-sites are not present in the rat PAI-1 promoter (Fig. 5), the data with respect to HIF-1 are in line with the results for the rat PAI-1 promoter in primary rat hepatocytes where the insulin effects were also mediated via HIF-1 binding to the HRE (99). Although the response to insulin in HepG2 cells was mediated via the MAPK signal transduction pathway (103) and that in primary rat hepatocytes was mediated via the PI3K/PKB pathway (99), this does not necessarily need to be conflicting. This can be explained by the fact that the tumor-derived HepG2 hepatoma cells would mainly require the growth promoting actions of insulin which are mediated via the MAPK pathway while the primary rat hepatocytes would resemble the more physiological stationary hepatocyte as apparent *in vivo* where the PI3K/PKB pathway has a key role in the metabolic actions of insulin (114). Thus, the insulin-dependent PAI-1 induction may be mediated via the MAPK or the PI3K/PKB pathway depending on the cell type.

While the involvement of HIF-1 and USF transcription factors in the insulin-dependent induction of PAI-1 expression

seems to be plausible, the involvement of FoxO transcription factors would be in contrast to the so far described action of FoxOs in response to insulin. Upon insulin treatment, the FoxO proteins become phosphorylated, which results in their nuclear export and cytoplasmic retention and subsequently in inhibition of target gene expression (115–119). Thus, a mutation of the FoxO1-like binding site in the human PAI-1 promoter should reduce basal promoter activity and should lead to a loss of the inhibitory effect of insulin. However, mutation of the FoxO1-like binding site within the human PAI-1 promoter did not reduce basal promoter activity. Further, if FoxO proteins would bind to that site, insulin should have mediated an inhibition of promoter activity, but this was not the case. Instead, insulin induced promoter activity and mutation of this site abolished the insulin effect only under normoxia (103). Thus, it seems likely that this element binds another or atypical FoxO protein which has not yet been identified. In addition, the insulin effect under hypoxia was not abolished, and thus this factor may not be the major regulator of insulin-dependent PAI-1 expression.

Although the results concerning the up-regulation of PAI-1 expression by insulin in different cell culture models show similar effects, the data concerning the up-regulation of human PAI-1 by insulin *in vivo* are somewhat contradictory. Some studies *in vivo* investigating the effects of insulin infusion found no effect on the levels of PAI-1 in blood or even a decrease of PAI-1 levels and activity (120–122). On the other hand, induction of PAI-1 by insulin was found when the perfused forearm model was used to study the local effects of insulin infusion (123). Furthermore, in combination with hypertriglyceridemia and hyperglycemia, hyperinsulinemia was shown to increase PAI-1 plasma levels (80). Thus, further investigations are still necessary to elucidate the

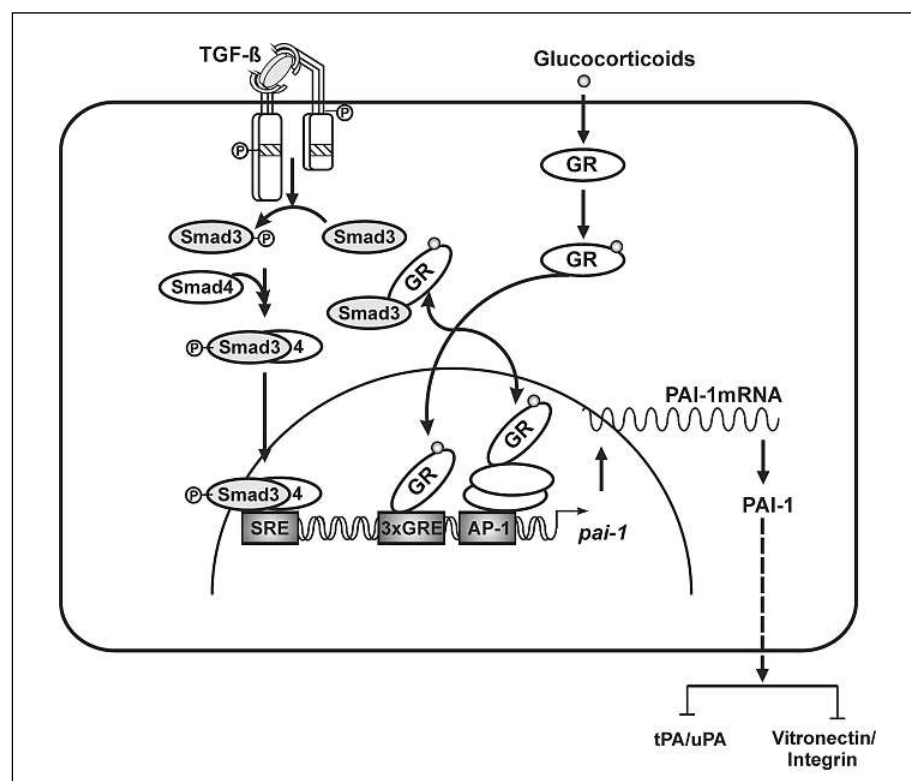


Figure 5: Regulation of PAI-1 expression in response to glucocorticoids. Glucocorticoids bind to their cytoplasmic glucocorticoid receptors (GRs) and the glucocorticoid-receptor-complex is translocated to the nucleus where the activated GR can directly bind to the three glucocorticoid response elements (GREs) identified by sequence similarity or other DNA bound transcription factors. Glucocorticoids can also repress the TGF- β -dependent human PAI-1 induction through direct binding of ligand-bound glucocorticoid receptor (GR) to Smad3 which acts in concert with Smad4. By contrast, an increase of TGF- β -induced PAI-1 expression can be mediated via the interaction of the GR with another protein like AP-1. The AP-1 activity is also supposed to be a result of a directly TGF- β -induced MAPK signalling. In addition, cooperation between GR and other factors like Sp1 is also possible to modulate PAI-1 expression.

complete physiological role of PAI-1 activation by insulin and its molecular mechanisms.

Similar to insulin it was found that IGF-1-dependent induction of PAI-1 gene expression occurred via a transcriptional mechanism involving HIF-1. IGF-1 enhanced HIF-1 α protein levels and HIF-1 DNA-binding to HRE2, and the classical E-boxes E4 and E5, and again the HRE played the major role while E4 and E5 had a supportive role. Inhibitor studies and expression of dominant-negative PDK1, dominant-negative Raf and the PKB inhibitor tribbles-3 (TRB3) then revealed that the PI3K and the MAPK (ERK) pathway but not PKB mediate the enhancement of HIF-1 α and PAI-1 by IGF-1 in HepG2 cells (124).

The enhancement of PAI-1 by IGF-1 may have consequences for several physiological and pathophysiological situations which require cell proliferation and angiogenesis. In conjunction with a variety of other molecules IGF-1-induced PAI-1 levels may promote vascularization of tumor tissue by contributing to prevent excessive degradation of cellular matrix which then enables endothelial cell sprouting. However, clear differences between certain types of tumors appear to exist and further knowledge of these variations may help to better understand the role of PAI-1 in these processes.

Glucocorticoids

Glucocorticoids regulate crucial functions in hepatocytes and the genes mainly controlled by glucocorticoid receptors (GR) are involved in increasing blood glucose levels via gluconeogenesis and mobilization of amino and fatty acids. The phosphoenolpyruvate carboxykinase (PEPCK) and tyrosine aminotransferase (TAT) are two prototypic genes regulated by the GR. Once bound to their cytoplasmic GRs the glucocorticoid-receptor-complex is translocated to the nucleus via the microtubule network. Within the nucleus, the activated GR can directly bind to DNA elements termed glucocorticoid response elements (GREs) or it can indirectly bind to DNA through its interaction with other DNA bound transcription factors. The GREs usually consist of two six base pair "half-sites" separated by a three-base-pair spacer and upon binding to the GRE as a homodimer, GR serves also as a scaffold for the recruitment of coactivators, chromatin remodeling factors and other factors that modulate the activity of the transcriptional machinery (125, 126).

A number of studies have shown that glucocorticoids can enhance PAI-1 expression in different cell types, among them liver and hepatoma cells (127–129). The first incidence that the human PAI-1 promoter could be activated by glucocorticoids came from a study in which it was shown that a fragment spanning 805 nucleotides of the 5' flanking and 72 of the 5' untranslated region contained all necessary information to respond to glucocorticoids and a GRE consensus site at -800/-549/-100 could be identified by sequence similarity (130). The next identification of promoter elements involved in transcriptional regulation by glucocorticoids came from a study with HTC rat hepatoma cells. Here it was shown by electrophoretic mobility shift assays, DNase-I protection assays and mutation analysis that the region at -1212/-1196 of the rat PAI-1 promoter bound a glucocorticoid receptor which could transactivate the PAI-1 promoter (129).

In addition to the activation of transcription, GRs have also been shown to reduce transcription. This occurs via binding to DNA sequences distinct from positive GREs or the interaction with other DNA-bound transcription factors such as AP-1, NF- κ B or Smads. All this may mainly account for the anti-inflammatory actions of glucocorticoids.

Interestingly, this mechanism seems to be important for the regulation of PAI-1 expression by other inducers like TGF- β . Although PAI-1 is considered to be a prototypical TGF- β induced gene, this induction may be at variance in response to glucocorticoids in the various types of liver cells. In the hepatocyte derived human tumor cell line Hep3B it was shown that the TGF- β -dependent human PAI-1 induction can be repressed by glucocorticoids through direct binding of liganded glucocorticoid receptor (GR) to the carboxyl terminal transactivation domain of Smad3 (131, 132).

Smad3 acted in concert with Smad4 which bound via their MH1 domain to a novel TGF- β response element (-732/-721) in the human PAI-1 gene (133). By contrast, in human keratinocytes it was shown that exposure to TGF- β induced human PAI-1 expression via E4 -566/-559 (134) whereas in the rat PAI-1 promoter it acted via HRE2 (-165/-160) (135).

Furthermore, glucocorticoid mediated repression was enhanced upon overexpression of steroid receptor coactivator-1 (SRC-1) and GR-interacting protein-1 (GRIP-1) whereas SRC-1 and GRIP-1 in the absence of glucocorticoids alone enhanced TGF- β -induced activation (136).

The latter effect may depend on the interaction of SRC-1 with the transcriptional co-activators p300/CBP indicating that SRC-1 may facilitate a functional link between Smad3 and p300/CBP (137). This suggests that, depending on the stimulus and the cell type, different PAI-1 promoter activation or repression models may be achieved by the GR coactivators, SRC-1 and GRIP-1. Similarly, studies from primary hepatic stellate cells and cirrhotic fat storing cells also showed that glucocorticoids decreased TGF- β -dependent induction (138).

By contrast, in primary rat hepatocytes dexamethasone significantly enhanced TGF- β -induced PAI-1 expression. Likewise, an increase of TGF- β -induced PAI-1 expression through dexamethasone was also observed in HTR-8 SV neo cells (139).

These findings implicate that the enhancement of TGF- β -mediated PAI-1 expression by dexamethasone is due to an indirect transcriptional mechanism like the interaction of the GR with another protein. It is possible that the positive cooperation between Smads and activator protein-1 (AP-1) contributes to this effect. The human and the rat PAI-1 promoter were shown to contain an AP-1 binding site (140–142); the presence of this site makes it likely that conditions leading to modified interactions between the GR and AP-1 proteins (Jun/Fos/Fra) regulate PAI-1 transcription via AP-1 complexes. The AP-1 activity is also supposed to be a result of a directly TGF- β -induced MAPK signaling (142). In line, pharmacological inhibition of the MEK/ERK pathway by PD98059 clearly suppressed TGF-induced PAI-1 expression in primary hepatocytes (138), which points to cooperative action between TGF- β and ERK signaling pathway. Thereby, this response seems to be mediated via ERK1 since overexpression of ERK2 in primary hepatocytes and HepG2 cells had almost no effect on PAI-1 expression (143). Further,

ERK1 seems then to act not only via AP-1 but may require also the co-activation of Sp1 which was shown to bind two Sp1 sites (Sp1a, -76/-71 and Sp1b, -46/-41) and also to be involved in the response to glucose and angiotensin II (144).

Together, the action of glucocorticoids on PAI-1 expression represents a new level of control since PAI-1 expression depends on the cell-type, the interactions between the GR with its DNA binding elements and the coactivators as well as GR interacting transcription factors.

Cytokines

In addition to its role in metabolism, the liver is a center of defence and largely responds to injury with the so called acute phase response. Thereby the liver synthesizes and secretes a large number of proteins, the so called acute phase reactants. PAI-1 was shown to be an important component of the acute phase response in humans since its levels were increased in patients with sepsis, surgery or trauma (145, 146). By contrast, the contribution of PAI-1 to the acute phase response was questioned in rats (147). The major mediators of the AP response have been shown to be cytokines, including IL-1, IL-6 and TNF α .

Although IL-1 β and TNF α appeared not to stimulate PAI-1 production in human primary hepatocytes (16), PAI-1 expression was strongly regulated by IL-1 and TNF α in HepG2 cells (148), whereas IL-6 alone had only a modest effect on PAI-1 levels (149, 150). However, IL-6 in combination with IL-1 caused a synergistic induction of PAI-1 expression (149–151). Although the cytokine-dependent regulation of PAI-1 expression occurred at transcriptional level, the data obtained for the corresponding DNA response elements seem to be different. While no STAT3 binding element participating in the IL-6 response could be mapped so far, it was shown in HepG2 cells that IL-6 increased hepatic PAI-1 expression via the -232- to -210-bp region of the promoter containing a C/EBPdelta binding site (152). One might speculate that a similar mechanism possibly applies also for other IL-6 type cytokines like oncostatin M (OSM). However, it was shown that the AP-1 element of the PAI-1 promoter mediated activation by OSM and also IL-1 in astrocytes, thus indicating that the response to OSM may be cell type-specific. Overexpression of dominant-negative STAT1, STAT3, STAT5 and an inhibitor of nuclear factor- κ B (I κ B) suppressed the OSM- and IL-1-induced expression of the PAI-1 reporter construct (153). This would suggest the possibility of a direct activation of PAI-1 expression by C/EBPdelta and an indirect activation of PAI-1 expression by the STAT pathway as well as an additional involvement of the NF- κ B pathway.

The IL-1 response as well as the TNF α response may in principle also be mediated via the so called NF- κ B-like sites (86). Importantly, a comprehensive analysis in a recent study identified a 5' distal TNF α -responsive enhancer of the human and mouse PAI-1 gene. This enhancer located 15 kb upstream of the transcription start site contains a conserved NF- κ B-binding site (5'-TGGAATTTCT-3') at -14889/-14880 that was able to bind the NF- κ B subunits p50 and p65 as well as mediated the response to TNF α (85); whether this element could mediate also the response to IL-1 was not studied. Interestingly, the fact that this newly identified TNF response element was only conserved

in human and mouse PAI-1 genes but not in the rat PAI-1 gene is in line with an earlier study showing that PAI-1 is not an acute phase reactant in rat liver (147). Interestingly, another study investigating the TNF α response of PAI-1 in HUVECs found that direct binding of Nur77/NAK-1 to the PAI-1 promoter was necessary to mediate the TNF α -induced PAI-1 expression (154). Together, these results indicate that induction of PAI-1 by cytokines may be cell-type and/or species specific and may involve different molecular mechanisms.

Hypoxia

Several diseases characterized by reduced delivery of oxygen to the liver lead to perivenous hypoxia and can be associated with perivenous damage. This occurs especially during heart failure (ischemic hepatitis) (155), obstructive lung dysfunction (sleep apnea / Pickwickian syndrome) (156), gut ischemia (157, 158) or cases of drug hepatotoxicity as observed with many xenobiotics like the industrial chemical carbon tetrachloride, the pharmacological agent acetaminophen or the "cultural poison" ethanol. The toxic metabolites are formed in the liver and cause perivenous damage (159, 160) which is associated with perivenous hypoxia (161–163).

A first incidence that hypoxia induces PAI-1 expression *in vivo* came from studies with mice placed in a hypoxic environment (5–6% O₂). Those mice which were exposed to hypoxia displayed enhanced PAI-1 plasma levels and PAI-1 mRNA as well as protein in the lungs when compared to the normoxic controls (164). The induction of PAI-1 expression by hypoxia was then confirmed in rat primary hepatocytes (165, 166) and in four human liver cell lines Chang, Hep3B, HuH7 and HepG2 (167). It was shown that the transcription factor responsible for the hypoxia-dependent PAI-1 activation was hypoxia-inducible factor-1 (HIF-1) acting via HREs within the rat and human PAI-1 promoter (165–167). Further, studies with the murine PAI-1 promoter have also shown that the hypoxic response is mediated via HREs [HRE-1 (-182/-178) and HRE-2 (-171/-167)]. In these studies with the macrophage-derived RAW cell line it was also shown that the hypoxia-dependent PAI-1 gene expression could be augmented by C/EBPalpha and early growth response gene-1 (EGR-1) binding the mouse PAI-1 promoter regions -209/-200 and -137/-129, respectively (168). Thus, although the involvement of HIF-1 appears to dominate the hypoxic response of the PAI-1 gene in hepatocytes and hepatoma cells, other factors like EGR-1 and C/EBPalpha may contribute to the hypoxia-dependent PAI-1 enhancement in other cell types. This appears to be even more complex since it was shown in the HTR-8/SVneo human trophoblast cell line (169) and in renal clear cell carcinoma cells (170) that in addition to HIF-1 also HIF-2 played an important and similar role in hypoxia-dependent PAI-1 expression.

The regulation of PAI-1 expression by HIFs appears not only to be important under conditions of hypoxia but also under conditions leading to the activation of HIF-1 α under normoxic conditions. This can be achieved by the action of hormones, growth- and coagulation factors, cytokines and conditions of mechanical-, physical- or chemical stress (for review see [171]). In liver, these events may be also of importance for situations associated with growth factors and cytokines which stimulate liver re-

generation and promote hepatoprotection. Thereby, both PKB and MAP kinases including p38 MAP kinase can contribute to the activation of the HIF pathway. While ERK-1 was also able to phosphorylate HIF-1 α and to promote its nuclear accumulation (172, 173), overexpression of PKB and the p38 upstream kinases MKK3 and MKK6 resulted in enhanced HIF-1 α levels and stimulated HIF-1-dependent PAI-1 expression (143). Although PKB induces HIF-1 α stabilization, HIF-1 α is not a direct substrate for PKB/Akt. However, inhibition and depletion of the PKB target glycogen synthase kinase-3 (GSK3) induced HIF-1 α , whereas overexpression reduced HIF-1 α levels. This regulation occurred in a VHL-independent fashion via phosphorylation of HIF-1 α within the oxygen-dependent degradation domain (174). Furthermore, GSK3 contributes to PAI-1 expression by phosphorylating and stabilizing Rev-erb alpha which is involved in regulating circadian gene expression. Interestingly, expression of a Rev-erb alpha variant mimicking a GSK3 phosphorylated variant negatively influenced circadian PAI-1 oscillations (175). Thus, GSK3 turned out to be a major determinant for the hypoxia-dependent and the circadian PAI-1 expression.

In addition to its prominent post-translational regulation, HIF-1 α can serve also as a bridge for inflammatory mediators and growth factors which transcriptionally regulate HIF-1 α via NF- κ B. Interestingly, in pulmonary artery smooth muscle cells it could be shown that hypoxia activated HIF-1 α transcription via PI3K signaling and binding of NF- κ B to a functional element in the HIF-1 α promoter (176, 177). These cell culture data were then corroborated also for the liver by an approach *in vivo* using mice lacking IKK- β , an upstream regulator of NF- κ B. These studies show that NF- κ B activity is required for HIF-1 α protein accumulation under hypoxia in the liver and brain of hypoxic animals (178). In addition, hepatocyte growth factor (HGF) and its receptor the oncogene Met appear also to be important in PAI-1 regulation. While HGF has been shown to enhance HIF-1 α via NF- κ B (179) it was also shown that hypoxia can enhance the levels of Met via HIF-1 α (180). When Met expressing lentiviral vectors were transduced into mice Met signalling in turn induces venous thrombosis due to enhanced PAI-1 expression and a tumorigenic process in hepatocytes (181). Noticeably, PAI-1(-/-) mice showed accelerated liver regeneration and higher levels of

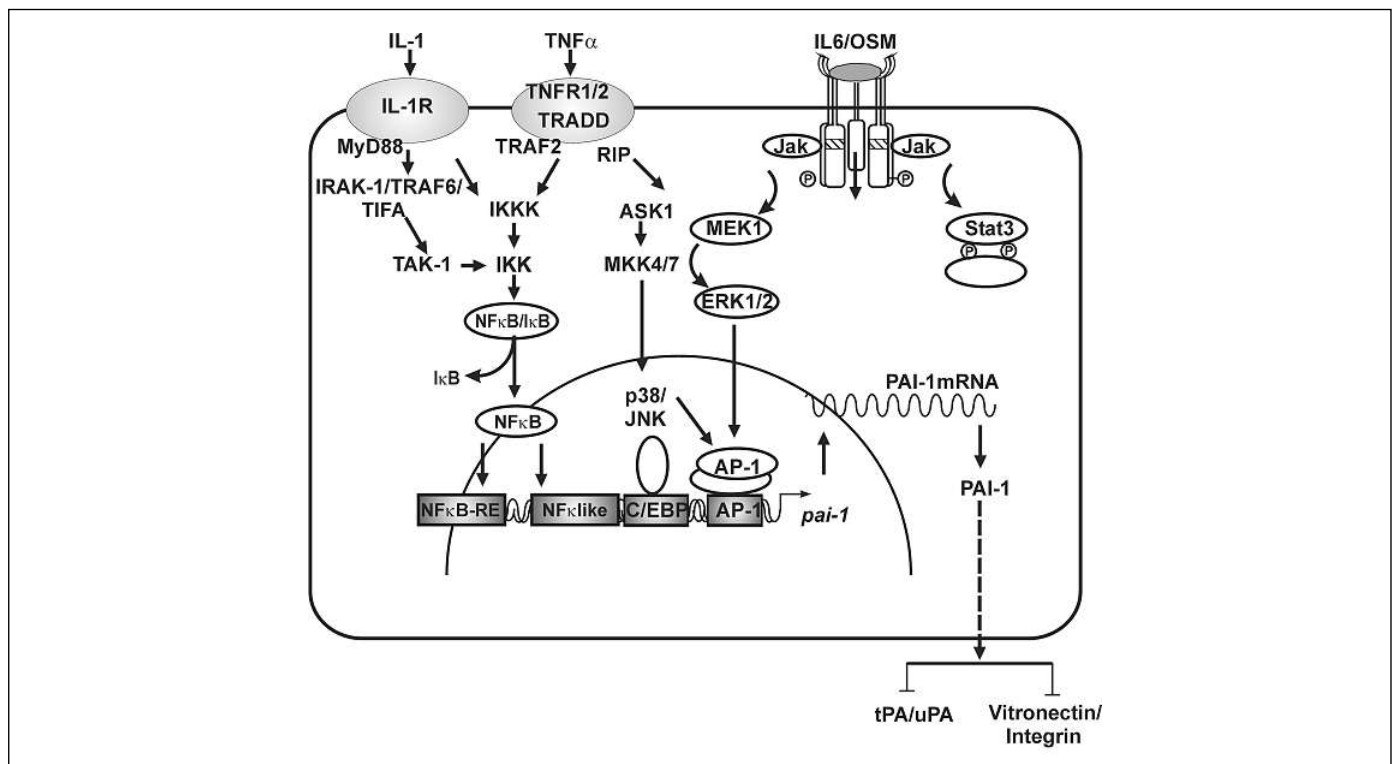


Figure 6: Regulation of PAI-1 expression in response to cytokines. PAI-1 is an important component of the acute phase response in humans the cytokines, interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α (TNF α) induce human PAI-1 expression. IL-1 binds to its receptor and IL-1R signalling recruits specific adaptor proteins like MyD88. Binding of MyD88 allows recruitment of IL-1R associated kinase (IRAK)-1 and IRAK-4. Active IRAK-1 interacts with TRAF6 and TIFA and the IRAK-1/TRAF6/TIFA complex interacts with the kinase TAK1 and the adaptor molecules TAB1 and TAB2. After phosphorylation of TAK1 the TRAF6-TAB2/3-TAK1-TAB1 complex migrates to the cytosol. Active TAK1 then leads to the downstream activation of I κ B kinases (IKK) and JNK or p38 MAPKs. IKKs activate NF- κ B by phosphorylating the NF- κ B inhibitory protein I κ B α , leading to its ubiquitination and proteasome-de-

pendent degradation, whereas JNK and p38 phosphorylate several other transcription factors. The IL-1 response as well as the TNF α response may in principle be mediated via the NF- κ B-like site and the NF- κ B-binding site in the far upstream enhancer. IL-6 and OSM-induced receptor clustering activates Janus kinases, mainly Jak1 which phosphorylate tyrosines within the gp130 receptor subunit leading to recruitment of other signaling proteins with matching SH2 domains such as signal transducers and activators of transcription (STATs) or adapter proteins for the mitogen activated protein kinases Erk1/2 and p38. While no STAT3 binding element could be mapped IL-6 increased hepatic PAI-1 expression via a C/EBPdelta binding site. The AP-1 element of the PAI-1 promoter may mediate activation by OSM.

mature HGF (78). Thus, agents that modulate the activity of the NF- κ B pathway may simultaneously contribute to the hypoxic response as well as to inflammatory processes and immunity.

Comparison between human and rat PAI-1 genes revealed strict conservation of the intron-exon structure (182, 183). In addition, two regions of the promoter showed a high degree of similarity: a 60 bp region – from –90 to the TATA box (90% identical), and the sequence located at –753 and –510 (> 80% identical) (Fig. 6) (183). Although partially conserved, a number of differences between the human, mouse and rat promoter exist and might account for some different regulatory patterns between these genes. Especially, the conservation for HIF-1 binding to the HRE was observed, whereas the USF-2-binding rHRE1 was absent from the human promoter. Instead, two classical E-boxes E4 and E5 (–566/–559 and –681/–674) were found which might function as putative USF-binding sites. Thus, due to these differences between the rat and human as well as the mouse PAI-1 promoter, it is conceivable that USF-2 might have a distinct effect on human PAI-1 gene transcription. In fact, it was shown that USF-2 could downregulate PAI-1 expression in primary rat hepatocytes via binding to the low-affinity HIF-1 but high-affinity USF-2 site HRE1 and to a lesser extent via the high-affinity HIF-1 and low-affinity USF-2 site HRE2 implicating a competition between HIF-1 and USF-2 (166). By contrast, in human HepG2 hepatoma cells USF-2 induced human PAI-1 expression. This occurred via binding of USF-2 to E4 and E5 within the promoter. In addition, the HRE contributed to the USF effect without binding it (110). These data suggested that PAI-1 expression depends on either the promoter context or USF activity which might be cell type-specific. Indeed, cotransfection of human or rat PAI-1 promoter luciferase constructs with expression vectors for wild-type USF-2 or USF-2 mutants in human HepG2 and rat H4IIE cells as well as in primary rat hepatocytes revealed that the effects of USF on PAI-1 expression depend on the cell type rather than the promoter context (110). This mode of action may be important during carcinogenesis when high levels of PAI-1 are found. This may be caused in part by defective USF proteins which can no longer downregulate PAI-1. Indeed, cell culture experiments with rat embryonic fibroblasts (REFs) showed that transfection of either USF-1 or USF-2 inhibited cellular transformation induced by c-Myc or activated Ras. In addition, USF-2 also inhibited transformation in REFs induced by the adenovirus oncoprotein E1A, while USF-1 did not, thus highlighting the broader inhibitory function of USF-2 (184). In line, many cancer cells including the prostate cancer cell line PC-3 (185, 186) displayed a loss of USF-2 transcriptional activity while it was active in non-tumorigenic cells. These findings imply that USFs and especially USF-2 may be important also as a suppressor of liver cell transformation and tumor progression.

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In addition to USF-2, Net which is together with Elk-1 and Sap-1 a member of the ternary complex factor family of Ets transcription factors, seems to be of importance for the regulation of PAI-1 by hypoxia and also for a number of other genes (187). In mouse embryonic fibroblasts, hypoxia downregulated Net protein levels by ubiquitination and proteasomal degradation. Thereby Net binding to the three Ets-binding sites located between –519 and –319 of the mouse PAI-1 promoter (188) was modulated and PAI-1 was downregulated. These findings seem to be very important for mouse fibroblasts and the regulation of HIF-1 and will most likely have more general impact; however, the role of Net in liver and for hypoxia-mediated PAI-1 gene regulation in human cells has not been determined yet.

Conclusion

At the moment it appears that a number of hormonal, metabolic and environmental stimuli exert an increase in PAI-1 expression in hepatocytes and hepatocyte-derived cell lines as well as in the liver *in vivo*.

Although a number of mechanistical details appear to be valid also for the situation *in vivo*, a careful interpretation should be made from study to study and between studies with cell lines and intact animals, since the mechanisms involved in PAI-1 expression may vary depending on the species, the cell type and the animals used. Often a number of cell types do not maintain the phenotype of the parent cell-type which is part of an entire organ or tissue.

Elevated PAI-1 levels and hypofibrinolysis are common during the development of alcoholic liver disease. Further, PAI-1 plays a critical role in both acute and chronic hepatic inflammation. These findings indicate a role of PAI-1 as a useful target for therapy to halt or blunt disease progression.

It is tempting to speculate that various stimuli induce PAI-1 expression via activation of different kinase signaling pathways. The transcription factors involved in positively transferring these responses are primarily NF- κ B, HIF-1, Sp1, AP-1 and Smad2/3. Thereby the hypoxia and PKB-MAPK signaling can merge at HIF-1 α . The negative regulation of PAI-1 expression can be exerted partially by GR antagonizing Smad3, USF-2, Rev-erb alpha and the new factor Net. Although quite a lot of progress has been achieved over the recent years a complete model for the regulation of PAI-1 gene expression in liver cannot be made, and numbers of issues have to be resolved in the future.

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