CREB binding to the hypoxia-inducible factor-1 responsive elements in the plasminogen activator inhibitor-1 promoter mediates the glucagon effect

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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 related to pathological conditions associated with hypoxia or hyperinsulinemia. In this study, we investigated the regulation of PAI-1 expression by glucagon and the cAMP/PKA/CREB signalling pathway in the liver. Stimulation of the cAMP/PKA/CREB signalling cascade by starvation in vivo or glucagon in vitro induced PAI-1 gene expression in liver. Furthermore, this response was associated with enhanced phosphorylation of CREB. By using EMSAs we found that three promoter elements, the HRE2, E-box 4 and E-box 5, were able to bind CREB but only the HRE2 and E5 appeared to be functionally active. Reporter gene assays confirmed that cAMP induced PAI-1 gene transcription via the same element in both human and rat promoters. Interestingly, although the HRE2 was involved, the glucagon/cAMP pathway had no influence on hypoxia-inducible factor-1 (HIF-1) mRNA and protein levels. Thus, CREB binding to the HIF-1 responsive elements in PAI-1 promoter mediates the glucagon effect in the liver.

Keywords
PAI-1, glucagon, cAMP, CREB, HIF-1

Introduction
A number of physiological and pathological processes, among them fibrinolysis and matrix degradation, are regulated by tissue-type (tPA) and urokinase-type (uPA) plasminogen activators which convert the inactive proenzyme plasminogen into plasmin. Plasmin in turn degrades fibrin and basement membrane components, such as laminin and fibronectin, as well as activates matrix metalloproteases and growth factors like HGF (hepatic growth factor) (1). The activity of tPA and uPA is tightly regulated by plasminogen activator inhibitors (PAIs). From the PAIs known so far, PAI-1 appears to be the most important. It can be produced by hepatocytes of the liver, vascular endothelial cells and vascular smooth muscle cells as well as by platelets (2–4). In addition, PAI-1 is a component of the extracellular matrix where it is bound mainly to vitronectin or to scavenger receptors from the low-density lipoprotein receptor family (5).

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 [6]). The hypoxia-dependent induction of PAI-1 gene expression was mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1) which was able to bind to E-box 4, E-box 5 and E-box 2 of the human PAI-1 promoter and E-box 2 of the rat promoter, respectively (7–9). With both promoters E-box 2 appeared to be most critical for the induction by hypoxia and was therefore renamed hypoxia responsive element-2 (HRE2).

Additionally, enhanced PAI-1 levels are found in patients with type 1 and especially type 2 diabetes mellitus (10, 11) where the overexpression of this protease inhibitor was considered to be responsible for the decreased basal membrane and extracellular matrix degradation and the resulting angiopathies (for review see [12]). These vessel pathologies as well as the associated metabolic changes result from defects in hormonal control systems which normally balance glucose and lipid homeostasis within a narrow range (13).
Under normal conditions, the pancreatic β-cell hormones insulin and glucagon contribute mainly to the balance of glucose and lipid metabolism. However, this balance is lost in type 2 diabetes where a defective insulin response in liver has been shown to contribute to the development of peripheral insulin resistance. This is associated with concomitant chronic activation of counter-regulatory hormones, in particular adrenal glucocorticoids and pancreatic glucagon. The 29 amino acid peptide glucagon acts via its receptor which belongs to the superfamily of G-protein-coupled receptors (14, 15). Although enhancement of Ca2+ and activation of protein kinase C have been described upon binding of glucagon to its receptor, the major signalling 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 signalling 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) (16–18). Thus, although the action of glucagon in carbohydrate metabolism appears to be clear, its contribution to the production of PAI-1 and the resulting development of peripheral microangiopathies remains obscure. Therefore, we investigated whether the glucagon/cAMP/PKA/CREB signalling pathway may contribute to enhanced PAI-1 expression.

Materials and methods

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers.

Animals

Male Wistar rats (200–260 g) were fed with a normal diet and received water ad libitum. Rats were anesthetized with pentobarbital (60 mg/kg body weight) prior to preparation of hepatocytes. Livers were isolated from rats fed the normal standard diet or after a 24-hour (h) fasting period, respectively.

Cell culture and transient transfections

Primary rat hepatocytes (PHC) were isolated by collagenase perfusion as described (8) and cultured in medium M 199 containing 0.5 mM insulin, 100 mM dexamethasone as permissive hormones and 5% fetal calf serum for the initial 5 h of culture. Cells were then cultured in serum-free medium from 5 h to 24 h under normoxia. HepG2 cells were propagated in MEM supplemented with 10% fetal calf serum (Invitrogen, Karlsruhe, Germany), 1% non-essential amino acids (Invitrogen) and 0.5% antibiotics. All cells were cultured under normoxia (16% O2, 79% N2, and 5% CO2 [by volume]) and/or under hypoxia (8% O2, 87% N2, 5% CO2 [by volume]). For stimulation, glucagon (dissolved in 0.9% NaCl containing 0.1% bovine serum albumin) (Sigma) or 8-(4-chlorophenylthio) adenosine 3',5'-cyclic monophosphate sodium salt (8-cpt-cAMP) (Sigma) were applied to the serum-free medium for the indicated time and concentrations; controls were treated with solvent and cells were cultured under normoxic and hypoxic conditions, respectively.

Freshly isolated rat hepatocytes (about 1x10^6 cells per dish) and HepG2 cells (about 4x10^4 cells per dish) were transfected by using the calcium phosphate coprecipitation method as described (19). In brief, cells were transiently transfected with 2.5 µg plasmid DNA containing 500 ng of pRL-SV40 (Promega) to control transfection efficiency and 2.5 µg of the appropriate PAI-1 promoter Firefly luciferase (FL) construct. Every culture experiment was done in duplicates. PHC and HepG2 cells were harvested 48 h after transfection and Luc activity from 20 µl cell lysate was recorded in a luminometer (Berthold) using the dual luciferase assay kit (Promega).

Plasmid constructs

The pGL3PAI-806 plasmids, containing the human PAI-1 promoter 5' flanking region from −806 to +19, pGL3PAI-806M5, pGL3PAI-806M4, pGL3PAI-806HREM and pGL3PAI-806 HREM45 have been already described (9). The pGL3pPAI-766 plasmid containing the rat PAI-1 promoter and the mutant pGI3pPAI-766HRE2m were described before (8). The reporter plasmid pCRE-Luc (Stratagene) contains four repeats of a CRE located upstream of the TATA box that controls the expression of the downstream firefly luciferase reporter gene.

RNA preparation and Northern blot analysis

Isolation of total RNA, Northern blot analysis and detections were performed essentially as described before (20). Blots were quantified with a videodensitometer (Biotech Fischer, Reiskirchen).

Western blot analysis

PAI-1 Western blot analysis was carried out as described (8). The primary rabbit antibody against PAI-1 (1:100) (American Diagnostics, Pfungstadt, Germany) was used. The monoclonal HIF-1α (Novus, Littleton, CO, USA), the monoclonal CREB-1 (Santa Cruz, Heidelberg, Germany) and the polyclonal phospho-CREB-1 (Ser133) antibodies (Santa Cruz) were used in a dilution of 1:1,000. The secondary antibody was either an anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase (1:5,000; Biorad, Munich, Germany). The enhanced chemiluminescence (ECL) system (Amersham, Freiburg, Germany) was used for detection.

Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as already described (9). The sequence of PAI-1 oligonucleotides used for EMSA are 5'-AGTCTGGACACGTGGGGA-3' (-689/-670), 5'-ACAATCACGTACACACA-3' (-199/-181). For supershift analysis 0.5 µg of the ATF-1/CREB cross-reactive (Santa Cruz) and non-cross-reactive CREB-1 (Santa Cruz) antibody was used.

Results

The cAMP response element binding protein (CREB) binds to the PAI-1 promoter

The transcription factor CREB is a central element in the cAMP/PKA signalling cascade and the cAMP/PKA/CREB signal can be antagonized by insulin. Insulin was shown to exert its effects on PAI-1 transcription via the transcription factor HIF-1 (21, 22).
HIF-1 could bind the human PAI-1 promoter at three different elements. These elements represent E-boxes, namely HRE2 (the previous E2) (-194/-187), E4 (-566/-559) and E5 (-681/-674) (7, 9). In addition to HIF-1, we detected that these elements can additionally be bound by a so far unknown factor/complex. Thus, it seems likely that this unknown complex may consist of proteins belonging to the ATF/CREB family which might then interfere with insulin signalling. Indeed, by comparing the HRE/E-box (5′-RCGTG-3′; 5′-CANNTG-3′) consensus sequence with putative binding sites for other transcription factors we found that the sequence 5′-ACGT-3′ of the PAI-1 boxes matches the core sequence of the cAMP responsive element (5′-TGACGTCA-3′) in 4 of 8 bp. Hence, it is likely that CREB can bind also to the PAI-1 HRE and E-boxes. Therefore, we analysed by EMSAs whether endogenous CREB present in nuclear extracts can bind to HRE2, E4 and E5. We found that oligonucleotides spanning HRE2, E4 and E5 were able to bind three DNA-protein complexes in line with previous studies (Fig. 1). When antibodies recognizing ATF-1 as well as CREB proteins were included in the binding reactions, one supershift was detected. The addition of a specific non-cross reactive ATF-1 antibody to the binding reactions did not interfere with the formation or mobility of the complexes. By contrast, addition of a specific antibody against CREB-1 supershifted one of the three DNA-protein complexes, confirming that this complex contains CREB-1 (Fig. 1). Further, mutation of HRE2, E4 and E5 eliminated binding of nuclear proteins, showing that the observed binding pattern is specific.

**Fasting enhanced PAI-1 expression in rat liver**
Since CREB appears to bind to the PAI-1 promoter, we aimed to examine whether a stimulus of the cAMP/PKA signalling cascade induces PAI-1 gene expression. One major stimulus for the cAMP/PKA/CREB signalling pathway is fasting, a process directed by the pancreatic hormone glucagon. Therefore, we investigated whether fasting has an impact on PAI-1 gene expression in the liver which is the major target organ for glucagon. We found that starvation increased the PAI-1 mRNA levels as well as the mRNA of the major rate-generating gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PCK) (Fig. 2).

**Hypoxia and glucagon /cAMP additively induced PAI-1 mRNA and protein levels**
Our data show that HRE2, E4 and E5 can be bound by CREB. In addition, these elements can bind HIF-1 (7, 9) which implicates that HIF-1 and the glucagon/cAMP/CREB cascade may cross-talk on the level of PAI-1 expression. To address that issue, we analysed endogenous PAI-1 mRNA and protein levels in primary rat hepatocytes, which were treated with glucagon or cAMP
under normoxia and hypoxia, respectively. Glucagon and cAMP were found to increase PAI-1 mRNA expression in a time- and concentration-dependent manner (data not shown). Treatment of the cells with 5 nM glucagon or 500 µM cAMP for 4 h induced PAI-1 mRNA by about five-fold under normoxia and by about seven-fold under hypoxia (Fig. 3). The increase in PAI-1 mRNA was followed by an increase in PAI-1 protein levels. Glucagon and cAMP increased PAI-1 protein levels by about five-fold under normoxia and by about 7.5-fold under hypoxia (Fig. 3).

**cAMP and CREB are critical mediators of the glucagon effect**

In order to examine the role of the cAMP pathway in the glucagon-dependent induction of PAI-1, hepatocytes were pretreated with pharmacological inhibitors of this pathway such as H7 and Rp-cAMPS before stimulated with glucagon and analysed for PAI-1 mRNA and protein levels. The glucagon-induced PAI-1 expression was attenuated by the pretreatment with H7 and Rp-cAMPS under both normoxia and hypoxia so that the control values were restored (Fig. 4A, B). This indicates that the glucagon-dependent PAI-1 gene expression is mediated via the cAMP signalling pathway. Further, glucagon and cAMP activate PKA which in turn phosphorylates CREB at serine 133. So we examined whether cAMP and glucagon can increase CREB phosphorylation. Therefore, cells were treated with cAMP and glucagon for 40 minutes under normoxia and hypoxia, and cellular proteins were subjected to Western blot analyses with an anti-phospho-S133-CREB antibody and total CREB antibody. We found that hypoxia, cAMP and glucagon (Fig. 4C) stimulated CREB phosphorylation at serine 133, albeit to a different degree. Although the effect of hypoxia on CREB phosphorylation is less pronounced, it may have an impact on the expression of HIF-1α which in turn can regulate PAI-1 expression. Therefore, we investigated whether cAMP and glucagon have an impact on HIF-1α expression. Neither cAMP nor glucagon had an effect on HIF-1α mRNA (Fig. 4D) and protein levels (Fig. 4C).

**The hypoxia-responsive elements are the major functional sites mediating the cAMP effect on PAI-1 gene expression**

To study whether HRE2, E4 and E5 within the PAI-1 promoter are also cAMP-responsive, transient transfection assays with human or rat PAI-1 promoter luciferase (Luc) reporter gene constructs were performed in HepG2 cells and primary rat hepatocytes. Treatment of cells with cAMP induced the human and the rat PAI-1 promoter by about three- and four-fold, respectively.
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(Fig. 5A, B). The cAMP-dependent induction of the human PAI-1 promoter was unaffected when we used pGL3hPAI-M4, while with pGL3hPAI-M5 construct the induction was reduced. By contrast, the cAMP effect was completely abolished when the HRE2 was mutated (Fig. 5A). Although the HRE is completely conserved between the human and the rat promoter, E4 and E5 are not present in the rat promoter which may indicate some more specialized functions of these elements in the human gene. In consensus with the conservation of the HRE, mutation of this element in the human and the rat promoter Luc construct (pGL3PAI-766HRE2m) abolished both the induction by hypoxia and the cAMP-dependent induction of Luc activity under normoxia and under hypoxia (Fig. 5C). These results demonstrated that activation of the human and rat PAI-1 promoters by cAMP and subsequently by CREB are mediated mainly via the conserved HRE (Fig. 5).

Discussion

In this study, we investigated the regulation of PAI-1 expression by glucagon and the cAMP/PKA/CREB signalling pathway. Our results demonstrate several new findings with respect to PAI-1 regulation. First, it was found that three promoter elements, the HRE-2, E-box 4 and E-box 5, were able to bind CREB, but only HRE2 and E5 appeared to be functionally active. Second, stimulation of the AMP/PKA/CREB signalling cascade by starvation in vivo or glucagon in vitro-induced PAI-1 gene expression, and this response was associated with enhanced phosphorylation of CREB and further enhanced by hypoxia. Third, although the HRE2 was involved, the glucagon/cAMP pathway had no influence on HIF-1α mRNA and protein levels.

Transcriptional and posttranscriptional regulation of PAI-1 expression

The present study showed that starvation and glucagon induce PAI-1 levels in liver. This is in line with an earlier study showing that treatment of rat hepatocytes with cAMP increases PAI-1 mRNA (23). In addition, increases of PAI-1 have also been observed in the livers of the rats upon injection of cAMP alone or in combination with dexamethasone (23). These findings are opposed by another study showing that dibutyryl-cAMP caused a decrease in PAI-1 mRNA in primary hepatocytes (23). In addition, HTC rat hepatoma cells responded with a decrease in PAI-1 levels upon stimulation with cAMP (25). Thus, the effect of cAMP or agonists that increase intracellular cAMP levels on PAI-1 expression appears to depend on the cell line or cell type. The different effects of cAMP on PAI-1 mRNA levels in the various cell types may in part be explained by a different 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 (26). It was then found by ultraviolet cross-linking analyses that three cytosolic proteins of about 38–76 kDa could bind to that region (27). Although one of these mRNA-binding proteins was cloned (28) the exact identity of the other PAI-1 mRNA-binding proteins remains unknown. However, our study clearly demonstrating a transcriptional effect of cAMP on PAI-1 gene expression does not rule out possible post-transcriptional regulations which could take place at the same time. Previous studies also indicated the presence of several elements within the PAI-1 promoter necessary for cAMP- or glucocorticoid-dependent regulation (29, 30). Interestingly, our analyses could not detect a cAMP-responsive element (CRE) completely matching the consensus CRE sequence. Instead, we found that E5, E4 and HRE2 were able to bind CREB. Further, sequence analyses revealed that the core sequence, which appears to be critical for CREB binding, and those resembling the core HRE are entirely

**Figure 5: Induction of human and rat PAI-1 promoter constructs by cAMP in HepG2 cells and primary rat hepatocytes.**

A, B, E) Cells were transfected with Luc gene constructs driven by the wild-type human PAI-1 promoter (pGL3-hPAI-806) or human promoter mutated at HRE2, E-box 4 and E-box 5. C, D) Cells were transfected with the wild type rat PAI-1 promoter (pGL3PAI-766) or the rat promoter mutated at HRE2 (pGL3PAI-766HRE2m). After transfection, cells were cultured with serum free culture medium for additional 16 h, then they were stimulated with cAMP and further cultured for the next 24 h. The luciferase activity was expressed as fold induction compared to the Luc activity, measured in the respective controls. Values represent means ± SEM of three independent experiments, each performed in duplicate. Statistics, Student t-test for paired values: *significant difference control versus cAMP; **significant difference 16% O\textsubscript{2} versus 8% O\textsubscript{2}; ***significant difference 8% O\textsubscript{2} versus 8% O\textsubscript{2} + cAMP.
conserved in the elements. In line with this, mutation of the HRE2 completely abolished the regulation by cAMP, whereas mutations of E5 and E4 were less effective. This suggests that these boxes might have an independent function in the regulation of PAI-1 expression by cAMP. Interestingly, E5 and E4 appeared to be involved also in the cell-type specific regulation of PAI-1 gene expression by USF2 (31). In addition to that, E5 and E4 were shown to be necessary elements for the PAI-1 regulation in response to TGF-β (32–35). This would indicate that TGF-β signalling and cAMP signalling might undergo a crosstalk at the level of these binding sites. However, it remains open whether the TGF-β and the cAMP response are antagonistic or agonistic, respectively.

**HREs as cAMP-responsive elements**

The results of this study using both human and rat PAI-1 promoter constructs suggest that the induction of PAI-1 expression by glucagon is regulated on the transcriptional level. This is further underlined by the fact that mutation of HRE2, which is conserved in both promoters, abolished the induction by cAMP (Fig. 5). The role of HRE2 in the cAMP response was at first glance unexpected. However, a previous study showed that transcription factors from the ATF/CREB family appear to be constitutively bound to several HREs. In addition, when used in an artificial system, i.e. cloned as enhancer element in front of the SV40 promoter, the HRE from hEpo gene was responsive to cAMP (36, 37). Thus, those findings and the findings of our study show that the binding of CREB to HREs does not only function as a "space holder" but also has an important function. Interestingly, these transcription factors appear not to compete against each other, since the glucagon/cAMP-dependent PAI-1 induction was additively enhanced under hypoxic conditions. Furthermore, hypoxia alone appears to enhance the phosphorylation of CREB (Fig. 4C), a phenomenon which was described to be present also in PC-12 cells (38). Thus, HREs might represent a new type of cAMP-responsive elements. The importance of these elements may consist in their ability to regulate both cAMP- and hypoxia-dependent induction. Further, we and others showed that insulin induces the level of HIF-1α and thus the activity of HIF-1 (21, 22, 39). The close cooperation between CREB and hypoxia, as seen in the present study where glucagon and hypoxia were additive with respect to PAI-1 gene regulation, and as seen in a previous study (22) where insulin and hypoxia were additive, might explain the phenomenon that insulin and glucagon are no longer antagonists with respect to PAI-1 gene expression. Thus, we propose a model in which glucagon/cAMP leads to CREB phosphorylation which in turn induces PAI-1 expression. The effect on PAI-1 gene expression can be additively enhanced under hypoxia and under conditions which stabilize HIF-1α like high insulin. Both factors may then be complexed by their interaction with CREB binding protein (CBP). This model may not only be valid for PAI-1 but also for VEGF which can be induced by hypoxia (40), insulin (39, 41) and cAMP (42). Thus, under certain circumstances the classical glucagon/cAMP-insulin antagonism may not be functional.

**Physiological and pathophysiological role of the cAMP-dependent PAI-1 expression**

Interestingly, the cAMP concentration is not only increased upon starvation but also during liver regeneration to stimulate DNA synthesis and the cell cycle (43, 44). In addition to its role in matrix remodelling and fibrinolysis, PAI-1 has been found to be an early response gene in the liver (45). Further, 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 (1) and TGF-β (46). Thus, the induction of PAI-1 by cAMP may represent 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. 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 (47). Thus, the decreased matrix degradation due to PAI-1-induced inhibition of matrix metalloproteinases may contribute to these vessel abnormalities during diabetes.

In summary we have shown that the glucagon/cAMP/PKA/CREB signalling pathway can induce PAI-1 expression in liver and primary hepatocytes. Thereby, binding of CREB to HRE2 and E5 within the PAI-1 promoter appears to be of special importance. This type of PAI-1 regulation may represent an important feedback cycle during liver regeneration as well as it can be a target to prevent diabetic angiopathy.

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**References**

8. Kietzmann T, Roth U, Jangermann K. Induction of...
the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia inducible factor-1 in rat hepatocytes. Blood 1999; 94: 4177–4185.


