ASPECTS OF T-PA AND PAI-1 GENE REGULATION IN HUMAN ENDOTHELIAL CELLS AND HEPATOCYTES

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STELLINGEN

1. Competitie met een consensus DNA-bindingselement in een bandshift assay is niet voldoende om de identiteit van een gebonden transcriptiefactor te bepalen.
Medcalf et al., J. Biol. Chem., 265: 14628, 1990. Dit proefschrift.

2. Gen-regulatie experimenten waarin een transcriptiefactor tot overexpressie gebracht wordt, leiden niet altijd tot fysiologisch relevante conclusies.

3. Gemfibrozil remt niet de EGF-geïnduceerde PAI-1 synthese in Hep G2 cellen.
Fujii and Sobel, Circulation, 85: 1888, 1992.

4. Het vinden van een correlatie tussen PAI-1 activiteit en triglyceride niveaus in een populatie met het 4G/4G PAI-1 promoter polymorfisme is geen bewijs voor een genotype-afhankelijk effect van triglyceriden op PAI-1
Panahloo et al., Diabetes, 44: 37, 1995.

5. Aangezien endotheelcellen geïsoleerd uit navelstrengvenen van zwarte amerikanen significant meer t-PA maken dan die van blanke amerikanen *(Frist et al., Thrombosis Res., 77: 279, 1995)*, kan bij zwarte amerikanen een hogere acute afgifte van t-PA verwacht worden.
Van den Eijnden-Schrauwen et al., Blood, 12: 3510, 1995.

6. De neiging van de politiek om controversiële wetgeving verder vast te laten stellen door de rechterlijke macht is ongrondwettelijk (Art. 16 Grondwet; Art. 11 Wet Algemene Bepalingen der wetgeving van het Koninkrijk).

7. Er wordt vaak onvoldoende rekening gehouden met het feit dat oestradiol receptoren ook gevoelig zijn voor andere stoffen dan 17ß-oestradiol.

8. De schaalvergroting van de farmaceutische industrie leidt ertoe dat de aandacht vooral gericht wordt op farmaca waar een grote markt voor is.

9. Als we Yves Rocher mogen geloven dat het extra DNA (deoxy-ribonucleïne zuur) in hun "ligne A.D.N. vegetal" essentieel is voor het celvernieuwingsproces, de zuurstofvoorziening en celactiviteit, dan zijn moleculair biologen zich onvoldoende bewust van de marktwaarde van hun reststoffen.

10. De exponentieel toenemende grofheid van de oudejaarsconferences van Youp van het Hek doet het ergste vrezen voor het jaar 2000.

ASPECTS OF T-PA AND PAI-1 GENE REGULATION

IN HUMAN ENDOTHELIAL CELLS

AND HEPATOCYTES

Proefschrift

ter verkrijging van de graad van Doctor
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door

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Aan mijn ouders

voor André

CONTENTS

CHAPTER 1

General Introduction

1.1 INTRODUCTION

The plasminogen/plasmin system represents a highly regulated enzymatic cascade for extracellular proteolysis. The system plays a key role in a number of biological and pathophysiological processes such as fibrinolysis (Collen, 1980; Bachmann, 1987), cell migration (Clowes et al., 1990; Sumi et al., 1992) and tissue remodelling (Danø et al., 1985). The inactive proenzyme plasminogen is present in high concentrations in blood and other body fluids. The conversion of the inactive proenzyme plasminogen into the active serine proteinase plasmin is catalyzed by plasminogen activators. A simplified scheme of the plasminogen/plasmin system is shown in Figure 1. The two main physiological plasminogen activators are tissue-type and urokinase-type plasminogen activator (t-PA and u-PA, respectively). Consistent with the important role of the plasminogen/plasmin system in so many processes, t-PA and u-PA activity are strictly regulated by, amongst others, their synthesis and the presence of specific plasminogen activator inhibitors (PAIs), of which PAI-1 is considered most relevant under physiological conditions (Sprengers and Kluft, 1987). A second inhibitor, PAI-2, has been demonstrated in the plasma of pregnant women and of patients suffering from leukemia or liver diseases (Kruithof et al., 1988; Belin, 1993). The subject of this thesis is the regulation of t-PA and PAI-1 synthesis by human endothelial cells and hepatocytes. These cell types are considered to be major contribuants of plasma t-PA and PAI-1. Relevant aspects of intravascular fibrinolysis and regulation of t-PA and PAI-1 expression will be introduced in the next paragraphs.

1.2 INTRAVASCULAR FIBRINOLYSIS

Intravascular fibrinolysis, i.e. the dissolution of the fibrin component of thrombi, is mediated by the intravascular plasminogen/plasmin system (Collen and Lijnen, 1991). Whereas inadequate dissolution of fibrin may result in the obstruction of a blood vessel, excessive premature fibrin degradation can lead to bleeding (Lijnen and Collen, 1989). Clearly, fibrin degradation needs to be finely regulated. The primary initiator of intravascular fibrin degradation, as concluded from *in vitro* experiments, is t-PA (Wun and Capuano, 1985, 1987), and the physiologically relevant inhibitor of t-PA activity in plasma is PAI-1 (Sprengers and Kluft, 1987). Consistent with an important role of PAI-1 in vascular fibrinolysis, genetic deficiencies of PAI-1 have been associated with bleeding

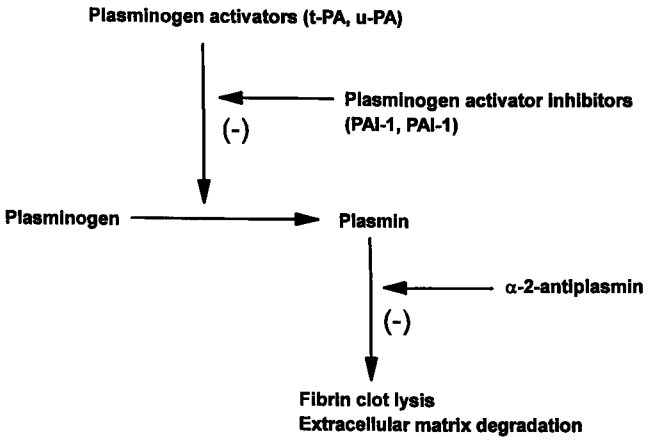

Figure 1. The plasminogen activation system. Schematic representation of the major steps of the cascade that generates fibrinolytic activity through plasminogen activation.

disorders (Schleef et al., 1989; Dieval et al., 1991; Fay et al., 1992), while high plasma PAI-1 levels are correlated with deep venous thrombosis (Nillson et al., 1985; Juhan-Vague et al., 1987; Nguyen et al., 1988), a high mortality in patients with septic shock (Juhan-Vague et al., 1985; Pralong et al., 1989), and an increased risk of recurrent myocardial infarction (Hamsten et al., 1987). Mice overexpressing PAI-1 develop venous occlusions (Erickson et al., 1990), while mice deficient in PAI-1 are in a mild hyperfibrinolytic state and have a greater resistance to endotoxin-induced thrombosis (Carmeliet et al., 1993). To date, no genetic deficiencies for t-PA in humans have been reported. Mice deficient for the t-PA gene appeared healthy and showed no spontaneous fibrin-depositions under control conditions, but a strongly elevated level of venous thrombosis occurred after challenge with endotoxin (Carmeliet et al., 1994). Endothelial cells overexpressing t-PA after retroviral transduction displayed enhanced local antithrombotic activity on vascular grafts in baboons (Dichek et al., 1996), indicating that increased levels of t-PA counteract the formation of a thrombus *in vivo*.

Given the importance of t-PA and PAI-1 expression in maintaining the intravascular fibrinolytic balance, the regulation of their synthesis is a critical feature which has been subject of many studies. t-PA in plasma is assumed to originate mainly, if not exclusively, from the endothelial cells which line the vessel wall (Kooistra et al., 1994). Endothelial cells *in vivo* contain large amounts of t-PA, and also t-PA mRNA was

localized in vascular endothelial cells using *in situ* hybridization technology (Levin and Del Zoppo, 1994). The origin of plasma PAI-1 is not known with certainty, but cell types likely to be involved in its synthesis are endothelial cells and hepatocytes (Loskutoff, 1991; Chomiki et al., 1994; Thornton and Gelehrter, 1995).

Cultured human endothelial cells and hepatocytes have been used to identify factors and signaling pathways to modulate t-PA and PAI-1 synthesis (for reviews see: Loskutoff, 1991; Kooistra et al., 1994). Some of the compounds found to stimulate t-PA or PAI-1 synthesis *in vitro*, for example retinoids and cytokines, were also shown to increase plasma levels of t-PA or PAI-1 *in vivo* in experimental animals (Kooistra et al., 1991; Sawdey and Loskutoff, 1991) and humans (Van Hinsbergh et al., 1990; Dootson et al., 1995), confirming the role played by endothelial cells and hepatocytes in determining plasma fibrinolytic activity.

1.3 REGULATION OF t-PA AND PAI-1 SYNTHESIS

Among the compounds reported to modulate t-PA and/or PAI-1 synthesis in cultured endothelial cells and hepatocytes are phorbol 12-myristate 13-acetate (Levin and Santell, 1988; Kooistra et al., 1991; Bosma and Kooistra, 1991), sodium butyrate (Kooistra et al., 1987), interleukin-1 (Emeis and Kooistra, 1986; Healy and Gelehrter, 1994), genistein (Van Hinsbergh et al., 1994), and fibrates (Fujii and Sobel, 1992). The work presented in this thesis was directed at gaining more insight into the regulatory mechanisms by which these changes in t-PA and PAI-1 expression are brought about. In the next paragraphs previous studies concerning the effects of these compounds on t-PA and PAI-1 synthesis are summarized.

1.3.1 Modulation of t-PA expression

Phorbol 12-myristate 13-acetate (PMA)

Many vasoactive compounds such as α-thrombin and histamine induce t-PA synthesis in cultured human endothelial cells through a mechanism that probably involves protein kinase C: inhibition of protein kinase C prevents the effect of α-thrombin and histamine on t-PA synthesis, while direct activation of protein kinase C by potent, stabile activators like the phorbol ester 4ß-phorbol 12-myristate 13-acetate (PMA) or by the physiological

activator diacylglycerol results in a strong increase in t-PA expression (Levin and Santell, 1988; Levin et al., 1989; Grulich-Henn and Müller-Berghaus, 1990; Kooistra et al., 1991). The induction of t-PA synthesis in PMA-treated human endothelial cells can, at least partially, be explained by an increase in t-PA transcription (Santell et al., 1992).

A very strong induction of t-PA transcription by PMA was found in HeLa cells (Medcalf et al., 1990). Two elements in the t-PA promoter (at -115 to -102 and +60 to +74) were shown to be essential for this induction. Both sites in the human t-PA promoter showed specific nuclear protein binding, as shown by bandshift assays with HeLa nuclear extracts. On the basis of sequence comparison and competition experiments, Medcalf et al. (1990) suggested these nuclear proteins to be "cAMP responsive element binding (CREB)" protein and activator protein-2 (AP-2). However, final proof for this suggestion (e.g. by performing supershift experiments) was not provided. In fact, binding of CREB and AP-2 to the t-PA promoter does not seem very likely, because these factors are activated by protein kinase A (PKA) (Imagawa et al., 1987; Lalli and Sassone-Corsi, 1994), while PKA activation does not induce t-PA expression (Kooistra et al., 1991). Furthermore, Ohlsson and Ny (1992) showed that the site at -115 to -102 has hardly any affinity for CREB. Whether or not the two promoter elements identified in HeLa cells also regulate t-PA transcription in human endothelial cells is not clear, mainly since transient transfection studies with t-PA promoter constructs in human endothelial cells proved to be unsuccessfull (Hanemaaijer et al., 1994). Also, the question remains whether or not additional elements are involved in t-PA transcriptional regulation, since in comparable studies with the very homologous murine t-PA promoter three additional regulatory sites were identified (Rickles., 1989; Darrow et al., 1990; Pecorino et al., 1991; Ohlsson et al., 1993).

Sodium butyrate

Short-chain fatty acids, in particular butyrate, are among the strongest stimulators of t-PA synthesis in cultured human endothelial cells (Kooistra et al., 1987). It is likely that butyrate itself is active rather than a metabolic product since other even-chain fatty acids, which are metabolized through the same pathway as butyrate, are much less effective. Subtle changes in the butyrate structure interfered with the capacity of butyrate to induce t-PA synthesis and optimal induction was shown to depend on a straight-chain C4 monocarboxylate structure with a methyl group at one end and a carboxy moiety at the other end (Kooistra et al., 1987). However, the regulatory mechanism by which butyrate

induces t-PA synthesis remained unknown.

Sodium butyrate is a pleiotropic agent, and several mechanisms have been proposed to explain its actions. First, one of the most evident and specific effects frequently reported to accompany sodium butyrate-stimulated gene expression is brought about by inhibition of the enzyme histone deacetylase (Riggs et al., 1977). The resulting increase in acetyl groups neutralizes positive charges in the histone molecules, which results in diminished interaction of the histones with the DNA. Consequently, there is increased accessibility of transcriptional factors to their DNA recognition motifs, leading to transcriptional activation (Workman and Roeder, 1987; Lee et al., 1993). A second effect of butyrate which may be relevant for the increase in t-PA synthesis, is an induction of the transcription factor c-*jun*. Sodium butyrate has been shown to increase c-*jun* mRNA levels in a variety of cell types (Chen and Allfrey, 1987; Tichonicky et al., 1990; Mollinedo et al., 1993; Nishina et al., 1993), and c-*jun* has frequently been suggested to be involved in t-PA transcription: an increase in c-*jun* mRNA levels precedes t-PA mRNA induction by butyrate and PMA in F9 cells and human endothelial cells, respectively (Nishina et al., 1993; Kooistra et al., 1991). Moreover, overexpression of c-*jun* in F9 cells has been associated with t-PA induction (Yang-Yen et al., 1990). Thirdly, induction of gene expression by butyrate often depends on the presence of glucose in the culture medium (Cox et al., 1987; Takano et al., 1988). Because glucose is not essential for butyrate-induced histone hyperacetylation (Cox et al., 1987), glucose apparently plays a role in a regulatory process independent of histone acetylation, but essential for the butyrate induction of gene expression. Which of these observations is relevant for the sodium butyrate-induced t-PA gene expression in cultured human endothelial cells requires further investigations.

1.3.2 Modulation of PAI-1 expression

Phorbol 12-myristate 13-acetate (PMA) and interleukin-1α (IL-1α)

A variety of agents known to stimulate the expression of the transcription factor activator protein-1 (AP-1) in HepG2 cells, including PMA, serum, IL-1α and transforming growth factor-ß (TGF-ß) (Angel et al., 1987; Pertovaara et al., 1989; Muegge et al., 1993; Chang and Goldberg, 1995), also strongly increase PAI-1 gene expression in these cells (Bosma et al., 1991; De Boer et al., 1991; Westerhausen et al., 1991; Healy and Gelehrter, 1994). AP-1 consists of a collection of structurally related transcription factors,

which belong to the Jun and Fos families. Jun proteins bind DNA as either Jun-Jun homodimers or Jun-Fos heterodimers, whereas the Fos proteins must heterodimerize with one of the Jun proteins, because they cannot form stable Fos-Fos homodimers (Angel et al., 1987; Angel and Karin, 1991). All AP-1 molecules recognize a specific DNA sequence, referred to as PMA-responsive element (TRE) (Angel et al., 1987). The 5'-flanking region of the human PAI-1 gene contains four putative AP-1-like binding sites which closely resemble the consensus TRE sequence, TGAg/cTCA (Bosma et al., 1988): two proximal sites, at -58 to -50 (TGAGTTCA) and at -79 to -72 (TGAGTGA), and two distal sites, at -721 to -714 (TGACACA) and at -662 to -656 (TGTATCA). Further experiments revealed that several of these TREs can play a role in the regulation of PAI-1 gene transcription (Descheemaeker et al., 1992; Westerhausen et al., 1991; Knudsen et al., 1994). However, depending on stimulus (and cell type) used, the different TREs were found to participate in the regulation of PAI-1 gene transcription to varying extents. For example, induction of PAI-1 by TGF-ß has been shown to involve the two distal TREs (Westerhausen et al., 1991), while the phorbol ester PMA induces PAI-1 transcription through the proximal TREs only (Descheemaeker, 1992; Knudsen et al., 1994). Furthermore, the involvement of a TRE in PAI-1 regulation does not necessarily imply a role for AP-1. Studies by Knudsen et al. (1994) demonstrated that the proximal TRE at -58 to -50 was required for PAI-1 induction by PMA in MCF-7 cells, but that this site was occupied by an as yet unidentified nuclear protein, despite the presence of AP-1 activity. Also, in human endothelial cells, PMA effectively induced c-*jun* and c-*fos* mRNA levels (Kooistra et al., 1991), but hardly stimulated PAI-1 expression (Dichek and Quertermous, 1989; Grulich-Henn and Müller-Berghaus, 1990; Scarpati and Sadler, 1989; Konkle et al., 1990; Kooistra et al., 1991). Therefore, it is critical to further understand how a productive interaction between AP-1 and the various PAI-1 TREs is controlled and fine-tuned.

One of the factors important for the activity and specificity of AP-1 as a transcriptional inducer is its composition. Combinatorial interactions between the Jun and Fos protein family members give rise to dimers with different activities with respect to DNA-binding activity and specificity, and transactivation capacity (Halazonetis et al., 1988; Nakabeppu et al., 1988; Chiu and Karin, 1989). Which forms of AP-1 are induced in HepG2 cells by the various stimuli and to which TRE(s) present in the PAI-1 promoter these (different) forms bind, is still largely unknown.

Genistein

Protein phosphorylation plays a key role in the transmission of extracellular signals to their intracellular targets and in the consequent regulation of transcription-factor activity (Hunter and Karin, 1992; Jackson, 1992). Several compounds known to stimulate PAI-1 expression, such as PMA and IL-1α, have been shown to increase protein tyrosine kinase activity and thereby protein tyrosine phosphorylation (Ternisien et al., 1995; O'Neill, 1995; Larner and Finbloom, 1995).

Two structurally unrelated inhibitors of protein tyrosine kinase activity, herbimycin A and genistein, interfered with basal and TNFα/IL-1α induced PAI-1 expression in cultured human endothelial cells (van Hinsbergh et al., 1994). The genistein analogue daidzein was ineffective, ruling out unspecific effects. It was concluded therefore, that basal as well as TNFα/IL-1α induced PAI-1 expression in endothelial cells is dependent on protein tyrosine kinase activation. However, the precise point of action at which herbimycin A and genistein interfered with the PAI-1 regulatory pathway remained unknown.

Fibrates

Fibrates are lipid-lowering drugs used in the treatment of diet-resistant hyperlipoproteinemia (Andersen et al. 1990; Avellone et al., 1992). Fibrates effectively lower plasma levels of triglycerides and low-density lipoprotein cholesterol, and enhance high-density lipoprotein cholesterol concentrations (Schonfeld, 1994). In addition to these favourable changes in lipid cardiovascular risk factors, fibrates have been reported to lower plasma PAI-1 levels, which could also contribute to their favourable effects on the prevention of coronary artery disease (Schonfeld, 1994). However, whereas the lipid-lowering effects of the various fibrates are comparable in different studies, variable results have been reported in lowering plasma levels of PAI-1 (Almér et al., 1986; Pazzuconi et al., 1992; Keber et al., 1994; Bröijersen et al., 1996). For example, gemfibrozil has been shown to lower PAI-1 levels in patients suffering from type IV hyperlipoproteinemia, whereas no change in PAI-1 levels by bezafibrate was observed (Almér et al., 1986; Pazzuconi et al., 1992; Keber et al., 1994). These studies also suggest that fibrates exert their PAI-1-lowering action independent of their lipid-lowering effect. In line with this, Fujii and Sobel (1992) showed that gemfibrozil decreased PAI-1 synthesis and PAI-1 mRNA levels in HepG2 cells cultured under standard culture conditions.

Fibrates have been reported to interfere with several signal transduction pathways which, independently, have also been shown to play a role in PAI-1 synthesis. First, fibrates have been reported to activate protein kinase C (Watanabe et al., 1992; Bieri, 1993) and to interfere with epidermal growth factor (EGF) receptor activity (Orellana et al., 1993). This is of interest since both protein kinase C activators and growth factors (like EGF and TGF-ß) are known to induce PAI-1 synthesis in human hepatocytes (Lucore et al., 1988; Bosma et al., 1991; Westerhausen et al., 1991). Secondly, fibrates have been demonstrated to activate the nuclear hormone receptor, peroxisome proliferator-activated receptor (PPAR) (Auwerx, 1992). One way in which PPAR could interfere with the regulation of PAI-1 expression is through squelching of transcription factors. For example, PPAR has been reported to downregulate gene expression of the rat glutathione transferase P gene through squelching of c-Jun (Sakai et al., 1995). The question whether or not such actions of fibrates are relevant for the downregulation of PAI-1 expression in hepatocytes has not been addressed yet.

1.4 OUTLINE AND AIMS OF THE STUDY

The work presented in this thesis was directed at gaining more insight into the regulatory mechanisms involved in the regulation of t-PA and PAI-1 synthesis in human endothelial cells and hepatocytes by a number of compounds, viz. PMA, IL-1α, sodium butyrate, genistein and fibrates. Because many of the effects on t-PA and PAI-1 expression are regulated at the level of gene transcription, an especial effort has been made to identify nuclear proteins and DNA regions involved in these regulatory processes.

In Chapter 2, the -135 to +100 region of the human t-PA promoter was analyzed for persistent and PMA-inducible DNA-protein interactions in cultured human vascular endothelial cells and HeLa cells, using a dimethyl sulphate *in vivo* footprinting approach and gel mobility shift assays. Previous studies in HeLa cells, using transient transfection experiments, showed this region of the t-PA promoter to be critical for basal and PMA-stimulated t-PA promoter activity.

In Chapters 3 and 4, the regulatory mechanism involved in the sodium butyrate-stimulated t-PA synthesis in cultured human endothelial cells was investigated. The effect of butyrate was compared to that of a specific histone deacetylase inhibitor, trichostatin A, and the role of histone H4 acetylation in t-PA gene transcription was evaluated. Furthermore, the possible involvement of the transcription factor c-Jun in the

effect of sodium butyrate on t-PA expression was studied. Finally, the role of glucose in t-PA induction by sodium butyrate was investigated by incubating human endothelial cells with butyrate in glucose-deprived medium and in the presence of the glucose-analogue, 2-deoxy-D-glucose.

Regulation of PAI-1 gene transcription by PMA, serum and IL-1α in hepatocytes is the main subject of Chapters 5 and 6. In Chapter 5, the involvement of c-Jun and/or c-Fos in basal and PMA-stimulated PAI-1 gene transcription in HepG2 cells was investigated by incubating HepG2 cells with antisense c-*jun* or c-*fos* oligodeoxynucleotides and by performing gel-shift experiments. In Chapter 6, the regulatory pathways transducting the inducing effects of PMA, serum and IL-1α on PAI-1 gene expression in HepG2 cells were compared by studying the expression of a stably transfected reporter gene under control of the -489 to +75 PAI-1 promoter region. These agents were also evaluated with respect to their capacity to induce AP-1 binding activity using bandshift analysis and inasmuch their PAI-1 stimulatory action could be inhibited by the tyrosine kinase inhibitor, genistein.

The studies described in Chapter 7 were directed at elucidating the regulatory mechanism by which fibrates suppress PAI-1 synthesis in hepatocytes. These investigations were performed with primary hepatocyte cultures from cynomolgus monkey (*Macaca fascicularis*). The potency of four different fibrates to decrease PAI-1 synthesis was determined and possible roles of protein kinase C, growth factor-receptor activity and the nuclear hormone receptors PPARα/RXRα in the fibrate-inhibited PAI-1 expression were evaluated.

In the final chapter (Chapter 8) the general conclusions that can be drawn from this work are discussed.

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CHAPTER 2

Cell-type specific DNA-protein interactions at the tissue-type plasminogen activator promoter in human endothelial and HeLa cells *in vivo* and *in vitro*

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Submitted

ABSTRACT

Tissue-type plasminogen activator (t-PA) gene expression in human endothelial cells and HeLa cells is stimulated by the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) at the level of transcription. To study the mechanism of transcriptional regulation, we have characterized a segment of the t-PA gene extending from -135 to +100 by *in vivo* footprinting analysis and gel mobility shift assay. *In vivo* footprinting analysis revealed changes in cleavage pattern in five distinct promoter elements in both endothelial cells and HeLa cells, including a PMA-responsive element (TRE), a CTF-NF-1 binding site and three GC-boxes, and an altered cleavage pattern of the TRE and CTF/NF-1 element after PMA treatment of HeLa cells. Although human endothelial cells and HeLa cells differed in the exact G residues protected by nuclear proteins, *in vitro* bandshift analysis showed that nuclear protein binding to the t-PA promoter was qualitatively and quantitatively very similar in both cell types, except for the TRE. Protein binding to the TRE under non-stimulated conditions was much higher in human endothelial cells than in HeLa cells and this difference was, at least partially, due to a higher binding affinity in human endothelial cells. The proteins bound were identified as SP-1 (GC-box II and III), CTF/NF-1 and the Jun/Fos heterodimeric form of AP-1 (TRE). *In vitro*, AP-1 and SP-1 binding with human endothelial cell nuclear extracts was increased two-fold after PMA treatment, while with HeLa nuclear extracts PMA strongly (over 20-fold) induced the binding of both AP-1 and SP-1 to the t-PA promoter. In the light of previous studies involving mutational analysis of the human and murine t-PA promoter our results underline an important role of the five identified promoter regions in basal and PMA-stimulated t-PA gene expression in intact human endothelial cells and HeLa cells.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) plays a key role in the dissolution of the fibrin matrix of thrombi and haemostatic plugs (1). t-PA catalyzes the conversion of the zymogen plasminogen into the active serine proteinase plasmin, the enzyme that digests fibrin. Gene targeting and gene transfer studies have confirmed the significant role of t-PA-mediated plasminogen activation in maintaining vascular patency (2). Regulation of t-PA expression, both *in vitro* and *in vivo*, has therefore been the focus of many studies (3).

t-PA in the circulation originates predominantly from the vascular endothelium (3). *In vitro* studies using cultured human endothelial cells have demonstrated that activation of protein kinase C (PKC) with vasoactive compounds such as thrombin or histamine, or with the phorbol ester 4ß-phorbol 12-myristate 13-acetate (PMA) stimulates t-PA expression (4-7). Based on nuclear run-on transcription assays, t-PA expression is modulated by PMA at the level of transcription (8). A very strong induction of t-PA gene transcription with PMA was found in HeLa cells (9). Transient transfection experiments in HeLa cells using deletion mutants of the t-PA gene promoter fused to the chloramphenicol acetyltransferase (CAT) reporter gene revealed that two regions in the t-PA promoter (between positions -102 to -115 and +60 to +74) are critical for basal and PMA-stimulated t-PA promoter activity (9). To pursue the physiological significance of these studies, we performed *in vivo* footprinting analysis in control and PMA-treated human endothelial cells and HeLa cells to reveal the pattern of protein-DNA interactions in the intact cell, where the nucleic acid is complexed with chromosomal proteins to form chromatin. In addition, such studies may reveal cell type-specific differences between primary human endothelial cells and the established human cervical carcinoma cell line, HeLa. Gel mobility shift assays were performed to identify the nuclear proteins which interact with the various binding sites in the promoter region of t-PA as revealed by *in vivo* footprinting.

MATERIALS AND METHODS

Materials

Dimethyl sulphate (DMS) and piperidine were obtained from Fluka (Bornem, The Netherlands). T_4 kinase and DNA ligase were obtained from Promega (Madison, WI). dNTPs (ultra pure), Taq polymerase and rATP were obtained from Pharmacia Biotech (Woerden, The Netherlands). 4ß-phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma (St. Louis, MO). A stock solution of PMA (100 μM) was prepared in ethanol and kept at -20°C. The anti-Jun and anti-Fos rabbit polyclonal antibodies were a gift from Dr. T. Oehler (Massachusetts Institute of Technology, Cambridge, MA) and Dr. H.J. Rahmsdorf (Kernforschungszentrum, Karlsruhe, Germany), respectively. These antibodies preferentially recognize c-Jun and c-Fos, but also other members of the Jun and Fos protein family. Anti-CTF/NF-1 rabbit polyclonal antibody (10) was a gift from Dr. W. van Driel (Laboratory for Physiological Chemistry, Utrecht University, The Netherlands). Anti-SP-1 and anti-AP-2 rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 5[γ-^{32}P]triphosphate (3 Ci/μmol), deoxycytidine 5[α-^{32}P]triphosphate (3 Ci/mol), uridine 5[α-^{32}P]triphosphate (>400 Ci/mol) and Sequenase (version 2) were obtained from Amersham Nederland BV ('s Hertogenbosch, The Netherlands). Bradford protein reagent was obtained from from Bio-Rad (Veenendaal, The Netherlands).

Oligodeoxynucleotides

The primers used for the *in vivo* DMS footprint analysis were synthesized and HPLC-purified by Isogen Bioscience (Amsterdam, The Netherlands), and had the following sequences:

elongation primer 1: 5'-CCCTTTTAAGCCTGGGACATAG-3';
PCR primer 2: 5'-GACTCTAAAGGAAGATGATTCTTAAGGTCCC-3';
elongation primer 3: 5'-GGAAGATGATTCTTAAGGTCCCATCCCACTCC-3';
25-mer linker: 5'-GCGGTGACCCGGGAGATCTGAATTC-3';
and 11-mer linker: 5'-GAATTCAGATC-3'.

The oligodeoxynucleotides used for the bandshift assays were synthesized by Isogen Bioscience (Amsterdam, The Netherlands), and correspond to the following regions of the t-PA promoter:
-120 to -98 (TRE-like): 5'-GATTCAATGACATCACGGCTGTG-3';
-95 to -72 (CTF/NF-1-like): 5'-TAATCAGCCTGGCCCGAAGCCAGGG-3';
-49 to -28 (GC-box I): 5'-TGAACTTCCTCCCCCTGCTTTA-3';
+30 to +52 (GC-box II): 5'-ACACAGAAACCCGCCCAGCCGG-3';
+57 to +78 (GC-box III): 5'-ACCGACCCCACCCCCTGCCTGG-3'.
As an aspecific competitor oligodeoxynucleotide, the following (random) sequence was used: 5'-CTGAGGATTCTCCACTGCA-3'. An AP-2 consensus sequence oligodeoxynucleotide was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and had the following sequence: 5'-GATCGAACTGACCGCCCGCGGCCCGT-3'. The consensus CTF/NF-1 oligodeoxynucleotide used in competition experiments, 5'-CCTTTGGCATGCTGCCAATATG-3', was obtained from Promega (Madison, WI).

Cell culture experiments

Endothelial cells from human umbilical cord veins (HUVEC) were isolated by the method of Jaffe et al. (11), and cultured as previously described (12). HUVEC were grown on fibronectin-coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (20 mM), newborn calf serum (heat-inactivated; 10% v/v), human serum (10% v/v), heparin (5 IU/ml), endothelial cell growth supplement (150 μg/ml) (13), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). HeLa cells were grown in DMEM supplemented with HEPES (20 mM), fetal bovine serum (heat-inactivated; 8% v/v), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). Both cell types were grown at 37°C under a 5% CO_2/ 95% air atmosphere, and the medium was replaced every 2-3 days. Subcultures were obtained by trypsin/ethylenedinitrilo-tetraacetic acid disodiumsalt-dihydrate (EDTA) treatment at a split ratio of 1:3 for HUVEC and of 1:10 for HeLa cells. HUVEC were cultured for maximally three passages.

For experiments, confluent cultures were used and the cells were always re-fed the day before the experiment with incubation medium, i.e. for HUVEC: DMEM supplemented with human serum (10%), L-glutamine, penicillin and streptomycin; and for HeLa cells: DMEM supplemented with L-glutamine, penicillin and streptomycin. After incubation of the cells with incubation medium containing the appropriate concentration of PMA (i.e. 10 nM for HUVEC and 162 nM for HeLa cells) or stock solvent, the cells were used for *in vivo* footprint analysis or the preparation of nuclear extracts.

DMS genomic footprinting

Confluent cultures of HUVEC (486 cm^2) or HeLa cells (162 cm^2) were washed once with phosphate-buffered saline (PBS) (0.15 M NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at room temperature, and then incubated with DMEM supplemented with 10 mM HEPES (pH 7.5) and 0.5% (v/v) DMS for 2 min. The cells were washed with ice-cold PBS and lysed in 10 mM Tris pH 7.7, 400 mM NaCl, 2 mM EDTA, and 0.2% (w/v) SDS. DNA was isolated by digestion with Proteinase K (300 μg/ml, 37°C overnight), followed by phenol/chloroform extraction, and ethanol precipitation (14). The DNA was dissolved in water to a final concentration of 1 μg/μl, and incubated with 10% (v/v) piperidine for 30 min at 90°C. After ethanol precipitation, the samples were processed for ligation-mediated PCR (LMPCR) analysis. *In vitro* controls were obtained by the reaction of purified DNA with DMS as described by Maxam and Gilbert (15).

LMPCR

LMPCR was performed by the method described by Mueller and Wold (16). Elongation primer 1 (0.6 pmol, see Materials section) was annealed to 10 μg of heat-denatured (3 min, 95°C) piperidine-cleaved DNA at 45°C for 30 min. Primer extension was then carried out with Sequenase version 2.0 for 15 min at 45°C by adding 8.8 μl of elongation mixture (20 mM $MgCl_2$, 20 mM DTT, 200 μM of dATP, dCTP, dGTP and dTTP, and 0.3 μl Sequenase version 2). The DNA polymerase was heat-inactivated by incubation at 67°C for 15 min. Ligation of the universal linker (100 pmol of annealed 25-mer and 12-mer oligodeoxynucleotide, see Materials section) to the primer-extended molecules was done overnight at 15°C by adding 20 μl of 17.5 mM $MgCl_2$, 42.3 mM DTT and 125 μg/ml bovine serum albumin (BSA) and 25 μl of ligation mix (10 mM $MgCl_2$, 20 mM DTT, 3 mM ATP, 50 μg/ml BSA and 0.4 U/μl T_4 DNA ligase). After ethanol precipitation, the pellets were dissolved in 60 μl water. PCR amplification was performed in 10 mM Tris (pH 8.8), 40 mM NaCl, 5 mM $MgCl_2$, 10 pmol primer 2, 10 pmol 25-mer linker primer (see Materials section), 10 U Taq polymerase, 0.2 mM of dATP, dCTP, dGTP and dTTP and 0.01% (w/v) gelatin in a total volume of 100 μl on a Perkin Elmer Thermocycler 9600. Twenty cycles of PCR (1 min 95°C, 2 min 64°C and 3 min 75°C) were performed. Subsequently, linear PCR was done with 2 pmol end-labelled elongation primer 3 (see Materials section), 5 U Taq polymerase, 2 μl 2.5 mM dNTP-mix. One PCR cycle (2 min 95°C, 2 min 66°C and 10 min 75°C) was performed. The PCR-amplified fragments were extracted with phenol/chloroform, ethanol precipitated, and then separated on a 6% (w/v) denaturating polyacrylamide-gel (15). The sequence gel was dried on Whatman-3MM paper, and radiolabelled DNA fragments were visualized by autoradiography.

Preparation of nuclear extracts

For gel shift experiments confluent cultures of HUVEC (324 cm^2) or HeLa cells (162 cm^2) were rinsed twice with ice-cold PBS and lysed in 2 ml of lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM DTT, 0.25 mM vanadate and 1 μg/ml of the protease inhibitors leupeptin, pepstatin and aprotinin). The lysates were homogenized in a potter (20 strokes); nuclei were collected by centrifugation (5 min at 1000 g, 4°C), and washed once with lysis buffer. The nuclear pellet was resuspended in 150 μl of 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.25 mM vanadate and 1 μg/ml of leupeptin, pepstatin and aprotinin. Suspensions were incubated for 15 min at 4°C while being continuously shaken, and then centrifuged at 1000 g, 4°C for 5 min. Supernatants were stored at -80°C until use. The protein concentrations in the nuclear extracts were determined using the Bradford protein assay.

Electromobility shift assay

Oligodeoxynucleotides were end-labelled using T_4-kinase and subsequently purified by phenol/chloroform extraction and ethanol precipitation. For the electromobility shift assay (EMSA), 25 fmol (about 10^4 cpm) of labelled double-stranded oligodeoxynucleotide was mixed with nuclear extract (5 μg protein) in a total volume of 20 μl of 20 mM HEPES (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$, 20% glycerol, 2.5 mM EDTA, 2 mM spermidine, 1 μg poly(dI-dC), 1 μg BSA and 1 mM PMSF. The mixture was incubated at 4°C for 30 min. All bandshifts were performed in the presence of a 100-fold excess of unlabelled nonhomologous DNA in order to prevent aspecific probe/protein interactions. Furthermore, all DNA-protein complexes were checked for the sequence specifity of the binding reaction by adding a 100-fold excess of the same, unlabelled, double-stranded oligodeoxynucleotide (100x competitor). For generation of supershifted complexes, the nuclear extracts were preincubated for 1 h at 4°C with the appropriate antiserum prior to the binding reaction. DNA-protein complexes were separated from the non-bound oligodeoxynucleotide by electrophoresis on a 5% polyacrylamide gel in 0.25xTBE buffer [22.5 mM Tris-borate, 0.5 mM EDTA] (14). Electrophoresis was carried out at room temperature at 150 V for 70 min, using 0.25xTBE as running buffer. The gel was dried on Whatman-3MM paper, and DNA-protein complexes were visualized by autoradiography.

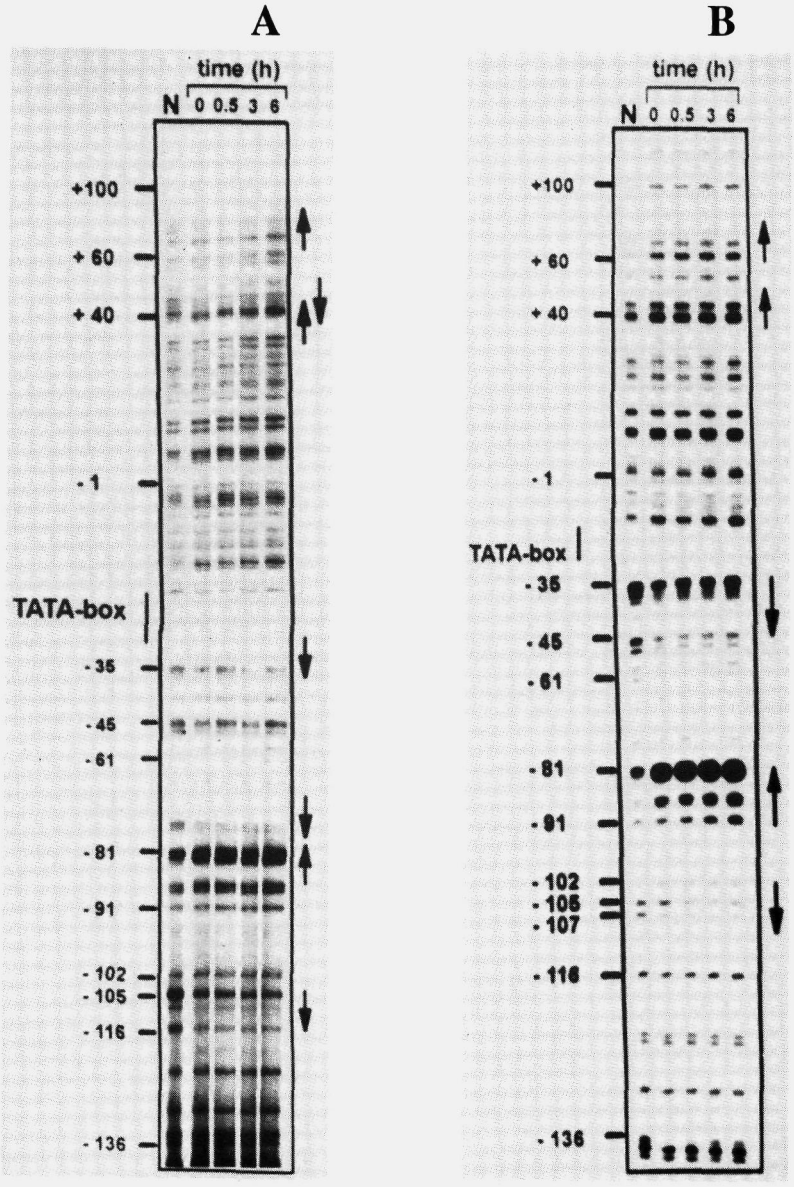

Figure 1. ***In vivo*** **DMS footprinting of the -136 to +100 5'-flanking region of the t-PA promoter in HUVEC and HeLa cells.** HUVEC (panel A) or HeLa cells (panel B) were incubated with PMA for various time periods (as indicated) and used for *in vivo* footprint analysis, as described in the Methods section. Regions containing hypersensitive and protected G residues, as compared to the naked DNA control (N), are indicated with arrows (↑ and ↓, respectively). Nucleotide positions of the t-PA promoter sequence are shown by the numbers to the left.

RESULTS

Genomic DMS footprinting of the t-PA promoter in HUVEC and HeLa cells

To study the interactions between nuclear proteins and the human t-PA promoter sequence *in vivo* in intact HUVEC and HeLa cells, we performed genomic DMS footprinting. Using the appropriate primers, the DNA-protein interaction sites of the t-PA promoter region between -130 and +100 were mapped. As shown in Figure 1 and summarized in Figure 2, only 9 out of the 16 affected residues coincide in HUVEC and HeLa cells. However, all affected residues are clustered around the same five consensus sites for transcription factor binding. These five boxes consist of a PMA responsive element (TRE) between positions -112 and -104; a consensus site for the family of CCAAT-binding transcription factors, also referred to as nuclear factor 1 (CTF/NF-1) binding site, between positions -92 and -77; and three GC-boxes between positions -43 and -34, +39 and +45, and +62 and +68, which have homology to SP-1 and activator protein-2 (AP-2) binding sites. Although the affected residues are clustered around the same five boxes in HUVEC and HeLa cells, the observed differences in the pattern of protection suggest that the proteins bound in HeLa and HUVEC may be similar but are not necessarily identical.

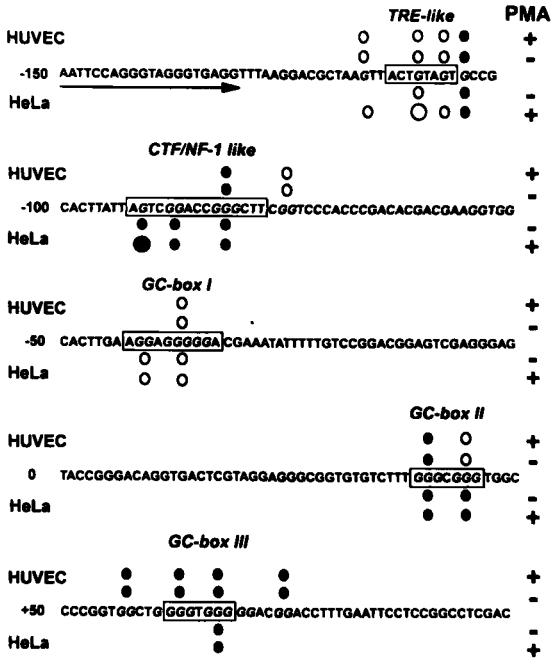

Figure 2. Summary of *in vivo* DMS footprinting analyses and consensus elements for transcription factor binding to the t-PA promoter. The data from Fig. 1 are summarized, and putative transcription factor binding sites in the t-PA promoter region are indicated with boxes. The numbers indicate the nucleotide positions of the t-PA promoter sequence. Clusters of affected G residues are given in *italics*. The protected and hyperreactive G residues are denoted by open and closed circles, respectively. Sizes of the circles indicate the relative extents of DMS protection or hypersensitivity.

PMA treatment of the cells did not markedly alter the DMS footprint pattern, except for that of the TRE-like region in HeLa cells (Fig. 1B). 30 min of PMA treatment slightly induced protection of the G residue at -113 and strongly increased protection of the G residue at -104. After 3 h also the protection of the G residue at -107 was further induced, and then remained unchanged up to 6 h. In contrast to HeLa cells, in HUVEC these residues were already contacted by protein under non-stimulated conditions, and PMA treatment had no marked effect on their methylation and subsequent cleavage. Finally, in HeLa cells PMA also affected binding in the CTF/NF-1-like region, reflected by an increase in hypersensitivity of the G residue at position -91 (Fig. 1B).

Identification of nuclear proteins that bind to the t-PA promoter

To identify the nature of the proteins bound to the t-PA promoter, the DNA-protein interactions were studied *in vitro* by using the electromobility shift assay (EMSA). All five regions identified with the *in vivo* footprinting assay bound nuclear protein, and protein binding was qualitatively and quantitatively comparable in HUVEC and HeLa cells, except for the TRE-like binding site (Figs 3-8). The specific DNA-protein complex formed with the TRE-like sequence was far more abundant with nuclear extracts from HUVEC than from HeLa cells (Fig. 3), which is in agreement with the higher protection of this region in HUVEC in the *in vivo* footprint analysis (Fig. 1).

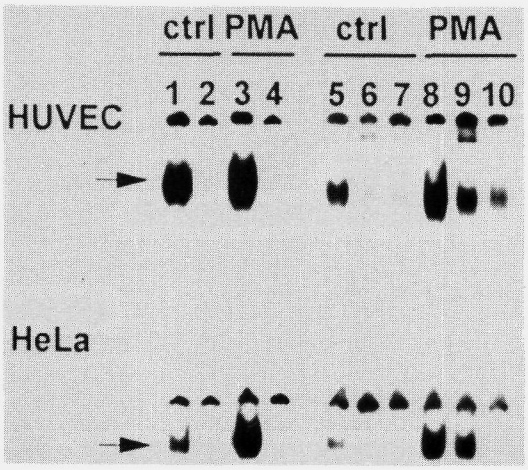

Figure 3. Gel-mobility shift assay of the -120 to -98 (TRE-like) t-PA promoter region. HeLa cells or HUVEC were incubated with control medium (ctrl) or medium supplemented with PMA (PMA) for 3 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a radiolabelled double-stranded -120 to -98 oligodeoxynucleotide. DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 1 day. Arrows mark the specific DNA-protein complex. Nuclear extracts were pre-incubated for 1 h in the absence (lanes 1, 3, 5 and 8) or presence of 100-fold excess unlabelled double-stranded -120 to -98 oligodeoxynucleotide (lanes 2 and 4), anti-Jun antibody (lanes 6 and 9) or anti-Fos antibody (lanes 7 and 10).

Dissociation experiments showed that the association of protein to the TRE-like site was stable over a 20 min period when using nuclear extracts from HUVEC, while with nuclear extracts from HeLa cells the protein rapidly dissociated from the DNA, indicating that at least part of the difference in binding can be explained by a difference in binding affinity of the protein (Fig. 4). PMA strongly induced protein binding with nuclear extracts from HeLa cells (more than 20-fold) but hardly further increased protein binding with nuclear extracts from HUVEC (about 2-fold) (Fig. 3). This is consistent with the *in vivo* footprint data, which showed a stronger induction of protection in PMA-treated HeLa cells than in PMA-treated HUVEC. PMA did not alter the protein dissociation rate with nuclear extracts of either cell type (Fig. 4), indicating that the strong induction of protein binding activity in the nuclear extracts from PMA-treated HeLa cells is probably the result of an increase in protein levels.

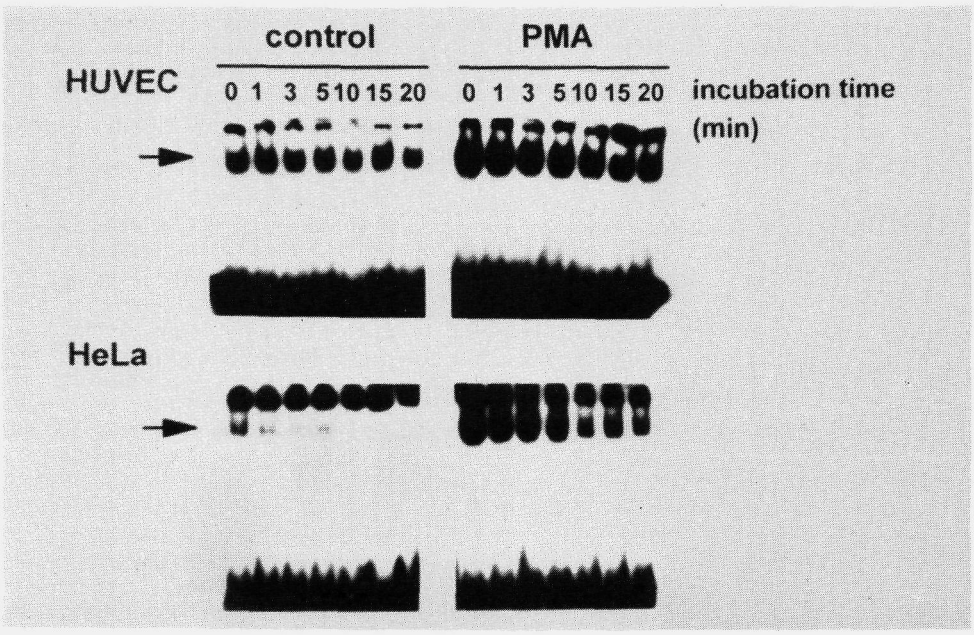

Figure 4. Protein dissociation from the TRE-like region in HUVEC and in HeLa cells. HeLa cells or HUVEC were incubated with control medium (control) or medium supplemented with PMA (PMA) for 3 h, and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a radiolabelled double stranded -120 to -98 oligodeoxynucleotide. After the binding reaction the mixtures were incubated in the presence of 100-fold excess unlabelled -120 to -98 region competitor oligodeoxynucleotide for the indicated times. DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 4 days. Arrows mark the specific DNA-protein complex.

TRE-like consensus sites bind transcription factors belonging to the families of the activator protein-1 (AP-1) or the cAMP responsive element binding (CREB) proteins, and also heterodimers formed between these two families like c-Jun/ATF2 (17). We found that protein binding to the TRE-like region of the t-PA promoter in both HUVEC and HeLa nuclear extracts was strongly inhibited with antibodies directed against the AP-1 family members Jun and Fos (Fig. 3). Apparently, this region of the t-PA promoter is bound by Jun/Fos heterodimers. Similar results were obtained with nuclear extracts of PMA-treated HUVEC (Fig. 3). The protein-DNA complex formed with nuclear extracts from PMA-treated HeLa cells was fully inhibited with anti-Fos antibody, but only partially (for about 50%) with anti-Jun antibody. This points at cell-type specific differences in the Jun family members bound.

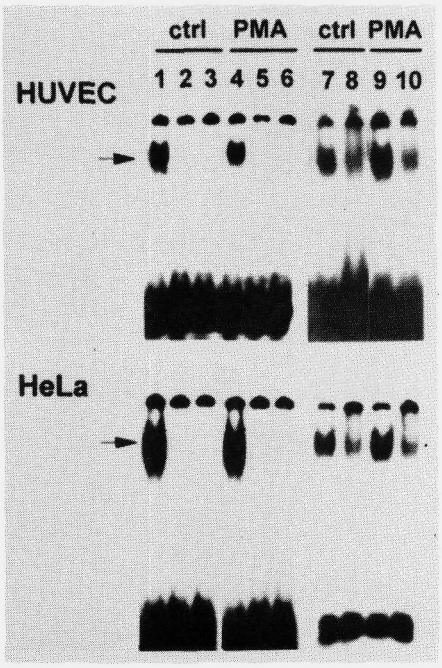

Figure 5. Gel-mobility shift assay of the -95 to -72 (CTF/NF-1 like) t-PA promoter region. HeLa cells or HUVEC were incubated with control medium (ctrl) or medium supplemented with PMA (PMA) for 3 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a double-stranded radiolabelled -95 to -72 oligodeoxynucleotide. DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 1 day. Arrows mark the specific DNA-protein complex. Nuclear extracts were pre-incubated for 1 h in the absence (lanes 1, 5, 7 and 9) or presence of 100-fold excess unlabelled double-stranded -95 to -72 oligodeoxynucleotide (lanes 2 and 5), 100-fold excess of a consensus CTF/NF-1 binding site (lanes 3 and 6) or anti-CTF/NF-1 antibody (lanes 8 and 10).

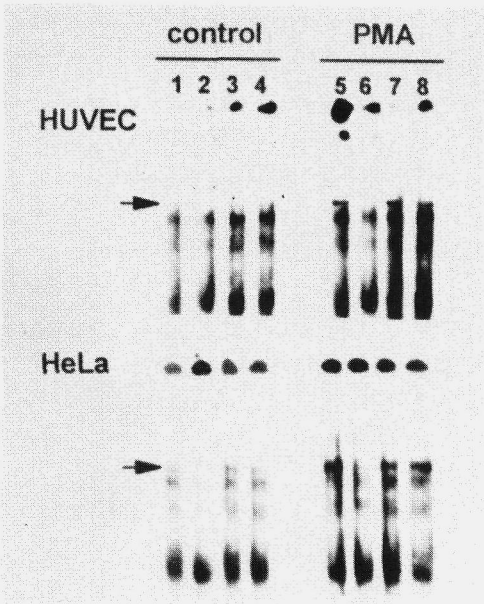

Figure 6. Gel mobility shift assay of the -51 to +35 (GC-box I) t-PA promoter region. HeLa cells or HUVEC were incubated with control medium (control) or medium supplemented with PMA (PMA) for 3 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a double-stranded radiolabelled -51 to +35 oligodeoxynucleotide (box I). DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 1 day. Arrows mark specific DNA-protein complexes. Nuclear extracts were pre-incubated for 1 h in the absence (lanes 1 and 5) or presence of 100-fold excess of unlabelled double-stranded -51 to +35 oligodeoxynucleotide (lanes 2 and 6), anti-SP-1 antibody (lanes 3 and 7) or anti-AP-2 antibody (lanes 4 and 8).

The CTF/NF-1-like binding site showed one DNA-protein complex with both HUVEC and HeLa nuclear extracts (Fig. 5). This complex consisted of DNA-bound CTF/NF-1 protein, and was not altered by PMA treatment in either cell type (Fig. 5).

Of the three GC-boxes identified, boxes II and III bound SP-1 and box I was occupied by an unidentified protein using nuclear extracts from HUVEC and HeLa cells (Figs. 6-8). Two SP-1 containing complexes were formed, except for GC-box II which, when incubated with nuclear extracts from HeLa cells, formed one SP-1 containing complex and one complex containing an unidentified protein. None of the GC-boxes bound any AP-2 protein although both HUVEC and HeLa cells expressed AP-2 (as assessed by EMSA with a consensus AP-2 binding site, data not shown). The protein-DNA complexes formed with all three GC-boxes were strongly induced when using nuclear extracts from PMA-treated HeLa cells, but hardly induced (upto 2-fold) with nuclear extracts from PMA-treated HUVEC (Figs. 6, 7 and 8).

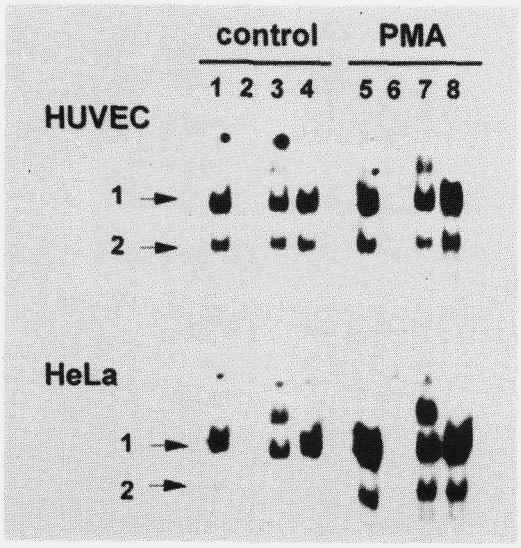

Figure 7. Gel mobility shift assay of the +30 to +52 (GC-box II) t-PA promoter region. HeLa cells or HUVEC were incubated with control medium (control) or medium supplemented with PMA (PMA) for 3 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a radiolabelled double-stranded +30 to +52 oligodeoxynucleotide (box II). DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled DNA-protein complexes were visualised by autoradiography for 1 day. Arrows mark specific DNA-protein complexes. Nuclear extracts were pre-incubated for 1 h in the absence (lanes 1 and 5) or presence of 100-fold excess of unlabelled double-stranded +30 to +52 oligodeoxynucleotide (lanes 2 and 6), anti-SP-1 antibody (lanes 3 and 7) or anti-AP-2 antibody (lanes 4 and 8).

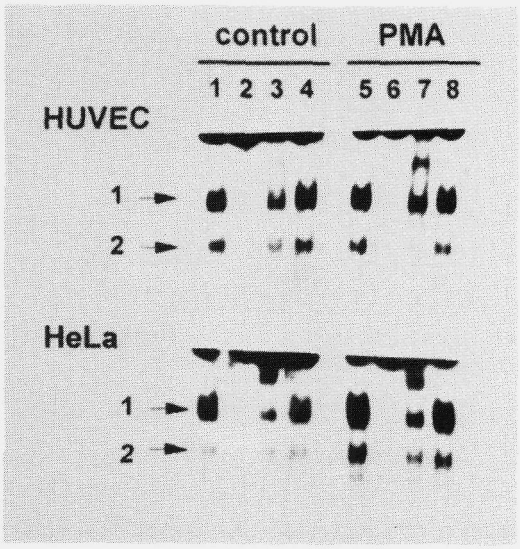

Figure 8. Gel mobility shift assay of the +57 to +78 (GC-box III) t-PA promoter region. HeLa cells or HUVEC were incubated with control medium (control) or medium supplemented with PMA (PMA) for 3 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a radiolabelled double-stranded +57 to +78 oligodeoxynucleotide (box III).
DNA-protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 1 day. Arrows mark specific DNA-protein complexes. Nuclear extracts were pre-incubated for 1 h in the absence (lanes 1 and 5) or presence of 100-fold excess of unlabelled double-stranded +57 to +78 oligo-deoxynucleotide (lanes 2 and 6), anti-SP-1 antibody (lanes 3 and 7) or anti-AP-2 antibody (lanes 4 and 8).

DISCUSSION

Previous investigations of the regulation of the human t-PA gene by transient transfection assays in HeLa cells employing deletion mutants of the t-PA gene promoter demonstrated that the DNA elements which regulate constitutive and PMA-stimulated expression are encoded by sequences downstream of position -115 of the t-PA gene (9). In this study, we further characterized the -135 to +100 region of the human t-PA promoter for persistent and PMA-inducible DNA-protein interactions in cultured vascular endothelial cells and HeLa cells. *In vivo* genomic footprinting analysis revealed five distinct protein binding elements in both endothelial cells and HeLa cells, corresponding to a PMA responsive element (TRE; -112 to -104), a CTF/NF-1 binding site (-92 to -77) and three GC-boxes (-43 to -34, +39 to +45, and +62 to +68). After PMA treatment of HeLa cells, the G residues of the TRE consensus sequence (-113, 107 and -104) were less susceptible to methylation, reflecting enhanced protein binding. In contrast to HeLa cells, in HUVEC these residues were already fully occupied by protein under non-stimulated conditions, and PMA treatment had no marked effect on their methylation and subsequent cleavage. In accordance with the *in vivo* genomic footprinting analysis, *in vitro* gel shift analysis revealed a considerable qualitative and quantitative similarity in binding of nuclear proteins from human endothelial cells and HeLa cells, except for the TRE sequence. This latter sequence was bound much more efficiently by nuclear factors from endothelial cells than from HeLa cells. The identified transcription factors bound to the TRE, the CTF/NF-1 site and GC-boxes II and III included Jun/Fos, CTF/NF-1 and SP-1, respectively, in both cell types.

Two of the five protein binding sites, the TRE and GC-box III, were also reported by Medcalf et al. (9) to be essential for basal and PMA-induced t-PA promoter activity in HeLa cells on the basis of mutational analysis. We found, using gel-shift assays, that nuclear protein binding to each of these sites was strongly induced in PMA-treated HeLa cells (about 20-fold) and about 2-fold in human endothelial cells, which parallels the difference in transcriptional induction of t-PA by PMA in HeLa cells and HUVEC (8, 9). In contrast to the increase in nuclear protein binding to the TRE, the enhanced nuclear protein binding to GC-box III was not reflected in a change in the cleavage pattern of the G residues of this region in the *in vivo* footprint, possibly because these G residues were already optimally accessible for methylation, or because of a high rate of exchange of bound proteins to this sequence.

Our observation that GC-boxes II and III bind SP-1 protein is in line with previous reports that human t-PA transcription predominantly initiates from a TATA-less promoter at position +110 (18). Such TATA-less promoters depend on SP-1 for the recruitment of the transcription initiation complex (19). Additional evidence for an important role of SP-1 in t-PA transcription is provided by the study of Medcalf et al. (9) who reported a strict correlation between nuclear protein binding to GC-box III (i.e. SP-1) and t-PA expression in different cell-types: a high nuclear protein binding and t-PA expression in Bowes melanoma cells, intermediate in HeLa cells and hardly detectable nuclear protein binding and no t-PA expression in HepG2 cells (9).

Our finding that the GC-box at +60 in the human t-PA promoter binds SP-1, is in contrast to reports suggesting that GC-box III is an AP-2 binding site. This suggestion, however, was based on experiments which showed competition of GC-box III nuclear protein binding by a consensus AP-2 binding site (9). Since a consensus AP-2 binding site is also capable of binding SP-1 protein (Arts and Kooistra, unpublished data), these experiments are not directly indicative of AP-2 binding. Similarly, the GC-boxes in the murine t-PA promoter have also been reported to lack affinity for AP-2 (20).

Our structural analysis of the human t-PA promoter extends previous studies by the identification of three additional protein binding sites (CTF/NF-1 and GC-boxes I and II), which were previously not detected by transfection and *in vitro* footprinting techniques (9, 21, 22). The exact role of these elements in human t-PA transcription remains unclear at present and needs to be established by mutational analysis and transfection experiments. They are likely to be important in t-PA expression, however, since deletion and/or mutation of these sites in the very homologous murine t-PA promoter hampered t-PA transcription (20, 23-25).

In conclusion, our studies on the identification of DNA-protein interactions at the t-PA promoter in intact human cells did not only confirm the *in vivo* presence of interactions previously detected *in vitro*, but also identified three additional protein binding sites. Although no clear cell-type specific differences in nuclear protein binding were found, the observed differences between HUVEC and HeLa cells in the exact G residues protected by nuclear proteins and differences in binding affinities suggest the existence of subtle differences in DNA-protein interactions between these cell types which may be essential for appropriate transcriptional control of t-PA in different physiological contexts.

ACKNOWLEDGEMENTS

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CHAPTER 3

Studies on the mechanism of sodium butyrate-stimulated t-PA expression in cultured human endothelial cells. Effects of trichostatin A and 2-deoxy-d-glucose.

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SUMMARY

We have found that the induction of tissue-type plasminogen activator (t-PA) by sodium butyrate in human umbilical vein endothelial cells (HUVEC) can be mimicked with a structurally unrelated, specific histone deacetylase-inhibitor, (R)-trichostatin A (TSA). Simultaneous addition of butyrate and TSA at concentrations approaching maximal induction by the compounds alone showed no additive effect on t-PA stimulation, indicating a common regulatory mechanism, i.e. histone acetylation. A decrease in butyrate-stimulated t-PA production with the glucose analogue, 2-deoxy-D-glucose, was part of a general decrease in protein secretion, possibly as a result of an impaired glycosylation. The altered glycosylation pattern of t-PA could be visualized by fibrin-underlay autography. The effects of 2-deoxy-D-glucose could be overcome by the simultaneous addition of mannose to the medium. Our results suggest an important role of histone acetylation in the butyrate-induced stimulation of t-PA expression in HUVEC.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a glycoprotein that converts plasminogen to plasmin in the presence of fibrin, the matrix structure of a blood clot; the plasmin thus formed then degrades fibrin. Plasma t-PA is continuously secreted into the blood by the endothelial cells (1). In a previous report we have shown that sodium butyrate is a strong and rather selective inducer of t-PA expression in cultured human umbilical vein endothelial cells (HUVEC) (2). This effect of butyrate on t-PA expression was confirmed at the mRNA level. However, the regulatory mechanism by which the stimulatory effect of butyrate is brought about, remained elusive. One of the most evident changes induced by butyrate is the acetylation of histones via inhibition of the enzyme histone deacetylase (3) which may lead to transcriptional activation (4). On the other hand, several reports suggest that butyrate can produce an effect on gene expression via a pathway that apparently depends on the presence of glucose in the medium. Takano et al. (5) showed that glucose deprivation of the pig kidney cell line, LLC-PK1, reduced the induction of the facilitated glucose transporter by butyrate. A study by Cox et al. (6) demonstrated that the induction of the glycoprotein hormone α subunit and its mRNA by butyrate in HeLa cells is inhibited by 2-deoxy-D-glucose, a glucose-analogue. These observations suggest that glucose and/or its metabolites may play an important role in butyrate-induced gene

expression. Since 2-deoxy-D-glucose has no effect on histone acetylation (6), this pathway is likely to be an alternative to chromatin modification.

To elucidate whether histone acetylation may play a role in the induction of t-PA by butyrate, we compared the effect of butyrate with that of (R)-trichostatin A (TSA). TSA is a compound structurally unrelated to butyrate, which has been shown to be a potent and specific inhibitor of histone deacetylase at very low concentrations (7). We explored a possible role of glucose in the regulation of t-PA synthesis by butyrate by depriving HUVEC of glucose and incubating the cells in the presence of 2-deoxy-D-glucose.

MATERIALS AND METHODS

Materials

N-butyric acid sodium salt, 2-deoxy-D-glucose and D-mannose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TSA was a generous gift from Dr M. Yoshida, Dept. of Agricultural Chemistry, The University of Tokyo, Japan. Stock solutions of TSA (10 mM) were prepared in DMSO and stored at -20°C until use. Experiments involving TSA were performed in subdued light, and the tubes containing the solutions were covered with aluminium foil. Culture media were from Flow Laboratories (Irvine, UK). Enzyme immunoassay kits for determination of human t-PA antigen ("Fibrinostika t-PA") or plasminogen activator inhibitor 1 (PAI-1) antigen ("Imulyse-kit") were from Organon Teknika (Boxtel, The Netherlands) and Biopool (Umeå, Sweden), respectively. [^{35}S]-methionine (> 1000 mCi/μmol) was obtained from Amersham International plc (Buckinghamshire, UK). Other materials used in the methods described below have been specified in detail in related references.

Cell Culture Experiments

Endothelial cells were isolated from human umbilical cord veins using collagenase (8). Cells were grown in fibronectin-coated dishes in DMEM supplemented with 20 mM HEPES (pH 7.4), 10% (v/v) human serum, 10% (v/v) newborn calf serum (heat-inactivated), 2 mM L-glutamine, 5 U heparin per ml, 150 μg endothelial cell growth supplement per ml (9), and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere, as described (10). The medium was replaced every 2 to 3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3. For experiments, confluent cultures were used at second or third passage, and cells were always refed the day before the experiment with incubation medium, i.e. DMEM supplemented with 20 mM HEPES (pH 7.4), 10% (v/v) human serum, glutamine and penicillin/streptomycin. Where indicated, DMEM was replaced with Leibovitz-L15 medium which contains pyruvate instead of glucose as energy source. Conditioned media (CM) were obtained by incubating HUVEC with incubation medium containing the appropriate concentration of the test compound or stock solvent, DMSO (final concentration maximally 0.1% (v/v)). CM were centrifuged for 2 min in a Beckman Microfuge centrifuge to remove cells and cellular debris, and samples were frozen at -20°C until use.

Assays

t-PA antigen and PAI-1 antigen determinations were performed using commercially available enzyme immunoassays. In fibrin autography, an observation by Granelli-Piperno and Reich (11) was exploited: complexes between t-PA and PAI-1 show activity on fibrin underlays after SDS-polyacrylamide gel electrophoresis and removal of SDS with Triton X-100 (2). Overall protein synthesis was determined by measuring the incorporation of [^{35}S]-methionine into the 10% (w/v) trichloroacetic acid-precipitable fraction of radiolabelled CM and cell extract (2).

RESULTS AND DISCUSSION

Dose-dependency and Time-course of the Stimulatory Effect of Butyrate and TSA on t-PA Synthesis in HUVEC

The effect of increasing concentrations of butyrate (0.1-10 mM) and TSA (0.1-10 μM) on t-PA production in HUVEC over 24 h is shown in Figure 1A. At the highest concentrations tested, cells became partly detached from the matrix, which was accompanied by a fall in t-PA production. At optimally stimulatory concentrations, butyrate (3 mM) and TSA (1 μM) increased t-PA production 5.6- and 3.9-fold, respectively, in the experiment shown. Between 4 different endothelial cell isolates, the stimulation of t-PA production over a 24 h incubation period varied between 5.2- and 12.2-fold for butyrate and between 3.1- and 7.9-fold for TSA. The stronger stimulatory effect of butyrate as compared to TSA as shown in Figure 1A was observed in each of the four experiments, and was on average 1.6 $\pm$ 0.11 (S.D.)-fold. The two compounds tested showed no marked effect on PAI-1 synthesis (data not shown). Figure 1B shows a representative time-course of the stimulatory effect of 3 mM butyrate and 1 μM TSA. t-PA antigen induction becomes detectable between 4 and 8 h, but whereas butyrate continues to enhance t-PA synthesis over the entire incubation period, the stimulatory effect of TSA levels off after 16 h.

Effect of the Combination of Butyrate and TSA on t-PA Synthesis

To determine whether or not butyrate and TSA exert their action by the same cellular pathway, we have incubated HUVEC with butyrate and TSA separately and in combination at varying concentrations for 24 h (Fig. 2). At suboptimal concentrations, a combination of the two compounds more than additively enhanced t-PA production. However, when butyrate and TSA approached maximally stimulating concentrations, the synthesis of t-PA in the presence of both compounds was not any higher than with butyrate alone. When a combination of maximally stimulating concentrations was used, t-PA synthesis slightly decreased, a phenomenon similar to that seen with supramaximal concentrations of butyrate or TSA (Fig. 1A). These results suggest that the stimulatory action of butyrate and TSA occur via the same regulatory pathway, probably involving histone acetylation.

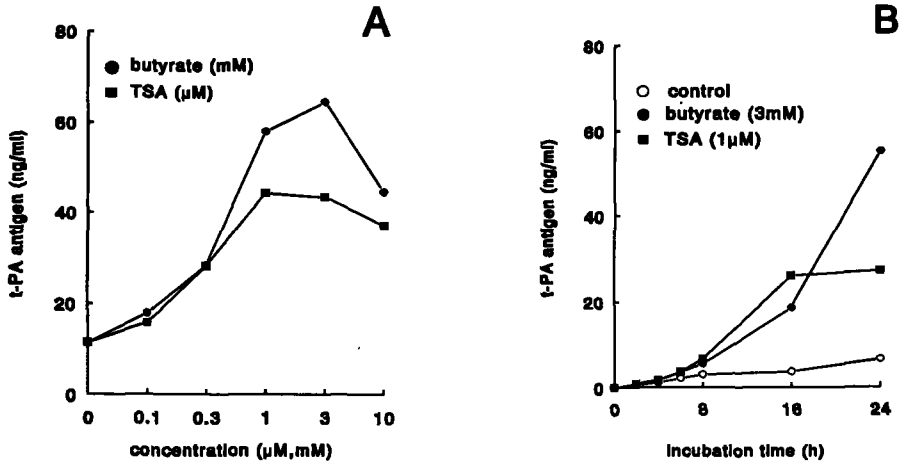

Fig.1 Dose-dependency and time-course of the stimulatory effect of butyrate and TSA on t-PA synthesis in HUVEC. HUVEC were incubated for 24 h with varying concentrations of butyrate (0.1-10 mM) or TSA (0.1-10 μM) (A) or for varying lengths of time up to 24 h with 3 mM butyrate or 1 μM TSA (B). Conditioned media were collected and analyzed for t-PA antigen as described in the Methods section. The data shown are mean values of duplicate incubations of a representative experiment (out of 4).

Effect of Glucose Deprivation on t-PA Induction by Butyrate

To investigate the effect of glucose deprivation on butyrate-stimulated t-PA production, HUVEC were grown in Leibovitz medium which contains no glucose but 5 mM sodium pyruvate as an energy source. We found little or no effect of glucose deprivation on basal and butyrate-induced t-PA synthesis. Furthermore, the addition of glucose to the Leibovitz medium did not have a modulating effect on t-PA expression (data not shown). To ascertain that no residual glucose (from the serum) or newly formed glucose was interfering in the above experiments, we supplemented the Leibovitz medium with the glucose analogue, 2-deoxy-D-glucose, a sugar known to interfere with glucose utilization. As illustrated in Figure 3A, 2-deoxy-D-glucose slightly suppressed basal production of t-PA and strongly inhibited the butyrate-induced t-PA production. This suppressive effect of 2-deoxy-D-glucose was not visible at the t-PA mRNA level (data not shown), but can be largely explained by a fall in overall protein synthesis and/or secretion (Figure 3B). An impaired glycoprotein synthesis in the presence of 2-deoxy-D-glucose has been observed before and ascribed to the sequestering of the nucleotides required for the formation of nucleotide sugars, particularly as GDP-2-deoxy-D-glucose (12,13).

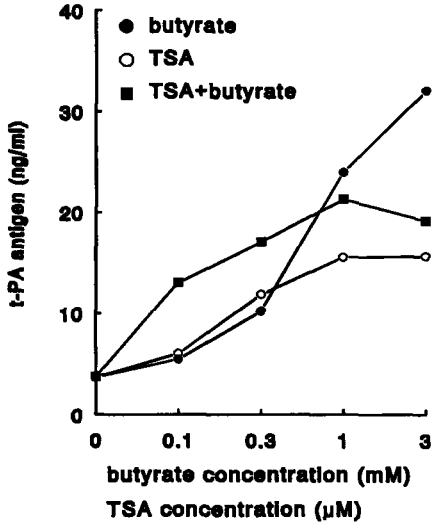

Fig.2 Effect of a combination of butyrate and TSA on t-PA synthesis in HUVEC. HUVEC were incubated in the presence of different concentrations of butyrate (0.1-3.0 mM) and TSA (0.1-3.0 μM), either alone or in combination, for 24 h. Conditioned media were collected and analyzed for t-PA antigen as described in the Methods section. The data shown are mean values of duplicate incubations of a representative experiment (out of 3).

To study whether the t-PA molecules synthesized in the presence of 2-deoxy-D-glucose have altered carbohydrate chains, and consequently altered molecular weights, we performed fibrin autography. As illustrated in Figure 4, 24 h CM from HUVEC predominantly show one lysis band using this technique, with an apparent molecular mass around 100 kD. Upon addition of butyrate, t-PA activity is induced, and a second lysis zone with a lower molecular weight appears. Both lytic zones are related to t-PA activity as demonstrated by inhibition by anti-t-PA IgG, and are the result of the activity of the complex between t-PA and PAI-1 (2). CM of 2-deoxy-D-glucose treated HUVEC showed a shift from the upper to the lower band on fibrin-underlay, both under basal and butyrate conditions, indicating impaired protein glycosylation in 2-deoxy-D-glucose-treated cells. A similar shift to a lower molecular weight was also observed when HUVEC (with or without butyrate) were treated with tunicamycin, a compound known to block the formation of protein N-glycosidic linkages (data not shown). Remarkably, the lysis zones obtained with CM from butyrate- and butyrate/2-deoxy-D-glucose-treated cells are very similar in size, although the latter CM contains approximately only half of the t-PA antigen present in the CM of the butyrate-treated cells. This suggests that glycosylation might influence t-PA activity and/or t-PA interaction with PAI-1, at least in the fibrin underlay assay. Changes in N-glycosylation have previously been reported to influence fibrin-dependent catalytic activity of t-PA (14,15).

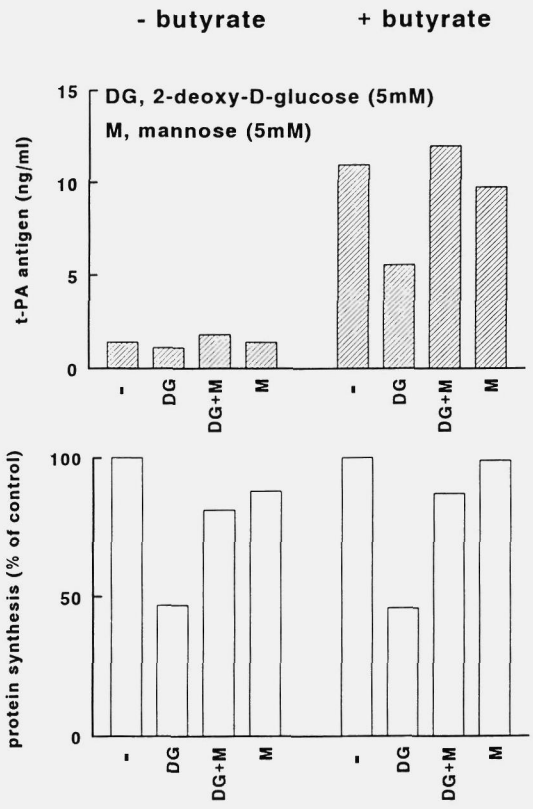

Fig.3 Effect of 2-deoxy-D-glucose and mannose on butyrate-stimulated t-PA synthesis and on overall protein synthesis in HUVEC. HUVEC were grown in Leibovitz medium and incubated for 24 h with or without butyrate (5 mM) and/or 2-deoxy-D-glucose (5 mM) and/or mannose (5 mM). Conditioned media were collected and analyzed for t-PA antigen as described in the Methods section (A). In a parallel experiment, overall protein synthesis was determined by measuring the incorporation of [^{35}S]-methionine into the 10% (w/v) *trichloroacetic acid-precipitable* fraction (B). Protein synthesis data are expressed as a percentage of control value. The data shown are mean values of duplicate incubations of a representative experiment (out of 5).

In line with other reports (16), we found that the impairment of protein glycosylation can be overcome by adding mannose to the medium (Figs 3A, 3B and 4), resulting in a restored overall protein synthesis rate (Fig. 3B) and a normalized t-PA/PAI-1 molecular weight on fibrin underlay autography (Fig. 4). The addition of mannose has been shown to cause a decrease in the amount of inhibitor, GDP-2-deoxy-D-glucose (13).

In conclusion, our data are consistent with a role of histone acetylation rather than of glucose in the induction of t-PA by butyrate. Further experiments are now underway to confirm the putative role of histone acetylation in the t-PA gene expression.

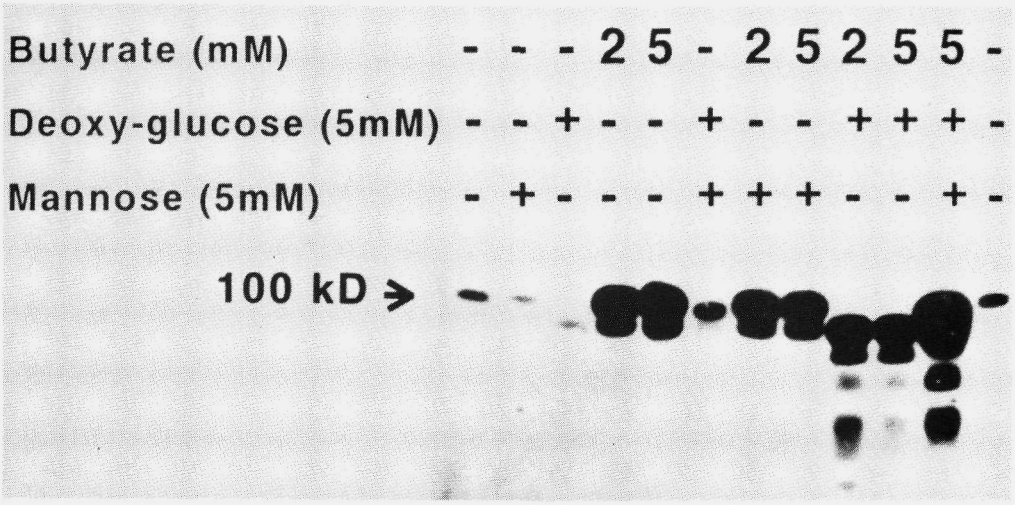

Fig.4 Analysis of conditioned media of HUVEC incubated with butyrate, 2-deoxy-D-glucose and/or mannose by fibrin autography. HUVEC were grown in Leibovitz medium and incubated for 24 h with or without butyrate (2 or 5 mM), 2-deoxy-D-glucose (5 mM) and mannose (5 mM). Conditioned media were collected and subjected to SDS/polyacrylamide-gel electrophoresis. t-PA activity was visualized by fibrin autography.

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We wish to thank Dr M. Yoshida for the gift of TSA and Karin Toet for expert technical assistance. This study was financially supported by the Netherlands Heart Foundation (grant 90.267).

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CHAPTER 4

Stimulation of tissue-type plasminogen activator gene expression by sodium butyrate and trichostatin A in human endothelial cells involves histone acetylation

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SUMMARY

We have previously shown that the pleiotropic agent sodium butyrate strongly stimulates tissue-type plasminogen activator (t-PA) expression in human umbilical vein endothelial cells (HUVEC). Here we provide evidence that the butyrate-induced t-PA expression in HUVEC involves histone H4 acetylation. (1) t-PA induction by butyrate occurs at the transcriptional level and does not require new protein synthesis, indicating a direct effect. (2) t-PA induction by butyrate can be fully mimicked by a specific, structurally unrelated, histone deacetylase inhibitor, trichostatin A. (3) At optimally stimulatory conditions, a combination of butyrate and trichostatin A does not enhance t-PA production more than each of the compounds alone, indicating that both compounds act through a common regulatory mechanism. (4) Induction of t-PA transcription by butyrate and trichostatin A was found to be preceded by histone H4 acetylation; at suboptimal inducing concentrations of butyrate and trichostatin A, the degree of acetylation of histone H4 caused by each agent was similarly reduced. These results are consistent with a role for histone H4 acetylation in t-PA induction by butyrate in HUVEC.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) plays a key role in the onset of the fibrinolytic process by converting the zymogen plasminogen into the active enzyme plasmin. Plasmin can degrade fibrin, the matrix structure of a blood clot. The importance of the role of t-PA *in vivo* has recently been demonstrated once more by Carmeliet et al. (1), who showed that t-PA-deficient mice have a reduced thrombolytic capacity. The activity of t-PA in plasma is regulated by, amongst others, a specific inhibitor, plasminogen activator inhibitor 1 (PAI-1). The vascular endothelium plays an important role in determining plasma t-PA activity by synthesizing both t-PA and PAI-1. Consequently, cultured human endothelial cells are frequently used as a model to gain insight into the regulation of t-PA and PAI-1 synthesis (2).

We have previously shown that sodium butyrate is a strong and rather selective inducer of t-PA production in cultured human umbilical vein endothelial cells (HUVEC), without markedly affecting PAI-1 synthesis (3). The effects of butyrate with respect to t-PA and PAI-1 synthesis were confirmed at the mRNA level. However, the regulatory mechanism by which butyrate stimulates t-PA expression remained unknown. Although

butyrate is a pleiotropic agent, one of the most evident changes brought about by butyrate is the acetylation of histones via inhibition of the enzyme histone deacetylase (4,5). The introduction of acetyl groups neutralizes positive charges in the histone molecules, which results in diminished interaction of the histones with the DNA. Consequently, there is increased accessibility of transcriptional factors to their DNA recognition motifs, leading to transcriptional activation (6-8). Besides having a direct effect via histone acetylation, butyrate may also affect t-PA gene expression by the induction of transcriptional factors such as c-Jun (9-13). For example, differentiation of the embryo carcinoma cell line F9 by butyrate or another differentiating agent, retinoic acid, is accompanied by an induction of t-PA gene expression and preceded by the induction of c-*jun*. Transfection of F9 cells with a c-Jun expression vector was shown to induce t-PA expression, suggesting that c-*jun* may be involved in the induction of t-PA by butyrate or retinoic acid (11).

In this paper we have sought evidence for a role for histone acetylation in the induction of t-PA gene expression by butyrate in HUVEC by comparing changes in t-PA transcription rate with the degree of histone H4 acetylation. Also, we have compared the effect of butyrate with that of (R)-trichostatin A (TSA), a compound structurally unrelated to butyrate, which has been shown to be a potent and specific inhibitor of histone deacetylase at very low concentrations (14). To examine a possible role for c-Jun in the butyrate-stimulated t-PA production, we have analysed c-*jun* mRNA levels in butyrate- and retinoic acid-treated HUVEC.

MATERIALS AND METHODS

Materials

All-*trans*-retinoic acid, n-butyric acid (sodium salt), and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TSA was generously provided by Dr. M. Yoshida, Department of Agricultural Chemistry, University of Tokyo, Japan. Stock solutions of retinoic acid (10 mM) and TSA (10 mM) were prepared in DMSO. A stock solution of butyrate (0.5 M) was prepared in 0.9% (w/v) NaCl. All stock solutions were stored at -20°C. The compounds were diluted with incubation medium immediately before use to the concentrations indicated. All experiments involving retinoic acid or TSA were performed in subdued light, and the tubes containing the solutions were covered with aluminium foil. Culture reagents were obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.), and plastics were supplied by Costar (Cambridge, MA, U.S.A.). Enzyme immunoassay kits for determination of human t-PA antigen ("Fibrinostika t-PA") were from Organon Teknika (Boxtel, The Netherlands). Bradford protein reagent was from Bio-Rad (Veenendaal, The Netherlands). Deoxycytidine 5[α-^{32}P]triphosphate (3 μCi/μmol), uridine 5[α-^{32}P]triphosphate (> 400 μCi/μmol) and Megaprime-kit were obtained from Amersham International. Other materials used in the methods described below have been specified in detail in related references.

Cell culture experiments

Endothelial cells were isolated from human umbilical cord veins by the method of Jaffe et al. (15), and cultured as previously described (16). Cells were grown on fibronectin-coated dishes in Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes, 10% (v/v) newborn calf serum (heat-inactivated), 10% (v/v) human serum, 5 i.u. of heparin/ml, 150 μg of endothelial cell growth supplement/ml (17), 2 mM glutamine, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml at 37°C under a 5% CO_2 atmosphere. The medium was replaced every 2-3 days. Subcultures were obtained by trypsin/EDTA treatment at a split ratio of 1:3. For experiments, confluent cultures were used at second or third passage, and the cells were always re-fed the day before the experiment with incubation medium, i.e. Dulbecco's modified Eagle's medium supplemented with 10% (v/v) human serum, glutamine, penicillin and streptomycin. Conditioned media (CM) were obtained by incubating cells at 37°C for various lengths of time with incubation medium containing the appropriate concentration of the test compound or stock solvent [DMSO; final concentration maximally 0.1% (v/v)]. CM were centrifuged for 2 min in a Beckman Microfuge to remove cells and cellular debris, and the samples were frozen at -20°C until use. The cells were washed twice with ice-cold PBS (0.14 M NaCl, 0.01 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.4), and were used for isolation of RNA or extraction of histones.

Extraction of histones

Histones were extracted from HUVEC according to the procedure described by Cousens et al. (5). About 8 x 10^6 cells were incubated for various lengths of time with or without various concentrations of butyrate, TSA or retinoic acid, and nuclei were isolated. All buffers contained 10 mM butyrate to minimize residual histone deacetylase activity. The nuclear pellet was suspended in 0.1 ml of ice-cold water, and concentrated H_2SO_4 was added to a final concentration of 0.2 M. In order to allow precipitation of nuclear membranes, the suspension was kept at 4°C for at least 1 h. Next, the sample was centrifuged for 5 min at 5000 *g*, and the supernatant was mixed with 1 ml of acetone. After keeping the preparation overnight at -20°C, the coagulated material was collected by centrifugation (15 min at 5000 *g*). This acid-soluble histone fraction was dissolved in 50 μl of water. Protein yields (quantified using Bradford protein reagent) were about 30 μg, 15 μg of which was used for an analysis by acid/urea/Triton (AUT) gel electrophoresis.

AUT gel electrophoresis

Acetylation of histones was analysed by gel electrophoresis using an AUT gel, basically as described by Cousens et al. (5), with some minor modifications as suggested by Hoshikawa et al. (18). The original procedure was modified by layering an upper gel [1 M acetic acid, 6.3 M urea, 4.4% (w/v) acrylamide] on to the separating gel [1 M acetic acid, 8 M urea, 0.5% (v/v) Triton X-100, 45 mM NH_3, 16% (w/v) acrylamide]. Before being applied to the gel, the histone preparations were incubated with an equal volume of loading buffer (7.4 M urea, 1.4 M NH_3, 0.01 M dithiothreitol) for 5 min at room temperature, and were then mixed with 1/8 volume of 0.1% (w/v) pyronine G in acetic acid. The histone preparations were electrophoresed in 0.2 M glycine/1 M acetic acid for 11 h at 13 mA. Gels were stained with Coomassie Brilliant Blue and subjected to densitometric analysis. The individual histones were designated by comparing the stained gel with published migration patterns of histones (5). In two experiments, control and butyrate-treated HUVEC were labelled for 6 h with [^{3}H]acetate. Histone preparations were obtained as described above and subjected to AUT gel electrophoresis. The gel was first stained and then fluorographed to determine the distribution of [^{3}H]acetyl groups among the various histones (5). The identity of the unacetylated H4 species was confirmed: the fastest running H4 species visible on the stained gel was not visible on the fluorogram, since it is not labelled by [^{3}H]acetate.

Preparation of RNA, Northern blot hybridization and run-on assay

Total RNA was isolated from about 5 x 10^6 HUVEC by the method of Chomczynski and Sacchi. (19). The cells were lysed directly in the plates. RNA samples were dissolved in water, and RNA concentrations were determined spectrophotometrically. RNA was fractionated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions using 1 M formaldehyde (20), and blotted to a Hybond-N filter according to the manufacturer's instructions. The filters were hybridized overnight at 63°C in NaPI hybridization mix [7% (w/v) SDS, 0.5 M NaPI (Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2), 1 mM EDTA] containing 3 ng of

[α-^{32}P]CTP-labelled probe/ml. The probes were labelled with a Megaprime kit, yielding an average activity of 0.2 μCi/ng. After hybridization, the filters were washed twice with 0.3 M NaCl, 0.03 M trisodium citrate and 1% (w/v) SDS, and twice with 0.15 M NaCl, 0.015 M trisodium citrate and 1% (w/v) SDS for 20 min periods at 63°C. The filters were then exposed to Kodak Hyperfilm with an intensifying screen at -80°C. The relative intensity of the bands present on the autoradiogram was read on a scanning densitometer.

Nuclear run-on assays were essentially performed according to Groudine et al. (21), with the modifications suggested by Twisk et al. (22).

cDNA probes

The following cDNA probes were used: a 2.5 kb *Eco*RI fragment of human PAI-1 cDNA (23); a 1.9 kb *Bgl*II fragment of human t-PA cDNA (24); a 1.2 kb *Pst*I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr. R. Offringa, University of Leiden, Leiden) (25); a 1.0 kb *Pst*I fragment of mouse c-*jun* cDNA (kindly provided by Dr. B. Hagmeyer, University of Leiden, Leiden) (26); and a 1.5 kb *Eco*RI fragment of the mouse c-*fos* cDNA (kindly provided by Dr. B. Hagmeyer) (27).

RESULTS

Effect of butyrate, TSA, retinoic acid and combinations thereof on t-PA synthesis

Figure 1 shows the effects of increasing concentrations of butyrate (0.1-3.0 mM), TSA (0.1-3.0 μM), retinoic acid (0.1-3.0 μM) and combinations thereof on t-PA production in cultured HUVEC over a 24 h period. In three separate experiments butyrate, TSA and retinoic acid maximally stimulated t-PA synthesis 7.7 ± 0.9-fold (mean±SD), 5.0 ± 0.5-fold and 2.4 ± 0.6-fold respectively. At higher test concentrations cells became partly detached from the matrix, which was accompanied by a fall in t-PA production. Whereas at suboptimal concentrations a combination of butyrate and TSA enhanced t-PA production more than did each compound alone, the synthesis of t-PA in the presence of both compounds was not any higher than with butyrate alone when butyrate and TSA approached maximally stimulatory concentrations (Figure 1a). These results suggest that butyrate and the specific histone deacetylase inhibitor TSA induce t-PA through a common mechanism. In contrast, retinoic acid more than additively enhanced butyrate-induced t-PA stimulation even at optimally stimulatory concentrations (Figure 1b). Similarly, retinoic acid had an additive effect on TSA-induced t-PA stimulation (results not shown). These data point to distinct mechanisms for t-PA induction by butyrate and TSA on the one hand and by retinoic acid on the other.

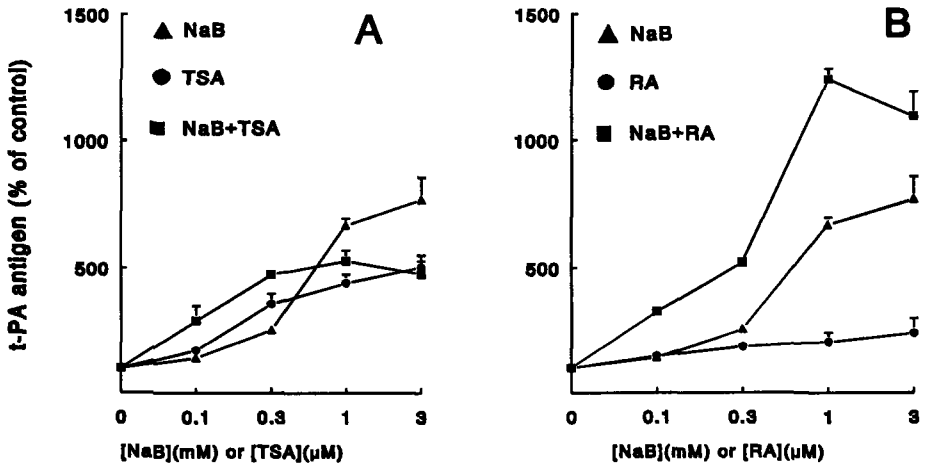


Figure 1 Effects of combinations of butyrate, TSA and retinoic acid on t-PA synthesis in HUVEC
HUVEC were incubated for 24 h with various concentrations of butyrate (0.1-3.0 mM), TSA (0.1-3.0 μM) and retinoic acid (0.1-3.0 μM), either alone or in combination. CM were collected and analysed for t-PA antigen as described in the Materials and methods section. Results are expressed as percentages of controls and are means ± S.D. for three independent experiments, with incubations in duplicate. (a) Combination of butyrate (NaB) and TSA; (b) combination of butyrate (NaB) and retinoic acid (RA).

Induction of t-PA mRNA and transcription by butyrate and TSA in HUVEC

Figure 2 shows a representative time course of the stimulatory effects of butyrate and TSA on t-PA mRNA in HUVEC. Induction of t-PA mRNA by butyrate (3 mM) or TSA (1 μM) became visible after 4 h, and t-PA mRNA levels continued to increase up to 16 h for both compounds. Upon further incubation, t-PA mRNA levels declined markedly in the TSA-treated cells but remained high in the butyrate-treated cells. To examine whether the observed t-PA mRNA induction profiles are dependent on the continuous presence of butyrate or TSA in the medium, HUVEC were treated with butyrate (3 mM) or TSA (1 μM) for 4 h to induce t-PA mRNA, then washed three times to remove the inducing compounds, and subsequently incubated with standard incubation medium. Total RNA was harvested at the indicated times after the wash-out, and analysed by Northern blotting. Autoradiograms were scanned and values were calculated relative to the corresponding GAPDH control. As shown in Figure 3 in a representative experiment, removal of butyrate or TSA from the incubation medium after 4 h resulted in a rapid decline in t-PA mRNA levels, pointing to a reversible action of the two compounds (compare the t-PA mRNA induction profiles in Figure 2). The slower decrease in t-PA

mRNA concentration in the butyrate-treated cells as compared with that in TSA-treated cells was consistently found in three separate experiments.

To investigate whether the induction of t-PA mRNA is regulated at the level of transcription, nuclear run-on experiments were performed. Under basal, non-stimulated conditions, t-PA transcription in HUVEC was found to be very low, and its signal was close to a prokaryotic control, pUC, which hampered an accurate assessment of the increases in t-PA transcription rate after treatment of the cells with butyrate or TSA. In two separate experiments we measured an increase in t-PA transcription rate as early as 4 h after the addition of butyrate (3 mM) or TSA (1 μM) to HUVEC (Figure 4). With both compounds a maximal 4-fold elevated transcription rate was observed after 6-8 h of incubation. In the following 16 h, t-PA transcription rates declined to control levels (with butyrate) or even below (with TSA). None of the experimental conditions resulted in a marked change in the PAI-1 transcription rate (Figure 4).

The induction profile of t-PA seen with butyrate at the transcriptional level deviates from that at the mRNA level beyond 8 h: whereas t-PA transcription declines to control levels, t-PA mRNA levels increase further (compare Figures 2 and 4), suggesting a post-transcriptional effect of butyrate, for example an increase in t-PA mRNA stability. An inhibition of t-PA mRNA degradation in butyrate-incubated HUVEC would be in line with the data presented in Figure 3, where the decline in t-PA mRNA was found to be slower in butyrate-pretreated cells than that in TSA-pretreated cells. Experiments aimed at direct measurement of t-PA mRNA stability by adding actinomycin D to the cells proved to be unsuccessful, since actinomycin D was found to significantly inhibit t-PA mRNA degradation (results not shown).

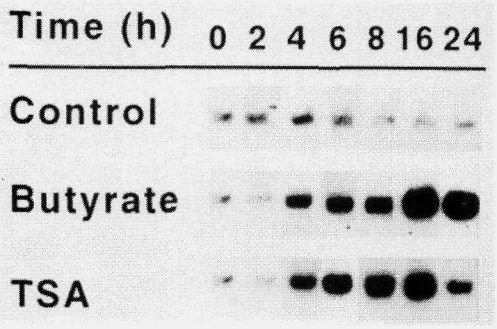


Figure 2 Time-course of the effects of butyrate and TSA on t-PA mRNA levels in HUVEC
HUVEC were incubated for various times up to 24 h with butyrate (3 mM), TSA (1 μM) or control medium, and RNA was isolated as described in the *Materials and methods section*. Northern hybridizations were performed using t-PA and GAPDH cDNA probes. Equal amounts of RNA, as checked by the GAPDH signal, were applied in each lane. The blot was exposed for 1 week at -80°C to Hyperfilm using an intensifying screen. A representative experiment (out of four) is shown.

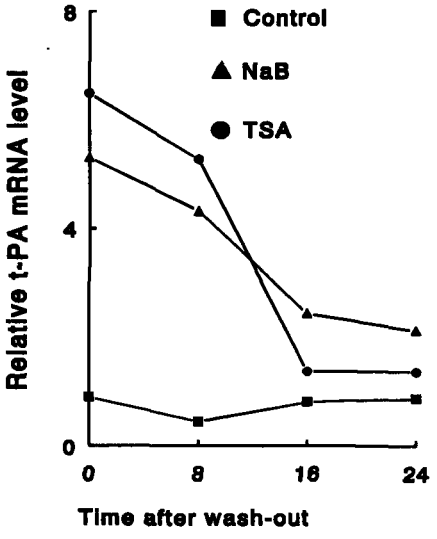


Figure 3 t-PA mRNA levels in butyrate- or TSA- pretreated HUVEC, following wash-out of inducers HUVEC were incubated for 4 h with butyrate (NaB; 3 mM), TSA (1 μM) or control medium, then washed three times to remove the inducing compounds, and subsequently incubated with control incubation medium. RNA was harvested at the indicated times after the wash-out, and analyzed for t-PA mRNA as described in Figure 2. Autoradiograms were scanned and t-PA mRNA levels were expressed relative to the t-PA mRNA level present in control cells immediately after the wash-out. A representative experiment (out of three) is shown.

Effects of butyrate and TSA on histone acetylation

To assess whether the pattern of butyrate- and TSA-induced changes in t-PA transcription rate corresponds to parallel changes in the acetylation of histone proteins, histone fractions were prepared from butyrate-, TSA-, and (for comparison) retinoic acid-treated HUVEC, and subjected to AUT gel electrophoresis. AUT gel electrophoresis allows separation of each individual cellular histone (H1, H2A, H2B, H3 and H4) and their variously acetylated species due to slower migration rates of the acetylated forms. Upon incubation of HUVEC with either butyrate (3 mM) or TSA (1 μM), the predominantly non-acetylated form of histone H4 present in control cells was converted into mono-, di- and tri-acetylated species of H4, whereas no changes in the gel mobility of histones H1, H2A, H2B and H3 were observed, as illustrated in Figure 5a for a 6 h incubation period. No effect of retinoic acid (1 μM) on histone acetylation was seen. Figures 5b and 6 show the time course and the dose-dependency of the changes in the acetylation of histone H4 upon incubation of HUVEC with various concentrations of butyrate and TSA. With both butyrate (3 mM) and TSA (1 μM), the disappearance of the non-acetylated H4 form was detectable as early as 2 h after the onset of the experiment (Figure 5b). However, whereas with butyrate the disappearance of the non-acetylated H4 form continued, to give almost negligible levels at 16 h, with TSA the maximal disappearance of the non-acetylated H4 form was reached at 2 h, with about 40% of H4 remaining in the non-

acetylated form (see also Figure 6). Also, with butyrate the amount of non-acetylated H4 remained very low beyond 16 h, whereas with TSA the amount of non-acetylated H4 had returned to control levels at 16 h. The increases in the amounts of mono-, di-, and tri-acetylated species of H4, along with the concomitant decreases in the non-acetylated form, in HUVEC exposed to 0.3, 1.0 or 3.0 mM butyrate or 0.1, 0.3 or 1.0 μM TSA are illustrated in Figure 6 at various time points. The degree of acetylation showed a similar butyrate- and TSA-dose-dependence as the induction of t-PA. In the case of 0.1 μM TSA, which barely stimulated t-PA synthesis, the H4 acetylation profile did not differ markedly from that of non-treated cells.

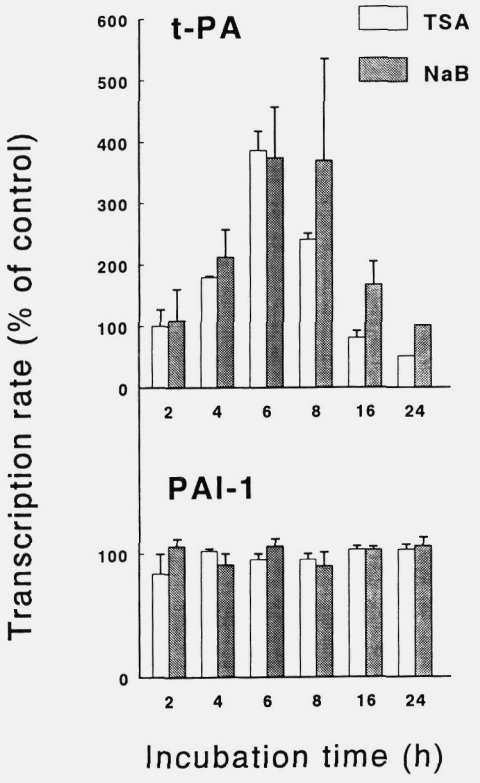


Figure 4 Effects of butyrate and TSA on t-PA gene transcription in HUVEC HUVEC were incubated for various times up to 24 h with butyrate (NaB; 3 mM), TSA (1μM) or control medium; nuclei were isolated and t-PA and PAI-1 transcription was determined by nuclear run-on assays as described in the Materials and methods section. Blots were exposed for 4 weeks at -80°C to Hyperfilm using an intensifying screen, and autoradiograms were scanned. Results are expressed as percentages of controls and are presented as means ± range for two independent experiments.

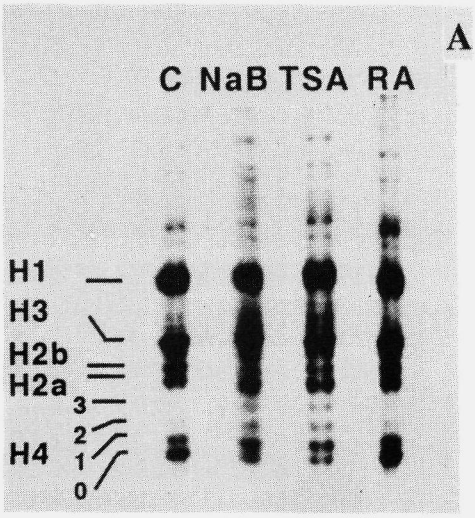
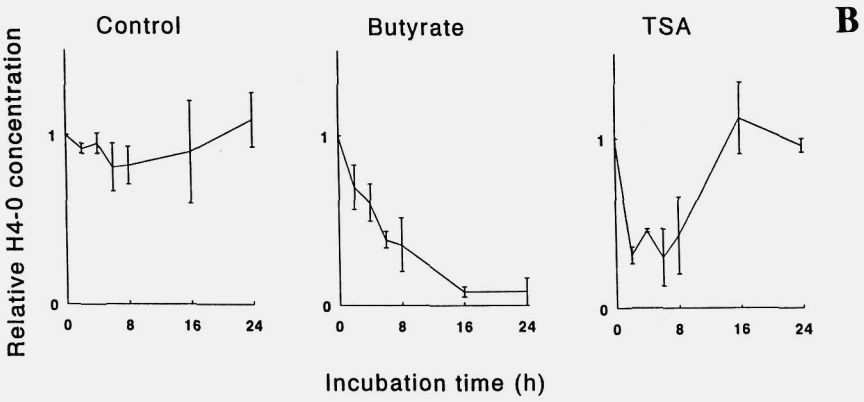


Figure 5 Time-course of the effects of butyrate, TSA and retinoic acid on histone acetylation in HUVEC HUVEC were incubated for various times up to 24 h with butyrate (3 mM), TSA (1μM), retinoic acid (1 μM) or control medium. Histones were extracted from the cells and analysed by AUT gel electrophoresis as described in the Materials and methods section. (a) A stained gel is shown after a 6 h incubation of HUVEC with control medium (C), butyrate (NaB), TSA or retinoic acid (RA). The numbers 0-3 indicate the numbers of acetyl groups present in histone H4. A representative experiment (out of 3) is shown. (b) The concentration of non-acetylated histone H4 (H4-0) versus incubation time is shown for control cells, butyrate-treated cells and TSA-treated cells. Concentrations were determined by scanning of stained acid/urea gels, and are expressed relative to the amount of non-acetylated H4 present at the start of the experiment. The data shown are means ± range for two independent experiments.



Effects of butyrate, TSA and retinoic acid on c-*jun* and c-*fos* expression

Although the above results are consistent with a role for histone acetylation in the induction of t-PA gene expression by butyrate, they do not exclude the possibility that this induction occurs via the expression of newly synthesized transcription factors. Indeed it has been reported that, upon differentiation of the embryo carcinoma cell line F9 by butyrate or retinoic acid, t-PA gene expression is induced, and that newly synthesized c-Jun may participate in this activation of t-PA expression (11-13). To determine whether c-Jun and/or c-Fos contribute to the induction of t-PA gene expression by butyrate, TSA or retinoic acid in HUVEC, we analysed RNA extracted from HUVEC for the expression of

c-*jun* and c-*fos* using Northern blot hybridization at different time points after addition of the compounds. Only retinoic acid was found to induce c-*jun* mRNA, and none of the three compounds induced c-*fos* expression (results not shown). These results exclude a role for c-*jun* and c-*fos* in the induction of t-PA by butyrate or TSA. Also, we found that the induction of t-PA mRNA by butyrate or TSA, in contrast to the induction by retinoic acid, does not require ongoing protein synthesis, as deduced from experiments in the presence of the protein synthesis inhibitor, cycloheximide (data not shown). These results point to a direct involvement of histone acetylation in the induced expression of t-PA by butyrate and TSA, rather than an indirect pathway involving the synthesis of new transcription factors. The results also confirm the difference in regulatory mechanism between butyrate/TSA and retinoic acid in inducing t-PA expression.

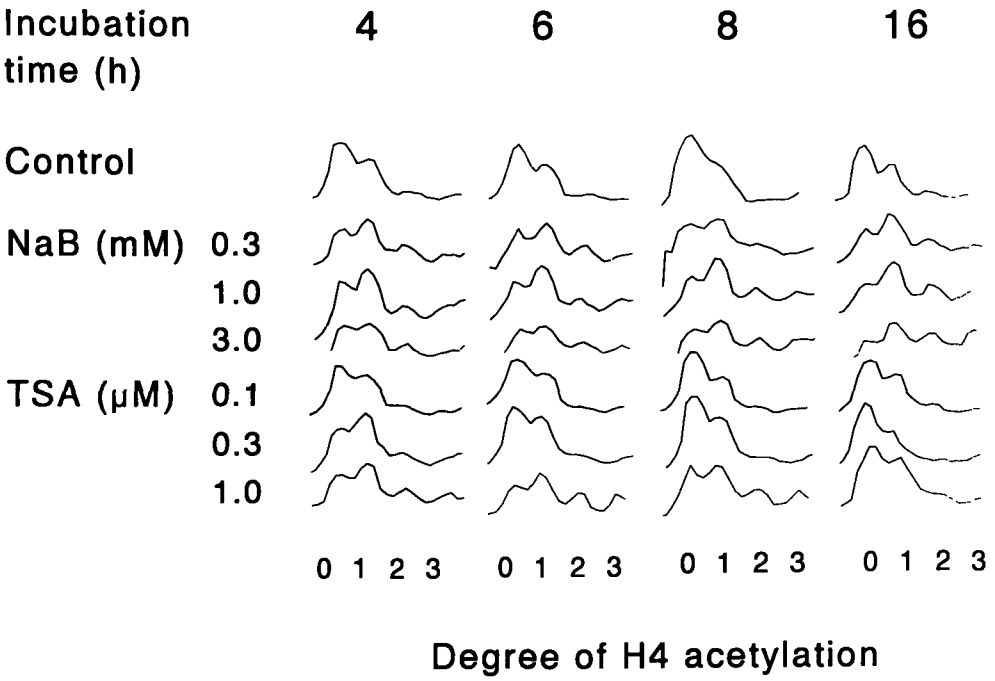


Figure 6 Time-course and dose-dependence of the effects of butyrate and TSA on histone H4 acetylation in HUVEC HUVEC were incubated for various times up to 16 h with various concentrations of butyrate (NaB), TSA or control medium, as indicated. Histones were extracted from the cells and analysed by AUT gel electrophoresis as described in the Materials and methods section. Scans are shown of the histone H4 regions of stained AUT gels. The numbers beneath the peaks of the gel scans indicate the numbers of acetyl groups present in H4.

DISCUSSION

In a previous paper we reported that the synthesis of t-PA in HUVEC is greatly increased by the pleiotropic agent butyrate (3). The following results in the present paper suggest that histone H4 acetylation is involved in the induction of t-PA in HUVEC by butyrate. (1) Butyrate-induced t-PA stimulation is a fast process, being visible at the transcriptional level within 4 h, as shown by run-on assay (Figure 4). (2) In contrast to F9 cells, in which butyrate and retinoic acid have been reported to induce c-*jun*, and c-Jun expression has been associated with t-PA induction (11-13), no c-*jun* (or c-*fos*) induction occurred in HUVEC upon butyrate treatment. Furthermore, t-PA induction by butyrate did not depend on ongoing protein synthesis, indicating a direct effect. (3) The induction of t-PA by butyrate can be mimicked by a specific, structurally unrelated, histone deacetylase inhibitor, TSA, but not by another differentiating agent, retinoic acid (Figures 1 and 4). In accordance with the much lower K_i value of TSA for the histone deacetylase (14), much lower concentrations of TSA (optimal concentration 1 μM) than of butyrate (optimal concentration 3 mM) were required for maximal induction of t-PA expression. (4) Simultaneous addition of both butyrate and TSA gave no additive effect on t-PA stimulation, indicative of a common regulatory mechanism (Figure 1). (5) Both butyrate and TSA induced a time- and dose-dependent increase in the amounts of mono-, di- and tri-acetylated species of histone H4, along with a concomitant decrease in the non-acetylated form; the onset of H4 acetylation preceded the increase in t-PA transcription (Figures 4 and 5), and the changes in the degree of H4 acetylation at differing concentrations of butyrate and TSA paralleled changes in t-PA expression (Figures 1 and 6).

Although the results obtained with the pleiotropic agent butyrate, resembled largely those obtained with the more potent and specific histone deacetylase inhibitor, TSA, they differed in their effects on t-PA mRNA stability. After 16-24 h of butyrate-treatment we found markedly elevated t-PA mRNA levels, despite the fact that t-PA transcription had already returned to basal levels. Similar effects of butyrate, namely modulation of both transcription and mRNA stability, have been reported for c-*fos* mRNA in F98 rat glioma cells and Caco-2 colon adenocarcinoma cells (28-29). The mechanism involved remains elusive.

Various lines of experimental evidence have shown a correlation between transcriptional activity and increased histone acetylation, including histone H4 acetylation. A direct link between histone acetylation and active genes was established in the studies

of Crane-Robinson and colleagues in which they used an antibody against the epitope *N*-ε-acetyl-lysine for the isolation of acetylated nucleosomes from embryonic chicken chromatin (30, 31). On probing with gene sequences, antibody-bound chromatin was found to be enriched in the actively transcribed α-globin gene and not enriched in the inactive ovalbumin gene. The importance of histone H4 acetylation in the control of transcription can be inferred from genetic experiments with *Saccharomyces cerevisiae* in which deletion of the histone H4 N-terminal region or substitution of the specific modifiable lysine residues resulted in a decrease in the activation of the GAL1 and PHO5 promoters (32). Similarly, the human female inactive X chromosome did not stain with antibodies to acetylated H4, in contrast to all the other chromosomes (33). Histone acetylation is believed to have a positive role in facilitating transcription factor access to promoter sequences, thus facilitating transcription. For example, *in vitro* reconstitution experiments by Lee et al. (8) showed that the presence of acetylated H4 facilitates the binding of the transcription factor TFIIIA to its target sequences without nucleosome displacement.

One might expect that such global modifications of chromatin structure induced by butyrate or TSA would lead to a general increase in the expression of many genes that were formerly silent. However, when SDS/PAGE was used to analyse the proteins synthesized after a 24 h incubation with 5 mM butyrate, very few differences were found from the normal proteins made (3). Also, Northern blot analysis showed no significant increases in mRNA levels of several genes tested, including urokinase-type plasminogen activator, PAI-1 and GAPDH (J. Arts and T. Kooistra, unpublished work). Similar results have been reported by others for different cell types (5). Apparently, high levels of histone acetylation are not sufficient for active transcription, but relate rather to the transcriptional competence of the gene (31). In this respect it should be noted that t-PA transcription in the presence of butyrate had returned to control levels even when histone H4 was still in a highly acetylated form (Figures 4 and 5). It remains to be determined which factors other than histone H4 acetylation itself are responsible for the selective switching of the t-PA gene in HUVEC in the presence of butyrate or TSA.

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CHAPTER 5

Role of c-Jun and proximal phorbol 12-myristate-13-acetate-(PMA)-responsive elements in the regulation of basal and PMA-stimulated plasminogen-activator inhibitor-1 gene expression in HepG2

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SUMMARY

Experiments were designed to clarify the role of c-Jun/c-Fos and of putative phorbol 12-myristate 13-acetate (PMA) responsive elements (TREs) in the induction of plasminogen-activator inhibitor 1 (PAI-1) gene transcription in the human hepatoma cell line HepG2 by activators of protein kinase C. Treatment of HepG2 cells with the phorbol ester PMA or serum rapidly and transiently increased c-Jun and c-Fos mRNA and protein levels prior to PAI-1 induction. This induction of PAI-1 gene transcription was found to be dependent on ongoing protein synthesis. An essential role of c-Jun and c-Fos in basal and PMA-stimulated transcription of the PAI-1 gene is demonstrated by our finding that antisense c-*jun* and c-*fos* oligodeoxynucleotides both strongly reduced basal and PMA-stimulated PAI-1 synthesis. Since it has already been shown that two TREs between positions -58 and -50 and between -79 and -72 of the PAI-1 promoter are essential for basal and PMA-induced PAI-1 promoter activity ([16]), we examined binding of nuclear proteins to these elements. The protein binding activity to the TRE between positions -79 and -72 shows a very strong PMA induction of an unknown factor, which is not related to c-Jun or c-Fos. The TRE binding between positions -58 and -50 forms two complexes, both containing c-Jun protein. The faster migrating complex primarily contains c-Jun homodimers. The amount of the faster migrating complex is enhanced more than 30-fold in PMA-treated cells, due to a strongly increased binding of c-Jun homodimers and, to a minor extent, to binding of c-Jun/c-Fos heterodimers. Dissociation experiments suggest that the c-Jun/c-Fos heterodimers bind with much lower affinity as compared to binding of c-Jun homodimers. Together with the finding that both antisense c-*jun* and antisense c-*fos* oligodeoxynucleotides reduced the amount of c-Jun homodimer, we conclude that binding of c-Jun homodimer to the TRE at positions -58 to -50 is important in the basal activity and PMA activation of the PAI-1 promoter in HepG2 cells.

INTRODUCTION

Plasminogen-activator inhibitor 1 (PAI-1), the major physiological inhibitor of tissue-type plasminogen-activator (t-PA) in plasma, is considered to be a critical component in the regulation of vascular fibrinolysis. High plasma levels of PAI-1 have been associated with deep venous thrombosis [1-3], a high mortality in patients with septic shock [4, 5], and an increased risk of recurrent myocardial infarction [6], while deficiency of PAI-1 in humans

results in an abnormal bleeding tendency [7-9]. Transgenic mice which overexpress PAI-1 develop venous thrombi [10] and PAI-1-deficient mice demonstrate an enhanced fibrinolytic capacity [11], which is consistent with an important role of PAI-1 in fibrinolysis. In addition, PAI-1 has been implicated in the regulation of several other plasminogen-dependent processes, including tissue remodelling and cell migration (for reviews, see [12, 13]).

Given the importance of PAI-1 as a regulator of many biological processes under both normal and pathological conditions, regulation of its synthesis is a critical feature which has been the subject of many studies. PAI-1 expression has been demonstrated in various cell types, and multiple factors have been identified that play a role in the regulation of PAI-1 synthesis and secretion [13, 14]. However, the exact regulatory mechanisms by which these factors alter PAI-1 expression are not yet clearly understood. A transcription factor that has been implied but not proved to be involved in the induction of PAI-1 gene expression by protein kinase C (PKC) activators such as phorbol 12-myristate 13-acetate (PMA), serum and growth factors, is activator protein-1 (AP-1) [15, 16]. AP-1 consists of either a homodimer of the Jun protein or a heterodimer of the Jun and the Fos proteins, and functions as a transcriptional activator for genes that contain a specific sequence in their promoter, TGAg/cTCA, referred to as a PMA-responsive element (TRE) [17]. The 5'-flanking region of the human PAI-1 gene contains four putative AP-1 like binding sites [18]: two proximal sites, at positions -58 to -50 (TGAGTTCA) and at positions -79 to -72 (TGAGTGA), and two distal sites, at positions -721 to -714 (TGACACA) and at positions -662 to -656 (TGTATCA). Depending on the cell type and stimulus used, different TREs have been found to be involved in the regulation of PAI-1 gene transcription. For example, induction of PAI-1 by TGFß has been shown to involve the two distal TREs [15], while the phorbol ester PMA induces PAI-1 transcription through the proximal TREs only [16, 19]. Involvement of a consensus TRE in PAI-1 transcription does not necessarily imply a role for AP-1, however. Knudsen *et al.* [19] demonstrated that the proximal TRE at -58 to -50 bp was required for PAI-1 induction by PMA in MCF-7 cells, but that this site was occupied by an as yet unidentified nuclear protein, despite the presence of AP-1 activity. In all, no direct evidence is available for a role for AP-1 in PAI-1 gene expression.

We have previously shown that PMA rapidly induces PAI-1 transcription in the human hepatoma cell line HepG2 [20]. We now show that serum and PMA induce the expression of the immediate-early response genes c-*jun* and c-*fos*, and that this induction precedes that of PAI-1 gene transcription. Experiments with antisense c-*jun* and c-*fos* oligodeoxynucleotides confirm the essential role played by AP-1 in PAI-1 gene transcription. Transient transfection experiments combined with electromobility shift assays identify the

TRE at positions -58 to -50 and c-Jun homodimers rather than c-Jun/c-Fos heterodimers as one of the nuclear targets of PMA in PAI-1 gene induction.

MATERIALS AND METHODS

Materials

Curcumin, n-butyryl Coenzyme A (lithium salt) and PMA were from Sigma Chemical Co. A stock solution of PMA (100 μM) was prepared in ethanol and kept at -20°C. The anti-c-Jun and anti-c-Fos rabbit polyclonal antibodies were a generous gift from Dr T. Oehler, Massachusetts Institute of Technology, Cambridge, USA. The anti-72 kD transcription factor rabbit polyclonal antibody was provided by Dr A. Belayew, Center for Thrombosis and Vascular Research, University of Leuven, Belgium. The anti-ATF2 rabbit polyclonal antibody was a gift from Dr N.C. Jones, Gene Regulation Laboratory, Imperial Cancer Research Fund, London, UK. Anti-SP-1 and anti-ATF2 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology. Deoxycytidine 5[α-^{32}P]triphosphate (3 Ci/mol), uridine 5[α-^{32}P]triphosphate (> 400 Ci/mol), 5[γ-^{32}P]triphosphate (3 Ci/μmol), [^{35}S]methionine (> 1 Ci/μmol), D-threo-[^{14}C]dichloroacetyl chloramphenicol (50 Ci/mol) and the Megaprime-kit were obtained from Amersham International plc. Human serum albumin (20% mass/vol.; pyrogen free) was obtained from the Central Laboratory of the Red Cross Blood Transfusion Service. Enzyme immunoassay kits for determination of human PAI-1 antigen (Imulyse) and human fibrinogen were obtained from Biopool and Organon Teknika, respectively. Other materials used in the methods described below have been specified in detail in relating references or were purchased from standard commercial sources.

Oligodeoxynucleotides

The (unmodified) antisense oligodeoxynucleotides were synthesized by MWG-Biotech, and had the following sequences: c-*fos*: 5'-CCGAGAACATCATCGTGG-3'; c-*jun*: 5'-GCAGTCATAGAACAGTCC-3'; and nonsense: 5'-ATCGCCTGTAGTGAACTG-3'. The oligodeoxynucleotides used for the bandshift assays were synthesized by Isogen Bioscience (Amsterdam, The Netherlands), and had the following sequences: TRE consensus: 5'-CGCTTGATGAGTCAGCCGGAA-3'; positions -66 to -43 PAI-1 promoter region: 5'-CTGGAACATGAGTTCATCTATTT-3'; positions -82 to -60 PAI-1 promoter region: 5'-GCCAGTGAGTGGGTGGGGCTGG-3'; aspecific competitor: 5'-CTGAGGATTCTCCACTGCA-3'.

cDNA probes and plasmids

The following cDNA fragments were used as probes in the hybridization experiments: a 2.5-kb *Eco*RI fragment of the human PAI-1 cDNA [21]; a 1.2-kb *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [22]; a 1.0-kb *Pst*I fragment of the mouse c-*jun* c-DNA [23]; and a 1.5-kb *Eco*RI fragment of the murine c-*fos* c-DNA [24]. PAI-1 promoter- chloramphenicol acetyl transferase (CAT) constructs were made by digestion of the PAI-1 promoter with *Eco*RI, ligation of HindIII linkers to the *Eco*RI site, and blunting by endfilling with Klenow. After digestion with *Bgl*II, the 4.4-kb *Bgl*II-*Hin*dIII fragment of the PAI-1 promoter was cloned into the *Hin*dIII/*Bam*HI sites of pKTCAT, a plasmid containing the *Bam*HI/*Hin*dIII CAT fragment of pSVO CAT [25] cloned into the *AAT*II-*Hin*dIII sites of pUC18. From the resulting plasmid, pKTPAI, smaller constructs were generated, using the *Ava*II sites at positions -114, -343, -489 and -1023, the *Apa*I sites at positions -524 and -594, and the NheI site at position -1561. After restriction enzyme digestion the sites were made blunt by endfilling with Klenow. All constructs have identical 3'-ends (the *Eco*RI site at position +75).

Cell culture

HepG2 cells were grown as monolayer cultures in a 5% CO_2/ 95% air atmosphere at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% (by vol.) fetal bovine serum (heat inactivated), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, as described previously [20]. For experiments, confluent cultures were used. Cells were serum-starved by incubating the cells for 16 h with incubation medium, Dulbecco's modified Eagle's medium supplemented with 0.1% (mass/vol.) human serum albumin, 100 IU/ml

penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Conditioned media were obtained by incubating cells at 37°C for various time periods with incubation medium containing the appropriate test compound or stock solvent (final concentration maximally 0.1%, by vol.). Conditioned media were centrifuged for 4 min at 5000 g in a Beckman Microfuge to remove cells and cellular debris, and samples were frozen at -20°C until use. Cells were washed twice with ice-cold phosphate-buffered saline (0.15 M NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and were used for isolation of RNA or preparation of nuclear extracts.

Northern blot analysis

Total RNA was isolated as described previously by Chomczynski and Sacchi [26] and electrophoresed in a 1% (mass/vol.) agarose gel under denaturing conditions using 1 M formaldehyde. The RNA was transferred to Hybond N by blotting and the filters were hybridized overnight at 63°C in hybridization mix [7% (mass/vol.) SDS, 0.5 M sodium phosphate pH 7.2, 1 mM EDTA] containing 3 ng [α-^{32}P]CTP-labelled probe/ml. The probes were labelled with a Megaprime kit to approximately 0.2 μCi/ng DNA. After hybridization with PAI-1 or glyceraldehyde-3-phosphate dehydrogenase probe, the filters were washed twice with 2xSSC (1xSSC being 0.15 M NaCl, 0.15 M trisodium citrate-dihydrate), 1% (mass/vol.) SDS and twice with 1xSSC, 1% (mass/vol.) SDS for 20 min time periods at 63°C. In the case of hybridization with c-*jun* or c-*fos* probes, the filters were washed with 2xSSC, 1% (mass/vol.) SDS for four 20 min periods at 63°C. The filters were then exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C. The intensity of the bands present on the autoradiogram was read on a scanning densitometer.

Nuclear run-on transcription assay

Nuclear run-on transcription assays were performed essentially as described by Greenberg and Ziff [27] and Nevins [28]. Isolated nuclei were resuspended in 50 mM Tris, pH 8.3, 40% (by vol.) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA (glycerol buffer), frozen in liquid nitrogen and stored at -80°C until use. Per run-on assay approximately $5x10^6$ nuclei were used. Nuclei were then incubated for 20 min at 30°C in 200 μl 20 mM Tris pH 7.9, 20% (by vol.) glycerol, 140 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of ATP, CTP and GTP, 2 μM [α-^{32}P]UTP, 10 mM creatine phosphate, 20 U/ml creatine kinase and 1000 U/ml RNAsin. The incubation was ended by adding 800 μl HSB buffer (10 mM Tris pH 7.4, 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 U/ml DNAse 1). As soon as the viscosity had disappeared, 1 ml of 10 mM Tris pH 7.4, 1% SDS, 20 mM EDTA was added. The RNA was isolated by hot phenol extraction. Contaminating DNA was further degraded by DNAse treatment for 20 min at 37°C in 20 mM Tris, pH 7.7, 1 mM $MgCl_2$. After phenol extraction, the RNA was partially degraded by incubation for 20 min with ice-cold 0.2 M NaOH, and neutralized with HEPES. To remove the unincorporated radiolabel, repeated ethanol precipitations with high salt content were performed. 2 μg linearized DNA plasmid (pGEM4Z) containing the appropriate cDNA was blotted onto Hybond N in alkali blotting solution (1.5 M NaCl, 0.25 M NaOH) using a minifold filtration apparatus. Prehybridization and hybridization were performed at 65°C in 0.5 M NaH_2PO_4-Na_2HPO_4 pH 7.2, 7% (mass/vol.) SDS and 1 mM EDTA. The RNA was hybridized for 65 h, after which the filters were washed several times with 2xSSC. Quantification was performed by exposing the filter to Hyperfilm, followed by densitometric scanning of the dots.

Immunoprecipitation of c-Jun and c-Fos protein

HepG2 cells (10 cm^2) were preincubated for 16 h in Dulbecco's modified Eagle's medium containing 0.1% (mass/vol.) human serum albumin, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine, then incubated in methionine-free Dulbecco's modified Eagle's medium supplemented with 0.1% (mass/vol.) human serum albumin, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 0.2 mCi/ml [^{35}S]methionine. Cell extracts were prepared as described by Kruijer et al. [29]. In brief, cells were washed twice with ice-cold phosphate-buffered saline, and harvested by scraping in 0.5 ml 50 mM Tris pH 8.0, 125 mM NaCl, 0.5% (by vol.) Nonidet P-40, 0.5% (mass/vol.) sodium deoxycholate, 0.1% (mass/vol.) SDS and the proteinase inhibitors leupeptin (1 μg/ml), pepstatin A (1 μg/ml), aprotinin (1 μg/ml) and phenylmethylsulfonyl fluoride (0.5 mM). The cell lysates were centrifuged in a Beckman TL-100 at 150,000 rpm (30 min, 4°C). The cleared cell lysates were then incubated for 1 h at 4°C with pre-immune rabbit serum coupled to protein A-Sepharose under continuous rotation. The protein A-Sepharose was removed (30 sec 10,000 g, 4°C) and the supernatant was subsequently used to immunoprecipitate c-Fos complexes by the same

procedure, using an anti-c-Fos rabbit polyclonal antibody which predominantly recognizes c-Fos, but has also a low affinity for FosB. Finally, the c-Fos depleted and c-Fos/c-Jun depleted extract was incubated with anti-c-Jun antiserum coupled to protein A-Sepharose. An anti-c-Jun polyclonal antibody was routinely used which had been shown to recognize c-Jun [30]. An anti-c-Jun monoclonal antibody (Santa Cruz Biotechnology) was also used, which gave identical results to those obtained with the polyclonal anti-c-Jun antibody. All immunoprecipitates were washed four times with 1 ml lysis buffer and once with phosphate-buffered saline, were then resuspended in Laemmli sample buffer [62.5 mM Tris pH 6.8, 10% (mass/vol.) glycine, 2% (mass/vol.) SDS, 5% (by vol.) 2-mercaptoethanol, 0.02% (mass/vol) bromophenolblue) [31] and boiled for 5 min. The samples were subjected to electrophoresis (10 mA, 16 h) on a 10% (mass/vol.) SDS-polyacrylamide gel. To enhance signals, the gel was impregnated with 22% (mass/vol.) 2,5-diphenyloxazole. The gel was dried on Whatman-3MM paper and exposed to Kodak XAR-5 film at -80°C in order to visualize labelled proteins.

Immunofluorescence of c-Jun and c-Fos protein

HepG2 cells were seeded at a density of $6x10^5$ cells/10 cm^2 dish one day before the experiment. Each 10 cm^2 dish contained three glass coverslips coated with fibronectin. After serum-starvation for 16 h, the medium was replaced with fresh incubation medium containing PMA (100 nM). After 1.5 h, the glass coverslips were washed twice with phosphate-buffered saline, and cells were incubated in 4% (by vol.) formaldehyde in phosphate-buffered saline at room temperature for 8 min. Nuclei were permeabilized by incubating for 10 min in 0.2% (by vol.) Triton X-100 in phosphate-buffered saline. The coverslips were then kept at room temperature for 1 h, turned upside down on top of a drop of 30 μl of anti-c-Jun or anti-c-Fos polyclonal antiserum or merely antibody dilution buffer [phosphate-buffered saline supplemented with 10% (by vol.) fetal bovine serum]. After washing four times with phosphate-buffered saline, the coverslips were incubated with a second antibody, tetramethyl rhodamine isothiocyanate-conjugated, affinipure goat anti-rabbit IgG (H+L) (Jackson Immunoresearch Laboratories) for 35 min and examined with a fluorescence microscope.

Transfection procedure

HepG2 cells were seeded at a density of $6x10^5$ cells/10 cm^2 dish one day before the transfection procedure. Calcium phosphate-DNA precipitates were prepared by the method of Graham and Van der Eb [32]. A total amount of 1 μg DNA per 10 cm^2 dish was used in all cases and, as a measure for transfection efficiency, cells were transfected with a ß-galactosidase expression plasmid. 4 h after the addition of the precipitates, the cells were incubated with 15% (by vol.) glycerol in Dulbecco's modified Eagle's medium for 1 min, then incubated with culture medium for 3 h. Cell lysates were prepared 8 h after serum starvation and analyzed for CAT activity.

Preparation of nuclear extracts for gel shift experiments

For gel shift experiments, confluent cultures of HepG2 cells (25 cm^2) were rinsed twice with ice-cold phosphate buffered saline and lysed in 2 ml 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.25 mM vanadate and 1 μg/ml of the protease inhibitors leupeptin, pepstatin and aprotinin. The lysate was homogenized in a potter (20 strokes); nuclei were collected by centrifugation (5 min at 1000 g, 4°C), and washed with lysis buffer once more. The dry nuclear pellet was resuspended in 150 μl of 20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM vanadate and 1 μg/ml leupeptin, pepstatin and aprotinin. This suspension was incubated for 15 min at 4°C while being continuously shaken and then centrifuged at 1000 g, 4°C for 5 min. Supernatants were stored at -80°C until use. The protein concentration in the nuclear extract was determined using the Bradford protein assay.

Gel shift experiments

Oligodeoxynucleotides were end-labelled using T_4-kinase and were subsequently purified by phenol/chloroform extraction, followed by precipitation. For EMSA, 25 fmol (about 10^4 cpm) radiolabelled double-stranded oligodeoxynucleotide was mixed with nuclear extract (5 μg protein) in a total volume of 20 μl 20 mM Hepes (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$, 20% (by vol.) glycerol, 2.5 mM EDTA, 2 mM spermidine, 1 μg poly(dI-dC), 1 μg bovine serum albumin and 1 mM phenylmethylsulfonyl fluoride. The mixture was incubated at 4°C for 30 min. In the case of a supershift experiment, the nuclear extract was incubated for 1 h with the appropiate antibody prior to the binding reaction. DNA/protein complexes were separated from the non-bound oligodeoxynucleotide by electrophoresis on a 5% polyacrylamide gel in 0.25x 22.5 mM Tris-borate, 0.5 mM EDTA [33]. Electrophoresis was carried out at room temperature at 150 V for 70 min, using the above buffer as running buffer. The gel was dried on Whatman-3MM paper and exposed to Kodak XAR-5 film at -80°C in order to visualize DNA/protein complexes.

Assays

For determination of human PAI-1 antigen in the conditioned media, the Imulyse-kit from Biopool was used. CAT activity was measured according to the method of Seed and Sheen [34], using equal amounts of cell extract protein. Protein was measured using the Bradford protein assay. Overall protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into the 10% (mass/vol.) trichloroacetic acid-precipitable fraction of radiolabelled conditioned medium and cell extract [35].

RESULTS

Time course of serum- and PMA-induced PAI-1 gene expression.

Incubation of serum-starved HepG2 cells with optimally inducing concentrations of serum [10% (vol./vol.); Fig. 1A) or PMA (100 nM; Fig. 1B) transiently enhanced PAI-1 mRNA levels after a lag period of 1 h, but with different time profiles and to different extents. After serum addition, total PAI-1 mRNA (i.e. the 3.2 kb form plus the 2.4 kb form) reached maximally nine-fold increased levels at 4 h, remained at this enhanced level up to about 8 h, then declined slowly with an apparent half life of about 4 h. The induction of PAI-1 mRNA by PMA was much stronger and shorter, reaching maximally a 32-fold increase after 4 h, followed by an immediate decline to prestimulatory levels with an apparent half-life time of about 3 h. The effects of serum and PMA were directed at the level of PAI-1 gene transcription as indicated by the results of nuclear run-on experiments (Fig 1). Serum induced PAI-1 transcription maximally about 10-fold at 2.5 h, transcription then decreased slowly, and was still five-fold enhanced at 7.5 h. PAI-1 transcription after PMA-treatment was maximally induced 36-fold after 2 h; this was immediately followed by a rapid decline to prestimulatory levels at 7.5 h. The increased transcription rates can thus account fully for the effects of serum and PMA on PAI-1 mRNA levels.

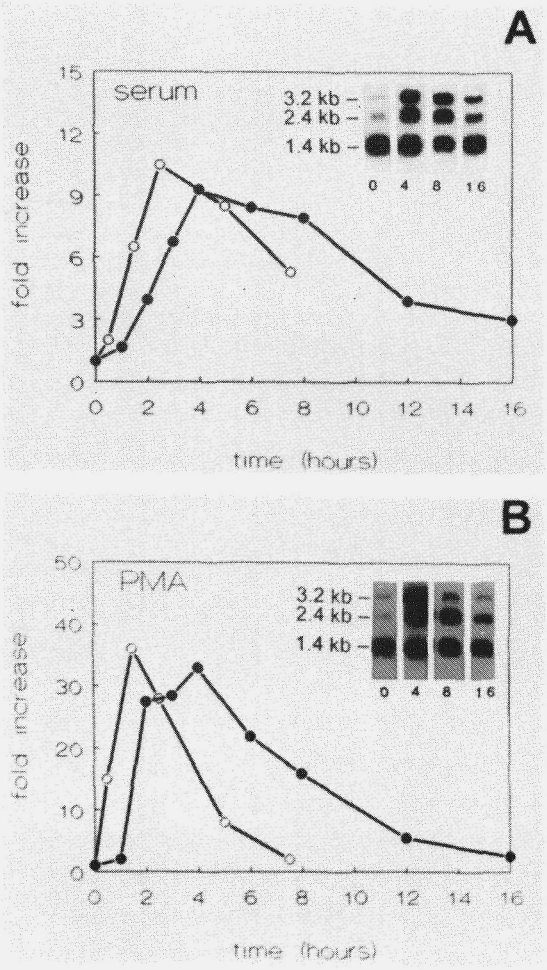

Fig. 1. Time course of the increase of PAI-1 mRNA levels (•) and gene transcription rate (o) by serum and PMA in HepG2 cells. HepG2 cells were preincubated with serum-free medium for 16 h, and then treated with serum (10%, panel A) or PMA (100 nM, panel B). At the indicated times, RNA or nuclei were isolated. The amount of PAI-1 mRNA present is given relatively to t=0, and is the mean out of four independent experiments. mRNA bands were analyzed by densitometric scanning. The insets show at various times the PAI-1 mRNA (3.2 and 2.4 kb bands) and the glyceraldehyde-3-phosphate dehydrogenase mRNA (1.4 kb band), the latter as a reference for the amount of RNA on the gel. To determine transcription rates nuclear run-on assays were performed. Radiolabelled RNA was hybridized to linearized plasmid DNA, containing the cDNA of PAI-1 and glyceraldehyde-3-phosphate dehydrogenase (as a standard). Blots were exposed to Amersham hyperfilm and bands were analyzed by densitometric scanning. The results for PAI-1 were standardized to glyceraldehyde-3-phosphate dehydrogenase and are the mean out of two independent experiments.

Effect of protein synthesis inhibition on PAI-1 mRNA induction.

To establish whether the induction of PAI-1 mRNA by serum and PMA required new protein synthesis, HepG2 cells were incubated with 10 μg/ml cycloheximide, a concentration sufficient to inhibit protein synthesis for over 95% (data not shown). When added 1 h prior to the inducers, the induction of PAI-1 mRNA seen at 3 h after the addition of serum or PMA was almost completely suppressed by cycloheximide (Fig. 2). These results indicate that the rapid induction of PAI-1 gene expression by serum and PMA is dependent on ongoing protein synthesis. In control cells incubated with cycloheximide alone, no effect was seen on PAI-1 mRNA levels (Fig. 2) nor on its transcription (data not shown).

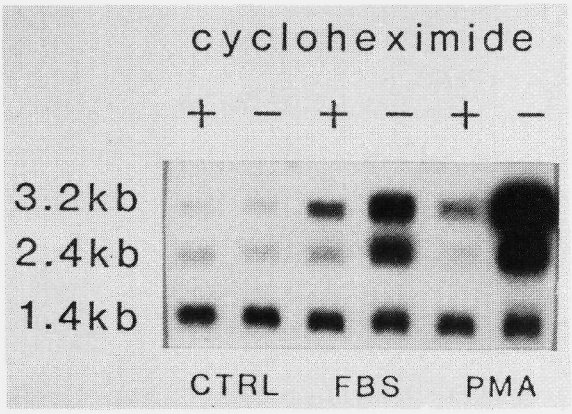

Fig. 2. Cycloheximide inhibits PAI-1 gene induction by serum and PMA in HepG2 cells. HepG2 cells were preincubated with serum-free medium for 16 h and then fresh serum-free medium with or without cycloheximide (10 μg/ml) was added. After 1 h, serum (10%, by vol.), PMA (100 nM) or serum-free medium (CTRL) was added. 3 h later, cells were rinsed twice with phosphate-buffered saline. RNA was isolated and analyzed by Northern blotting for PAI-1 mRNA levels (3.2 and 2.4 kb bands) and, as a reference for the amount of RNA in each lane, for glyceraldehyde-3-phosphate dehydrogenase mRNA levels (1.4 kb band).

Induction of c-jun and c-fos by serum and PMA.

To determine whether c-*jun* and c-*fos* were increased following treatment of HepG2 with serum or PMA, their mRNA levels were measured over an 8 h period (Fig. 3). Under basal, serum-deprived conditions, c-*jun* and c-*fos* mRNA levels in HepG2 were at low, almost undetectable levels (Fig. 3). After the addition of serum or PMA, a transient increase of c-*jun* and c-*fos* mRNA was seen, but with different timeprofiles and to different extents. c-*jun* mRNA, after addition of serum, started to increase after 1 h, reaching a maximal level at 2.5 h, then returned to prestimulatory levels at 6 h. Upon PMA addition, c-*jun* mRNA increased more rapidly, reaching maximal levels as early as at 1 h, followed by a rapid decline. c-*jun* mRNA levels, however, were still elevated 8 h after PMA addition. The maximal level of c-*jun* mRNA induced by PMA was 2-3 times higher than the maximal level reached with serum. The addition of serum resulted in only a minor increase of c-*fos* mRNA, whereas with PMA a strong and rapid increase of this messenger was seen, reaching its peak value after 1 h, then returning to basal levels at 3 h.

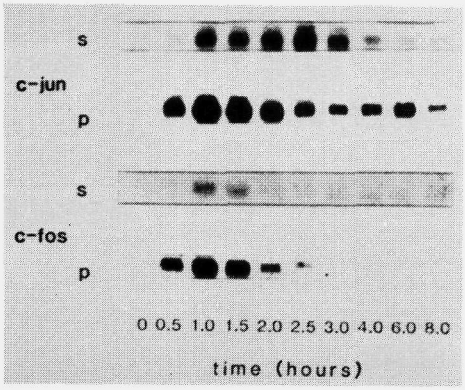

Fig. 3. Time-profile of c-*jun* and c-*fos* mRNA induction by serum (S) and PMA (P) in HepG2 cells. HepG2 cells were preincubated with serum-free medium for 16 h, and then fresh medium containing serum (10%, by vol.) or PMA (100 nM) was added. At the times indicated, RNA was isolated and analyzed by Northern blotting. The amount of RNA in each lane was equal according to a reference probe, glyceraldehyde-3-phosphate dehydrogenase (not shown).

To confirm that the c-*jun* and c-*fos* mRNA induction is reflected at the protein level, we analyzed c-Jun and c-Fos protein induction by PMA by performing immunoprecipitation of radiolabelled cell extracts using anti-c-Jun and anti-c-Fos polyclonal antibodies (Figs 4A and B). In order to distinguish between complexes consisting of c-Jun/c-Fos heterodimers or c-Jun/c-Jun homodimers, cell extracts were first incubated with anti-c-Fos, then with anti-c-Jun polyclonal serum.

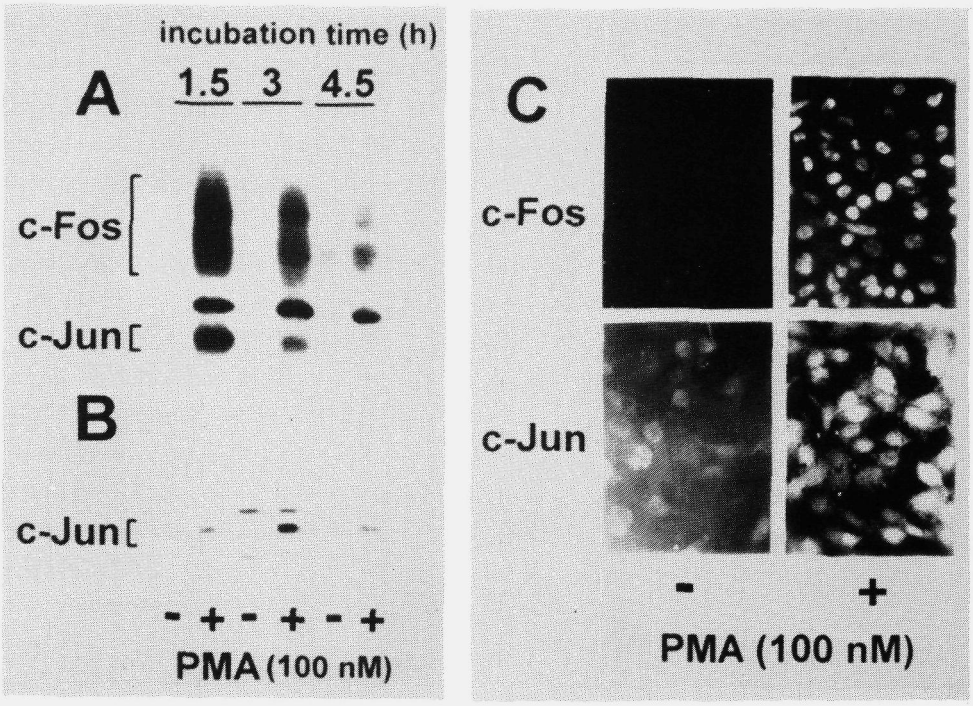

Fig. 4. Time-profile of c-Jun and c-Fos protein induction by PMA in HepG2 cells. HepG2 cells were preincubated with serum-free medium for 16 h and then fresh medium with (+) or without (-) PMA (100 nM) was added. [^{35}S]methionine was added 30 min after the start of the experiment and cell extracts were harvested at the different time points indicated. Immunoprecipitations were performed successively with anti-c-fos (A) and anti-c-jun (B) polyclonal antiserum. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis and radiolabelled proteins were visualised by autoradiography for 7 days (A) or 5 days (B). Brackets indicate the positions of c-Jun and c-Fos proteins on the basis of their molecular weights reported in the literature (30). (C): 1.5 h after PMA addition cells were immunostained for c-Jun and c-Fos as described in the Methods section.

Radiolabelled c-Fos (a broad band around 55 kD) and coprecipitated c-Jun (39 kD) levels were strongly increased by PMA after 1.5 h, followed by a rapid decline after 3 and 4.5 h (Fig. 4A), which is in agreement with the c-*fos* mRNA induction profile. The identity of the additional radiolabelled protein observed after immunoprecipitation with the anti-c-Fos polyclonal antibody is not clear. This protein was not present after precipitation with pre-immune serum (data not shown). Levels of radiolabelled c-Jun not complexed to c-Fos also strongly increased upon PMA addition, reaching maximal induction after 3 h (Fig. 4B). All other precipitated radiolabelled proteins (Fig. 4B) were also present in the pre-immune serum control (data not shown). c-Jun and c-Fos protein levels in HepG2 were also analyzed by immunostaining (Fig. 4C). Under control conditions, fluorescence seen with anti-c-Jun and anti-c-Fos polyclonal antibodies was comparable to that observed using non-specific polyclonal antibodies. After a 1.5 h incubation period with PMA (100 nM), a strong increase in fluorescence intensity was noted in the nucleus with both c-Jun and c-Fos polyclonal antiserum, thereby confirming the above immunoprecipitation experiments.

Effect of antisense c-jun and c-fos oligodeoxynucleotides on PAI-1 induction.

To investigate whether AP-1 is indeed involved in PMA induction of PAI-1 expression, HepG2 cells were exposed to antisense oligodeoxynucleotide (18 nucleotides) complementary to the c-*jun* and c-*fos* mRNA translation initiation sites, thereby inhibiting c-Jun and c-Fos protein synthesis. This approach has previously been shown to be successfull in inhibiting c-Fos protein synthesis in NIH 3T3 cells [36]. With 30 μM antisense c-*jun* oligodeoxynucleotide, the PMA-induced increase in c-Fos protein levels was unaffected. However, c-Jun complexed to c-Fos was reduced by about 50% (Fig. 5A) and non-c-Fos-bound c-Jun was no longer detectable (Fig. 5B), reflecting the tendency of c-Jun to preferentially form c-Jun/c-Fos heterodimers [37]. The addition of a nonsense oligodeoxynucleotide (with a comparable pyrimidine/purine ratio present in the antisense c-*jun* oligodeoxynucleotide) did not markedly affect radiolabelled c-Jun or c-Fos levels. In two parallel, independent experiments, the presence of 30 μM antisense c-*jun* oligodeoxynucleotide decreased basal and PMA-stimulated PAI-1 synthesis over an 8 h period by $61\pm12\%$ and $50\pm6\%$ (mean$\pm$range), respectively (Fig. 5C). PAI-1 synthesis was not inhibited in the presence of 30 μM or 60 μM nonsense oligodeoxynucleotide (data not shown).

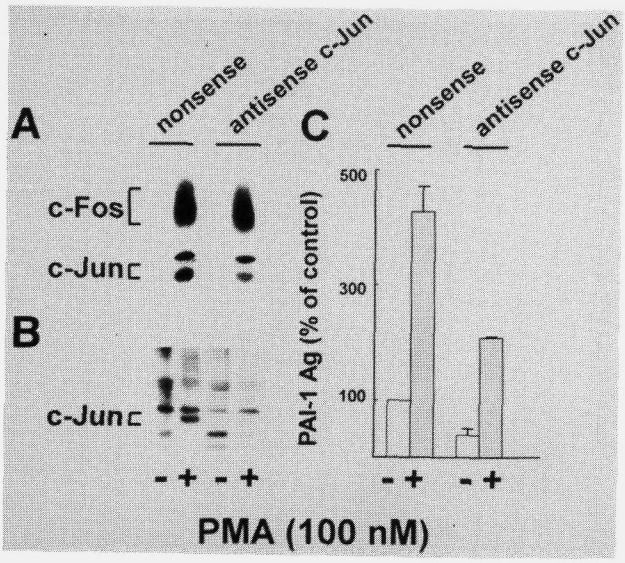

Fig. 5. Effect of antisense c-*jun* oligodeoxynucleotides on c-Jun, c-Fos and PAI-1 induction by PMA. HepG2 cells were incubated with control medium (-) or PMA (100 nM) containing medium (+) for 1.5 h as described in the legend to Figure 4. 1 h prior to the addition of PMA nonsense (30 μM) or antisense c-*jun* (30 μM) oligodeoxynucleotides were added. Immunoprecipitations were performed successively with an anti-c-Fos (A) and an anti-c-Jun (B) polyclonal antiserum. The experiment shown is representative of 2 independent experiments. In parallel experiments, cells were incubated with oligodeoxynucleotides for 8 h and the conditioned media were analyzed for PAI-1 antigen (Ag) levels (C). Values are given as mean $\pm$ variation (% of control).

Addition of 60 μM antisense c-*fos* oligodeoxynucleotide resulted in about a 30% decrease in PMA-induced radiolabelled c-Fos levels and co-precipitated c-Jun levels (Fig. 6A). Unexpectedly, the level of radiolabelled c-Jun subsequently immunoprecipitated with the anti-c-Jun polyclonal antibody decreased also by about 30% (Fig. 6B). How the antisense c-*fos* oligodeoxynucleotides also decrease c-Jun protein levels is unclear at present. The decrease in c-Fos and c-Jun levels after incubation with the antisense c-*fos* oligodeoxynucleotide was accompanied by a decrease of $56\pm7\%$ and $44\pm5\%$ (mean$\pm$range) in basal and PMA-stimulated PAI-1 synthesis, respectively, over an 8 h period in two independent experiments (Fig. 6C).

To check that the inhibition of PAI-1 synthesis by the antisense c-*jun* and c-*fos* oligodeoxynucleotides was not the result of a general decrease in protein synthesis, the fibrinogen level was measured. Neither the antisense c-*jun* nor antisense c-*fos* oligodeoxynucleotides inhibited fibrinogen production (data not shown). These antisense experiments indicate that c-Jun and, directly or indirectly, c-Fos are necessary for basal and PMA-induced PAI-1 expression in HepG2. This conclusion is also supported by experiments performed with curcumin, a compound which has been reported to prevent the induction of c-*jun* mRNA and to interfere with the interaction between (existing) AP-1 and TREs [38]. We found that curcumin (100 μM) reduced both basal and serum-induced or PMA-induced PAI-1 mRNA levels in HepG2 (data not shown).

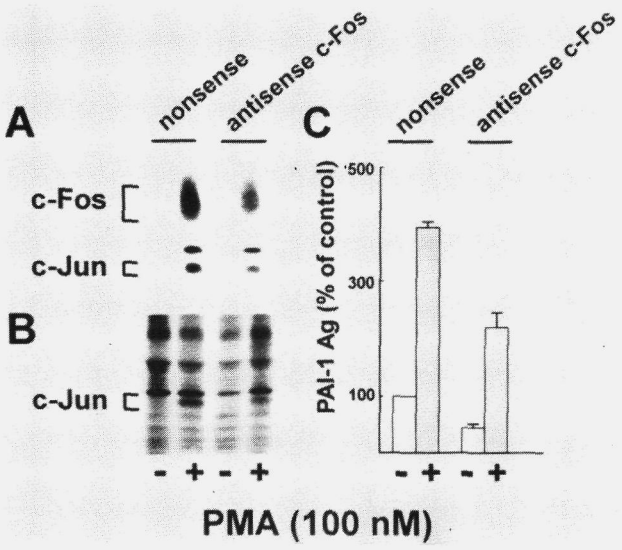

Fig. 6. Effect of antisense c-*fos* oligodeoxynucleotides on c-Fos, c-Jun and PAI-1 induction by PMA. HepG2 cells were incubated with control medium (-) or PMA (100 nM) containing medium (+) for 1.5 h as described in the legend to Figure 4. 1 h prior to the addition of PMA, *nonsense* (60 μM) or *antisense c-fos* (60 μM) oligodeoxynucleotides were added. Immunoprecipitations were performed successively with an anti-c-Fos (A) and an anti-c-Jun (B) polyclonal antiserum. The experiment shown is representative of 2 independent experiments. In parallel experiments, cells were incubated with oligodeoxynucleotides for 8 h and the conditioned media were analyzed for PAI-1 antigen (Ag) levels (C). Values are given as mean $\pm$ variation (% of control).

Binding of AP-1 to the -58/-50 TRE but not to the -79/-72 element.

In transient transfection studies, previous studies by Descheemaeker *et al.* [16] were confirmed and it was shown that a CAT reporter construct containing the PAI-1 promoter region from positions -489 to +75 was sufficient for maximal basal and PMA-stimulated CAT expression in HepG2 cells (data not shown). This region contains the two proximal TREs, each of which is important for PMA induction of PAI-1, as shown by mutational analysis [16, 19]. To analyse whether c-Jun/c-Fos complexes bind to these putative TREs, electromobility shift assays (EMSA) were performed. The bandshifts were performed in the presence of an excess of free double-stranded probe. Several DNA-protein complexes were observed upon incubation of either the -66 to -43 bp region (box A) or the -82 to -60 bp region (box B) with nuclear extracts from HepG2 cells (Fig. 7). With box A, two complexes (referred to as 1 and 2) were found, of which predominantly the lower one (complex 2) increases after PMA treatment (Fig. 7A). Competition experiments with an excess of a consensus TRE oligodeoxynucleotide (as present in the collagenase promoter), but not random oligodeoxynucleotide, inhibited the formation of both complexes (Fig. 7A). The formation of the two protein-DNA complexes could also be effectively inhibited by incubation of the nuclear extracts with anti c-Jun polyclonal antibodies. Incubation with

anti-c-Fos polyclonal antibodies only partly prevented the increase in complex 2 formation seen with nuclear extracts from PMA-treated HepG2 cells while not interfering with complex 1 formation (Fig. 7A). These experiments indicate that c-Jun is involved in the formation of both complexes while c-Fos is only present in complex 2 if the nuclear extract is from PMA-treated HepG2 cells. Since a complex containing c-Jun homodimers runs to the same position as that containing c-Jun/c-Fos heterodimers, it is likely that complex 2, besides c-Jun/c-Fos, consists of c-Jun homodimers.

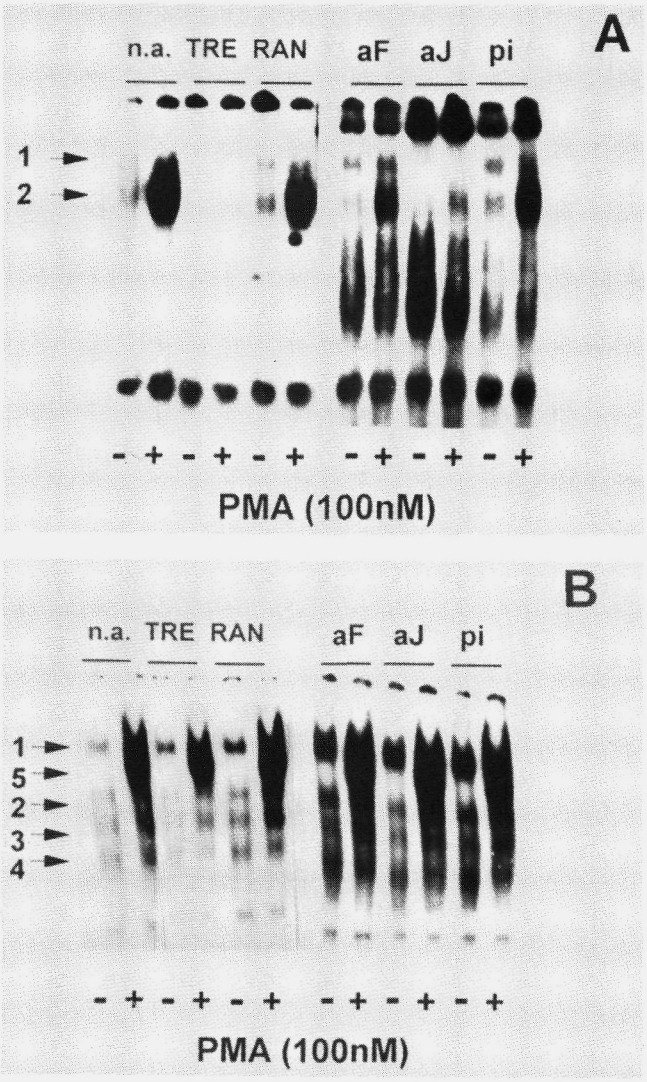

Fig. 7. HepG2 nuclear protein binding to box A and box B of the PAI-1 promoter region as assayed by electromobility shift assay (EMSA). HepG2 cells were incubated with control medium (-) or PMA (100 nM) containing medium (+) for 1.5 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with radiolabelled -66 to -43 region oligodeoxynucleotide (A) or with radiolabelled -88 to -60 region oligodeoxynucleotide (B). Nuclear extracts were pre-incubated for 1 h in the absence (no addition, n.a) or presence of 100-fold excess unlabelled doublestranded TRE (TRE), 100-fold excess aspecific competitor oligodeoxynucleotide (RAN), anti-c-Fos antibody (aF), anti-c-Jun antibody (aJ) or preimmune serum (pi). The bandshifts were performed in the presence of an excess of free double-stranded probe. To improve the separation of the DNA/protein complexes, the running time was extended, as a result of which no free probe is visible. DNA/protein complexes were separated on a 5% (mass/vol.) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 7 days (A) or 5 days (B). Arrows mark specific DNA/protein complexes.

EMSA experiments with box B (positions -82 to -60) show four different complexes with control nuclear extracts; PMA induces an additional binding activity (Fig. 7B, complexes 1-5). In contrast to the gel-shift experiments involving box A, competition with excess consensus TRE oligodeoxynucleotide or treatment of the nuclear extracts with anti-c-Jun or anti-c-Fos polyclonal antibodies did not change the EMSA pattern, indicating that box B of the PAI-1 promoter is not recognized by proteins of the AP-1 family (Fig. 7B). In an attempt to identify the nuclear proteins binding to box B, antibodies directed against transcription factors previously reported to bind to this box or to similar sequences were used. No detectable binding of the 72kDa transcription factor previously reported to bind to box B in HeLa cells [16]. Similarly, we found that box B binds SP-1 in nuclear extracts from human umbilical vein endothelial cells, but not from control or PMA-treated HepG2 (data not shown). Another candidate, activator protein-2, is not present in HepG2 cells [39]. Therefore, the identity of the box B binding nuclear proteins from HepG2 cells remains to be determined.

Further analysis of c-Jun and c-Fos binding activities towards box A.

As described above, PMA induces binding of both c-Jun and, to a lesser extent, of c-Jun/c-Fos heterodimers to box A of the PAI-1 promoter (complex 2). Since the relative amount of c-Jun/c-Fos heterodimer bound to box A is remarkably low compared to the high induction of c-Jun/c-Fos heterodimers at the protein level (Fig. 4), binding experiments were also performed with the collagenase TRE which has been reported to bind c-Jun/c-Fos heterodimers with high affinity. The collagenase TRE sequence complexes with a nuclear protein present in HepG2 nuclear extracts (Fig. 8), and this is strongly induced after PMA treatment of HepG2. Competition experiments with an excess of a collagenase TRE oligodeoxynucleotide but not random oligodeoxynucleotide displaced the binding to the collagenase TRE (Fig. 8). Incubation of nuclear extracts from control HepG2 cells with anti c-Jun polyclonal antibodies, but not with anti c-Fos polyclonal antibodies, completely prevented this DNA binding (Fig. 8). In contrast, incubation with an anti c-Jun or with an anti c-Fos polyclonal antibody strongly inhibited complex formation with nuclear extracts from PMA-treated HepG2. These data indicate that, in contrast to box A, the collagenase TRE is predominantly bound by c-Jun/c-Fos heterodimers in cell extracts of PMA-treated HepG2 cells. Furthermore, the amount of complex seen with the collagenase TRE is much higher than that seen with box A (Figs 7A and 8; note the difference in exposure times).

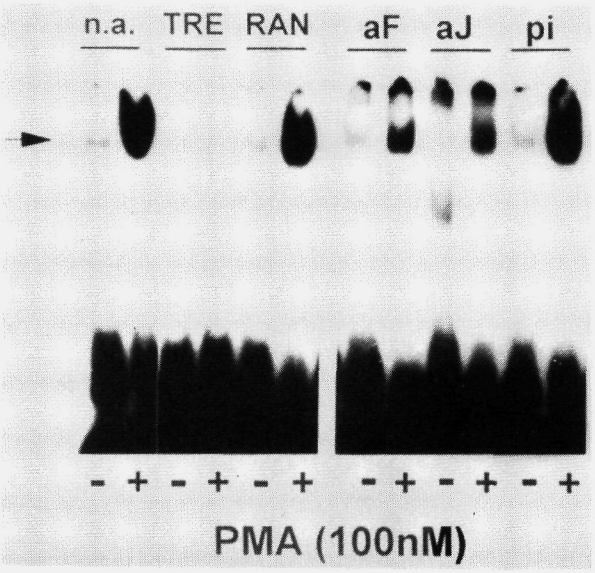

Fig. 8. Nuclear protein binding to the collagenase TRE as assayed by electromobility shift assay (EMSA). HepG2 cells were incubated with control medium (-) or PMA (100 nM) containing medium (+) for 1.5 h and nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with radiolabelled consensus TRE oligodeoxynucleotides. Nuclear extracts were pre-incubated for 1 h in the absence (no addition, n.a.) or presence of 100-fold excess unlabelled *doublestranded* TRE (TRE), 100-fold excess aspecific competitor oligodeoxynucleotide (RAN), anti-c-Fos antibody (aF), anti-c-Jun antibody (aJ) or preimmune serum (pi). DNA/protein complexes were separated on a 5% polyacrylamide gel and radiolabelled DNA-protein complexes were visualised by autoradiography for 1 day. Arrows mark specific DNA/protein complexes.

Figure 9 shows the results of experiments in which the dissociation of different proteins complexed to box A is assessed. With nuclear extracts from non-stimulated HepG2 cells, complexes 1 and 2 dissociate at a comparable rate, and protein binding is reduced to background levels after a 15 min dissociation period. After PMA treatment, part of complex 2 rapidly dissociates during the first minute but the remainder of the complex remains intact during the next 20 min. After treatment of the nuclear extract with anti-c-Fos polyclonal antibody, only the stable complex remained (Fig. 9B), indicating that the labile complex that dissociated during the first minute contains c-Jun/c-Fos heterodimers and that the stable complex contains c-Jun only. These results show that PMA treatment of HepG2 cells increases the amount of c-Jun complexed to box A of the PAI-1 promoter; the slower dissociation rate of the c-Jun complexes bound to box A after PMA treatment may reflect a higher affinity if the association rates have remained identical.

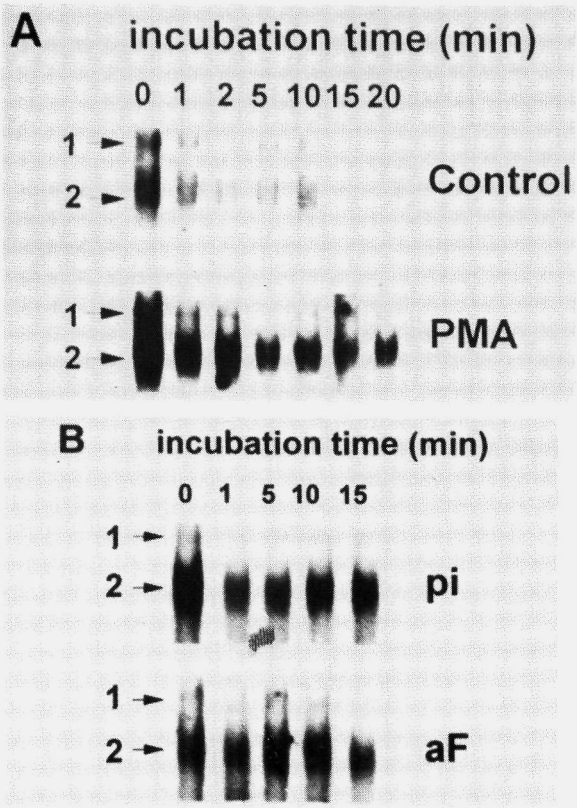

Fig. 9. Dissociation of the proteins bound to box A of the PAI-1 promoter region.
HepG2 cells were incubated with control medium (Control) or PMA (100 nM) containing medium for 1.5 h and nuclear extracts were prepared as described in the Methods section. Panels A and B: Extracts were incubated for 30 min with radiolabelled -66 to -43 bp region oligodeoxynucleotide. After the binding reaction the mixtures were incubated in the presence of 100-fold excess cold -66 to -43 bp region competitor oligodeoxynucleotide for the indicated times. In panel B nuclear extracts from PMA-treated HepG2 cells were preincubated with anti-c-Fos polyclonal antiserum (aF) or preimmuneserum (pi). The bandshifts were performed in the presence of an excess of free double-stranded probe. DNA/protein complexes were separated on a 5% (mass/vol.) polyacrylamide gel and radiolabelled complexes were visualised by autoradiography for 4 days. Arrows mark specific DNA/protein complexes.

DISCUSSION

Previous studies have suggested that c-Jun/c-Fos (AP-1) complexes play an important role in the upregulation of PAI-1 gene expression following the activation of membrane receptors and of protein kinase C in the response to growth factors such as TGF-ß [15, 40] or chemicals such as the phorbol ester PMA [16, 19]. The PAI-1 promoter contains four putative AP-1 binding sites, the sequence of which differs only by 1 bp from the consensus AP-1 binding sequence (TRE) as reported by Angel *et al.* [17]. However, attempts to delineate the importance of each AP-1 binding site have been confusing, whereas Keeton *et al.* [40] reported an 11-fold induction by TGF-ß mediated through the proximal promoter region at positions -87 to -49 of PAI-1 in Hep 3B, Westerhausen *et al.* [15] did not find an involvement of this region in the induction of PAI-1 by TGF-ß in HepG2. Since the same *cis*-acting elements were assayed in both of these cell types, the differences in regulation presumably depend on celltype specific *trans* acting elements and factors. Studies by Knudsen

et al. [19] even question the existence of a role for AP-1 in PAI-1 gene expression, since these investigators found that the proximal PAI-1 TRE was bound by an as yet unidentified transcription factor, despite the presence of AP-1 binding activity in cell extracts from the human breast carcinoma cell line, MCF-7. These studies illustrate that the putative role of AP-1 in PAI-1 gene expression is considerably more complex than previously suggested.

The present study was, therefore, directed at analyzing more comprehensively the role of c-Jun/c-Fos and the various TREs in PMA-induced PAI-1 gene expression. For this purpose, the human hepatoma cell line, HepG2 was stimulated with the phorbol ester, PMA. Stimulation of HepG2 with PMA (or serum) resulted in a rapid and strong induction of c-*jun* and c-*fos* which preceded a strong increase in PAI-1 gene transcription. Both basal and PMA-stimulated expression of the human PAI-1 gene in HepG2 are strongly dependent on c-Jun and the TRE motif located between positions -58 and -50 of the PAI-1 promoter on the basis of the following observations. Firstly, transfection studies, including mutational analysis, demonstrated that the two TREs between positions -58 and -50 and between positions -79 and -72 were crucial for basal and PMA-induced PAI-1 expression ([16]; this paper). Secondly, both basal and PMA-stimulated PAI-1 synthesis could be inhibited with either antisense c-*jun* or antisense c-*fos* oligodeoxynucleotides. With both antisense oligodeoxynucleotides a decrease in c-Jun induction was observed, whereas only antisense c-*fos* oligodeoxynucleotides suppressed c-Fos induction. Thirdly, gel-shift experiments with nuclear extracts from non-stimulated and PMA-treated HepG2 cells showed that only box A (positions -66 to -43), but not box B (positions -82 to -60) formed c-Jun containing complexes (referred to as complexes 1 and 2). With control extracts, complex 1 consisted of c-Jun and an unidentified protein and complex 2 contained predominantly c-Jun homodimers. With nuclear extracts from PMA-treated cells, complex 2 formation increased more than 30-fold, largely as a result of an increase in c-Jun homodimer binding, but also, albeit to a lesser extent, of c-Jun/c-Fos heterodimer binding. These results point at an important role of c-Jun homodimers and the region at positions -58 to -50 in the regulation of basal and PMA-stimulated PAI-1 transcription.

Our finding that box A represents a high affinity site for c-Jun homodimers is in agreement with results obtained by Knudsen *et al.* [19] who reported specific binding of purified c-Jun homodimers to box A (TGAGTTCA). Box A thus clearly differs from the heptameric collagenase TRE (TGAGTCA), which preferentially binds c-Jun/c-Fos heterodimers and resembles the octameric TREs found in, for example, the u-PA promoter [41] and the c-Jun promoter (TGATGTCA and TGAGGTAA, respectively) [42]. Our data on the importance of c-Jun homodimers in PAI-1 regulation may explain why some previous

reports failed to demonstrate a role for AP-1 in PAI-1 expression. For example, Knudsen *et al.* [19] found a strong increase in AP-1 binding activity with a consensus TRE upon treatment of MCF-7 cells with PMA, but no binding of AP-1 to box A could be demonstrated. Similarly, TGF-ß, an effective inducer of AP-1 activity [43], failed to stimulate the proximal PAI-1 promoter region in HepG2 cells [15]. In these studies, the composition of the AP-1 proteins present was not examined. Since c-Jun preferentially heterodimerizes to c-Fos [37], it is possible that no c-Jun homodimers were formed. It can also be anticipated that agents which suppress c-Jun homodimer formation will decrease PAI-1 expression. In this respect, it may be significant that cAMP-raising compounds have been shown to lower PAI-1 transcription in a variety of cell types [44, 45], although cAMP induces AP-1 activity, cAMP is known to induce c-Fos, but not c-Jun (for review, see [37]), which will result in a decrease in the amount of c-Jun homodimers because of the formation of c-Jun/c-Fos heterodimers. Such a mechanism indeed has been described for u-PA expression in HepG2, which requires c-Jun homodimer binding to an octameric TRE in the u-PA promoter: overexpression of c-Fos resulted in transcriptional inhibition of u-PA expression [41].

It was observed that PMA treatment increased the amount of c-Jun complexed to the PAI-1 proximal TRE, and slowed down their dissociation, possibly as a result of an increased binding affinity of the c-Jun homodimers (Figs 4 and 9). The results of the present study do not allow us to assess the relative importance of these two factors in PAI-1 gene induction. Previous work has demonstrated that PMA treatment of cells leads to dephosphorylation of the inhibitory sites in the c-Jun DNA-binding domain, which would increase its binding activity [46]. However, cycloheximide, a protein synthesis inhibitor, abolished the effect of PMA (and serum) on PAI-1 mRNA levels in our studies, indicating that activation of existing c-Jun through post-translational modification alone is insufficient to induce PAI-1 expression.

Although the human PAI-1 promoter contains four potential AP-1-binding sites, the combined results of transfection experiments and bandshift assays show that only box A mediates PAI-1 transcriptional induction by AP-1. The evolutionary conservation of the octameric TRE of box A between rodents and humans [47] underscores its functional significance. Small differences in base pair sequence may alter the nature of the nuclear proteins bound. The effect of subtle sequence differences on nuclear protein binding is illustrated by our observation that box A did not bind c-Jun/ATF-2. Box A (TGAGTTCA) closely resembles the consensus binding sequence for c-Jun/ATF-2, TGA CG T(T/C)A [48]. Other similar octameric TREs bound c-Jun/ATF-2, for example the c-Jun promoter (TGATGTCA and TGAGGTAA) [42] and the u-PA promoter (TGAAGTCA) [41].

Furthermore, c-Jun/ATF-2 is involved in u-PA induction by PMA in HepG2 [41]. These sequences differ from the A box with respect to the G at position 5.

Our finding that the antisense *c-jun* oligonucleotides inhibited basal and PMA-stimulated PAI-1 synthesis only to about 50% is in agreement with the results obtained by Descheemaeker *et al.* [16] who showed a comparable decrease in PAI-1 promoter activity in HeLa cells and HT1080 cells after site-directed mutagenesis of the region at positions -59 to -50; the remainder of the PMA-response was found to be mediated through the region at positions -79 to -72. We observed that this region bound a factor that was stongly induced after PMA treatment of the cells. The identity of this DNA-binding protein remained unknown, but was different from proteins previously demonstrated to bind the region at positions -79 to -72 in other cell types, including AP-2 and a 72-kD transcription factor in HeLa cells [16] and SP-1 in human endothelial cells (this study).

In conclusion, our results demonstrate that the binding of c-Jun, most likely c-Jun homodimers, to the region at position -58 to -50 of the PAI-1 promoter is an important factor in basal and PMA-stimulated PAI-1 gene expression in HepG2. Previous studies have shown that, in addition to the region at positions -58 to -50 bp, the region between positions -79 and -70 is important for basal and stimulated PAI-1 expression; we found a massive induction of the binding of an unknown factor to this latter region. This is a new finding and further work should be carried out to characterize this DNA binding protein.

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CHAPTER 6

On the role of c-Jun in the induction of PAI-1 gene expression by phorbol ester, serum and interleukin-1α in HepG2 cells

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ABSTRACT

We have characterized the regulation of plasminogen activator inhibitor-1 (PAI-1) gene expression by phorbol 12-myristate 13-acetate (PMA), serum and interleukin-1α (IL-1α) in the human hepatoma cell line HepG2. PMA, serum and IL-1α induced a rapid and transient 28-fold (PMA), 9-fold (serum) and 23-fold (IL-1α) increase in PAI-1 mRNA, peaking between 4 and 6 h. These inductions of PAI-1 mRNA accumulation can be fully explained by comparable, transient increases in PAI-1 gene transcription and were dose-dependently reduced by pretreatment of the HepG2 cells with the protein tyrosine kinase inhibitor genistein. Conversely, stimulation of tyrosine phosphorylation by sodium orthovanadate, an inhibitor of protein tyrosine phosphatases, caused an increase in PAI-1 mRNA levels. The effects of PMA, serum and IL-1α on PAI-1 mRNA expression have been compared with their ability to modulate the expression of a chloramphenicol acetyltransferase (CAT) reporter plasmid, which was under control of the -489 to +75 region of the PAI-1 promoter, and stably transfected into HepG2 cells. This region of the PAI-1 promoter was previously found to contain a TRE (between -58 to -50) necessary for PMA responsiveness and with a high affinity for c-Jun homodimers. Whereas incubation of these transfected HepG2 cells with PMA and, to a lesser extent, serum showed an induction profile of CAT mRNA very similar to that of PAI-1 mRNA, hardly any induction of CAT mRNA was found with IL-1α. In line with these findings, IL-1α poorly induced c-Jun homodimer binding to the PAI-1 TRE in gel mobility shift assays. We propose that IL-1α induces PAI-1 gene transcription in HepG2 cells via a signalling transduction pathway distinct from that of PMA and serum, and not involving c-Jun homodimer binding to the PAI-1 TRE. Both the PMA/serum and the IL-1α signal transducing pathway involve protein tyrosine kinase activation.

INTRODUCTION

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type and urokinase-type plasminogen activator (t-PA and u-PA, respectively) *in vivo* (Sprengers and Kluft, 1987). Elevated levels of PAI-1 have been implicated in the pathogenesis of thromboembolic disease and may contribute to the risk of reinfarction in patients that have suffered a previous myocardial infarction (Hamsten et al., 1985; 1987). Also, increased PAI-1 gene expression has been observed in atherosclerotic human arteries (Schneiderman et al., 1992). On the other hand, PAI-1 deficiencies are associated with a bleeding tendency

in humans (Schleef et al., 1989; Dieval et al., 1991; Fay et al., 1992).

Multiple factors have been identified that play a role in the regulation of PAI-1 synthesis. PAI-1 behaves as an acute-phase reactant in humans in that plasma levels of PAI-1 are increased in patients during septicemia (Engebretsen et al., 1986) and after surgery or trauma (Aillaud et al., 1985; Kluft et al., 1985; Juhan-Vague et al., 1985). Furthermore, PAI-1 synthesis in cultured human endothelial cells and human hepatocytes has been shown to be inducible by cytokines and inflammatory mediators such as endotoxin (Emeis and Kooistra, 1986; Van Hinsbergh et al., 1988; Van den Berg et al., 1988), interleukin-1 (IL-1) (Emeis and Kooistra, 1986; Van Hinsbergh et al., 1988; De Boer et al., 1991; Healy and Gelehrter, 1994) and tumor necrosis factor (TNF) (Schleef et al., 1988; Van Hinsbergh et al., 1988; Van den Berg et al., 1988); growth factors like insulin (Alessi et al., 1988; Kooistra et al., 1989), insulin-like growth factor (Schneider and Sobel, 1991), transforming growth factor ß (Saksela et al., 1987; Westerhausen et al., 1991) and epidermal growth factor (Lucore et al., 1988); and the PKC-activating phorbol ester, phorbol 12-myristate 13-acetate (PMA) (Santell and Levin, 1988; Pertovaara et al., 1989; Grulich-Henn and Müller-Berghaus, 1990; Bosma and Kooistra, 1991).

The human hepatoma cell line HepG2 is often used as a model of human hepatocytes (Kooistra et al., 1989). Many of the stimulators of PAI-1 synthesis in HepG2 cells, including PMA, serum and IL-1, may be classified as agents that increase the abundance and/or activity of the transcription factor activator protein-1 (AP-1) (Angel et al., 1987; Pertovaara et al., 1989; Muegge et al., 1989; Muegge et al., 1993). AP-1 is a collection of homodimeric and/or heterodimeric complexes composed of the Jun and Fos gene products (Angel et al., 1987). These complexes interact with a common DNA binding site, the PMA responsive element (TRE) and activate gene transcription in response to activators of protein kinase C, growth factors and cytokines (Angel et al., 1987; Brenner et al., 1989). Several studies have been directed at elucidating the mechanism by which PMA stimulates PAI-1 gene transcription in HepG2 cells. Transfection studies, including mutational analysis, combined with experiments with anti-sense c-*jun* and c-*fos* oligonucleotides and electromobility shift assays point to an important role of c-Jun homodimer binding to the TRE at position -58 to -50 of the PAI-1 promoter in the regulation of basal and PMA-stimulated gene transcription in HepG2 cells (Descheemaeker et al., 1992; Knudsen et al., 1994; Arts et al., submitted).

In this study, we have examined whether IL-1α and serum stimulate PAI-1 gene transcription through the same regulatory pathway as found for PMA. To that end, we have used stably transfected HepG2 cells, in which the expression of a reporter gene, chloramphenicol acetyltransferase (CAT), is under control of the -489 to +75 region of the

PAI-1 promoter. This promoter region contains the c-Jun homodimer binding site essential for PMA-induction of PAI-1 gene transcription (Descheemaeker et al., 1992; Knudsen et al., 1994; Arts et al., submitted). In addition, we have compared PMA, IL-1α and serum on their capacity to induce c-*jun* mRNA expression and to increase c-Jun homodimer binding to the -58 to -50 region of the PAI-1 promoter, using gel-shift analysis. Finally, we evaluated the effect of the protein tyrosine kinase inhibitor genistein on these processes. Genistein has previously been shown to suppress rather selectively the basal and IL-1α stimulated PAI-1 gene expression in cultured human endothelial cells (Van Hinsbergh et al., 1994), and has frequently been reported to inhibit the induction of c-*jun* (Zwiller et al., 1991; Rizzo et al., 1995).

MATERIALS AND METHODS

Materials.
4ß-phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO). A stock solution of PMA (100 μM) was prepared in ethanol and kept at -20°C. Human recombinant IL-1α was a gift from Dr. S. Gillis (Immunex Corporation, Seattle, WA). A stock solution of IL-1α (10^5 U/ml, specific activity 10^8 U/mg) was kept at -80°C. Fetal calf serum (FCS) was from Flow Laboratories (Irvine, UK). Tyrphostin A47 was from Brunschwig (Amsterdam, The Netherlands). Genistein and sodium orthovanadate were from LC Laboratories (Woburn, MA). Stock solutions of genistein and tyrphostin (100 mg/ml) were prepared in dimethyl sulfoxide (DMSO) and kept at -20°C. A stock solution of sodium orthovanadate (500 mM) was freshly prepared in phosphate-buffered saline (0.15 M NaCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) (PBS) at the start of each experiment. Anti-c-Jun and anti-c-Fos polyclonal antibodies were a gift from Dr.T. Oehler (Massachusetts Institute of Technology, Cambridge, MA). Deoxycytidine 5[α-^{32}P]triphosphate (3 Ci/mol), 5[γ-^{32}P]triphosphate (3 Ci/μmol), [^{35}S]methionine (> 1 Ci/μmol) and the Megaprime-kit were from Amersham Nederland BV ('s Hertogenbosch, The Netherlands). Bradford protein reagent was from Biorad (Veenendaal, The Netherlands). Human serum albumin (HSA) [20% (w/v), pyrogen free] was from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Other materials used in the methods described below have been specified in detail in relating references, in the text, or were purchased from standard commercial sources.

Cell culture.
HepG2 cells were grown as monolayer cultures under 5% CO_2/ 95% air atmosphere at 37°C in Dulbecco's modification of Eagle's medium (DMEM), supplemented with 10% (v/v) FCS (heat-inactivated), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine as described previously (Bosma and Kooistra, 1991). For experiments, confluent cultures were used, and cells were always refed the day before the experiment with incubation medium, viz. serum-free DMEM containing 0.1% (w/v) HSA, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Conditioned media were obtained by incubating cells at 37°C for various times upto 24 h with incubation medium containing the appropriate concentration of the test compound or stock solvent. After incubation, the cells were washed twice with ice-cold PBS and were used for isolation of RNA or preparation of nuclear extracts.

Northern blot analysis.
Total RNA was isolated as described by Chomczynski and Sacchi (1987) and electrophoresed in a 1% (w/v) agarose gel under denaturing conditions using 1 M formaldehyde (Sambrook et al., 1989). The RNA was

transferred to Hybond-N filter by blotting and the filters were hybridized overnight at 63°C in NaPI hybridization mix [7% (w/v) sodium dodecyl sulfate (SDS), 0.5 M NaPI pH 7.2, 1 mM ethylenediamine tetraacetic acid (EDTA)] containing 3 ng of [α-^{32}P]CTP-labelled probe/ml. The probes were labelled with a Megaprime kit, yielding an average activity of 0.2 μCi/ng DNA. After hybridization with PAI-1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or chloramphenicol acetyltransferase (CAT) probe, the filters were washed twice with 2xSSC (1xSSC being: 0.15 M NaCl, 0.0.15 M Na_3citrate-dihydrate), 1% SDS and twice with 1xSSC, 1% SDS for 20 minute time periods at 63°C. In the case of hybridization with c-*jun* or c-*fos* probe, the filters were washed with 2xSSC, 1% SDS for 4 successive 20 min periods at 63°C. The filters were then exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C.

cDNA probes.
The following cDNA probes were used: a 2.5 kb *Eco*RI fragment of the human PAI-1 cDNA (Van den Berg et al., 1988); a 1.2 kb *Pst*I fragment of the rat GAPDH cDNA provided by Dr.R. Offringa (Fort et al., 1988); a 1.0 kb *Pst*I fragment of the mouse c-*jun* c-DNA (De Groot et al., 1990); a 1.5 kb *Eco*RI fragment of the murine c-*fos* c-DNA (Curran et al., 1982); and a 0.6 kb *Not*I fragment of the pOPRSVICAT expression vector (Stratagene, Cambridge, MA).

Preparation of nuclear extracts.
For gel shift experiments HepG2 cells (25 cm^2) were rinsed twice with ice-cold PBS and lysed in 2 ml of lysis buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40, 1 mM dithiothreitol (DTT), 0.25 mM sodium orthovanadate and 1 μg/ml of the protease inhibitors leupeptin, pepstatin and aprotinin). The lysate was homogenized, nuclei were collected by centrifugation (5 min at 1000 g, 4°C), and washed with lysis buffer once more. The dry nuclear pellet was resuspended in 150 μl of 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (Hepes, pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), 1 mM DTT, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.25 mM sodium orthovanadate and 1 μg/ml of leupeptin, pepstatin and aprotinin. This suspension was incubated for 15 min at 4°C while being continuously shaken, and then centrifuged at 1000 g, 4°C for 5 min. Supernatants were stored at -80°C until use. The protein concentration in the nuclear extract was determined using the Bradford protein assay.

Gel shift experiments.
For electromobility shift assays (EMSA) an oligodeoxynucleotide representing the PAI-1 promoter region between -66 and -43 (5'-CTGGAACATGAGTTCATCTATTT-3') was synthesized by Isogen Bioscience (Amsterdam, The Netherlands). The oligodeoxynucleotide was end-labelled using T_4-kinase and subsequently purified by phenol/chloroform extraction and precipitation. For EMSA, 25 fmol (about 10^4 cpm) of labelled double-stranded oligodeoxynucleotide was mixed with nuclear extract (5 μg protein) in a total volume of 20 μl 20 mM Hepes (pH 7.9), 20 mM KCl, 2 mM $MgCl_2$, 20% (v/v) glycerol, 2.5 mM EDTA, 2 mM spermidine, 1 μg poly(dI-dC), 1 μg bovine serum albumin (BSA), 1 mM PMSF and 2.5 pmol aspecific competitor oligodeoxynucleotide (5'-CTGAGGATTCTCCACTGCA-3'). The mixture was incubated at 4°C for 30 min. DNA/protein complexes were separated from the non-bound oligodeoxynucleotide by electrophoresis on a 5% polyacrylamide gel in 0.25xTBE buffer [22.5 mM Tris-borate, 0.5 mM EDTA (Sambrook et al., 1989)]. Electrophoresis was carried out at room temperature at 150 V for 70 min, using 0.25xTBE as running buffer. After the gel had been dried on Whatman-3MM paper, DNA/protein complexes were visualized by autoradiography.

Immunoprecipitation of c-Jun and c-Fos protein.
HepG2 cells (10 cm^2) were incubated in methionine-free culture medium supplemented with 0.1% (w/v) HSA, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.2 mCi/ml [^{35}S]methionine. Cell extracts were prepared as described by Kruijer et al. (1984). Cells were washed twice with ice-cold PBS and harvested by scraping in 0.5 ml of lysis buffer [50 mM Tris pH 8.0, 125 mM NaCl, 0.5% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS and the proteinase inhibitors leupeptin (1 μg/ml), pepstatin A (1 μg/ml), aprotinin (1 μg/ml) and PMSF (0.5 mM)]. The cell lysates were centrifuged in a Beckman TL-100 centrifuge at 150,000 rpm (30 min, 4°C). The cleared cell lysates were then incubated for 1 h at 4°C with pre-immune

rabbit serum coupled to protein A-Sepharose under continuous rotation. The protein A-Sepharose was removed (30 sec at 10,000 g, 4°C) and the supernatant was subsequently used to immunoprecipitate c-Fos complexes by the same procedure, using an anti-c-Fos rabbit polyclonal antibody. Finally, the c-Fos and c-Fos/c-Jun depleted extract was incubated with anti-c-Jun antiserum coupled to protein A-Sepharose (Oehler and Angel, 1992). All immunocomplexes were washed four times with 1 ml lysis buffer and once with PBS, resuspended in Laemmli sample buffer (62.5 mM Tris pH 6.8, 10% (w/v) glycine, 2% (v/v) SDS, 5% (v/v) ß-mercaptoethanol, 0.02% (w/v) bromophenolblue) (Laemmli, 1970) and boiled for 5 min. The samples were subjected to electrophoresis (10 mA, 16 h) on a 10% (w/v) SDS-polyacrylamide gel. The gel was dried on Whatman-3MM paper and labelled proteins were visualized with a Fujix Bas-1000 phosphoimager.

Selection of stable HepG2 transfectants.
HepG2 cells (78 cm^2) were transfected with the calcium phosphate coprecipitation procedure (Graham and Van der Eb, 1973) with 10 μg of a -489 to +75 PAI-1-promoter CAT construct (Bosma, 1991) and 2.5 μg of the pSV2NEO-plasmid, which conveys neomycine resistance under control of the SV40 promoter. Stable transfectants were obtained by selection with 0.4 mg/ml G418-sulfate (Life Technologies, Breda, The Netherlands). Individual clones were isolated, amplified and characterized for expression of the CAT construct.

Assays.
Overall protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into the 10% (w/v) trichloroacetic acid-precipitable fraction of radiolabelled conditioned medium and cell extract (Kooistra et al., 1987).

RESULTS

Regulation of PAI-1 mRNA accumulation and activity of a c-Jun binding region of the PAI-1 promoter by PMA, IL-1α and serum

In HepG2 cells, PMA-induced PAI-1 gene expression involves c-Jun homodimer binding to the -58 to -50 region of the PAI-1 promoter (Descheemaeker et al., 1991; Arts et al., submitted). As a first approach to evaluate the role of this c-Jun binding region in the induction of PAI-1 by serum and IL-1α, we have used stably transfected HepG2 cells, in which the expression of a reporter gene, chloramphenicol acetyltransferase (CAT), is under control of the -489 to +75 region of the PAI-1 promoter. This system allowed the simultaneous analysis of the expression of PAI-1 mRNA and the activity of the c-Jun binding proximal region in the PAI-1 promoter. Figure 1A shows autoradiograms of Northern blots of RNA isolated from HepG2 cells incubated for various times with PMA (100 nM), serum (10%, v/v) or IL-1α (300 U/ml) and probed with PAI-1 and CAT. In human cells, two PAI-1 mRNA species of 3.2 and 2.4 kb, reflecting different polyadenylation sites, are expressed. With all three agents, induction of the 3.2 kb PAI-1 mRNA was evident after 2 h, maximal after about 3 to 4 h, and then rapidly declined. Induction of the 2.4 kb mRNA started more slowly, peaked after about 4 to 6 h, and then gradually decreased. Maximal accumulation of PAI-1 mRNA (3.2 and 2.4 kb species) when normalized for the amount of

glyceraldehyde-3-phosphate dehydrogenase mRNA and expresssed as the ratio of experimental: control at t=0 , was 28-fold with PMA (at 4 h), 9-fold with serum (at 6 h), and 23-fold with IL-1α (at 4 h).

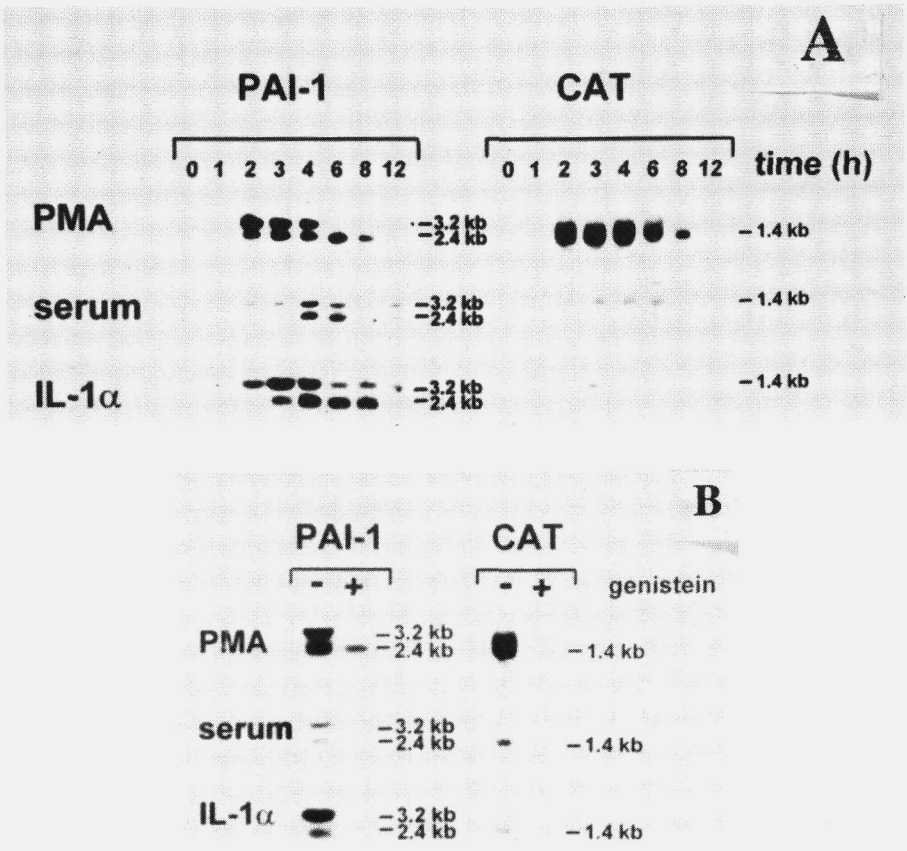

Fig. 1. Effect of PMA, serum, IL-1α and genistein on PAI-1 and CAT mRNA levels. HepG2 cells were preincubated with serum-free medium for 16 h, and then fresh medium containing PMA (100 nM), serum (10%, v/v) or IL-1α (300 U/ml) was added. (A) At the indicated times, total RNA was isolated and analyzed by Northern blotting for PAI-1 and CAT mRNA expression. (B) 1 h prior to the start of the experiment, serum-free medium with (+) or without (-) genistein (60 μg/ml) was added. Total RNA was isolated 3 h after induction and analyzed by Northern blotting for PAI-1 and CAT mRNA expression. (A and B) The amount of RNA in each lane was equal according to a reference probe, GAPDH (not shown). Blots were exposed to Amersham hyperfilm for 2 days (PAI-1) or 3 days (CAT). The experiment shown is representative for 3 independent experiments.

The induction of CAT mRNA shows a similar time profile as that of PAI-1 mRNA. However, whereas PMA stimulates CAT mRNA accumulation to a similar extent as that of PAI-1 mRNA (27-fold at 4 h), serum is a slightly weaker inducer of CAT mRNA (maximally 7-fold at 6 h), and IL-1α hardly induces CAT mRNA at all (maximally about 2-fold at 3 h).

Effect of genistein, tyrphostin A47 and vanadate on the induction of PAI-1 mRNA and promoter activity

Genistein, at 60 μg/ml, a concentration that almost completely prevents the increase in PAI-1 synthesis induced by IL-1α in cultured human endothelial cells and partly reduces the basal PAI-1 production by these cells (Van Hinsbergh et al., 1994), did not affect basal levels of PAI-1 mRNA in HepG2 cells over a 4 h period (data not shown). Genistein (60 μg/ml), when added 1 h before the addition of PMA, serum or IL-1α to HepG2 cells, almost fully blocked the induction of PAI-1 and CAT mRNA by these agents at 3 h (Fig. 1B). This inhibiting effect of genistein was concentration dependent, and became detectable at concentrations as low as 10 μg/ml (data not shown). One of the possible mechanisms of genistein action on the induction of PAI-1 gene transcription may be inhibition of protein tyrosine kinase activity. To further define the role of tyrosine phosphorylation in the regulation of PAI-1 gene expression, the effects of tyrphostin A47, an inhibitor of receptor-linked protein kinase activity (Levitski, 1992), and vanadate, an inhibitor of protein tyrosine phosphatases (Evans et al., 1994; Fantus et al., 1995), were evaluated. Tyrphostin A47 at 60 μg/ml, a concentration at which both genistein and tyrphostin completely blocked bFGF-induced mitogenesis in cultured human endothelial cells (Fotsis et al., 1993; Van Hinsbergh et al., 1994), failed to inhibit PAI-1 mRNA accumulation induced by PMA, serum and IL-1α in HepG2 cells (data not shown). Vanadate, at 250 μM, slowly, but continuously increased PAI-1 and CAT mRNA levels in HepG2 cells, as illustrated in Figure 2 for a 12 h incubation period. Remarkably, only the accumulation of the 3.2 kb PAI-1 transcript was observed with vanadate. Since the induction of PAI-1 gene expression by PMA, serum and IL-1α is dependent on ongoing protein synthesis (Bosma and Kooistra, 1991; Arts and Kooistra, unpublished results) and inhibition of protein synthesis by cycloheximide has been reported to result in the accumulation of PAI-1 mRNA in human endothelial cells (Van den Berg, 1988), we checked genistein, tyrphostin A47 and sodium orthovanadate for their effect on overall protein synthesis. Overall protein synthesis, as measured by [^{35}S]methionine incorporation, was not markedly affected by genistein, tyrphostin A47 or sodium orthovanadate at the concentrations used (data not shown), indicating that their effects on PAI-1 expression are directly related to their protein tyrosine kinase- or phosphatase-inhibiting activities.

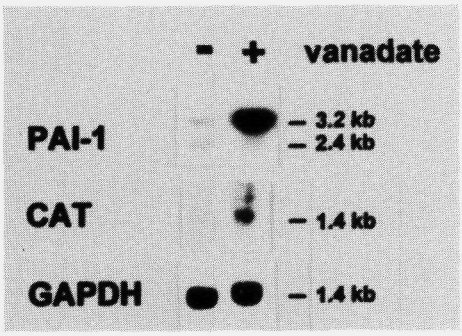

Fig. 2. Effect of vanadate on PAI-1 and CAT mRNA levels. HepG2 cells were preincubated with serum-free medium for 16 h, and then fresh medium containing sodium orthovanadate (250 μM) was added. After 12 h, total RNA was isolated and analyzed by Northern blotting for PAI-1, CAT and, as a reference, GAPDH mRNA expression. Blots were exposed to Amersham hyperfilm for 2 days. The experiment shown is representative for 2 independent experiments.

Effect of PMA and IL-1α on c-*jun* expression and c-Jun DNA binding activity

Previous studies indicated that PMA, serum and IL-1α transiently induce the accumulation of c-*jun* mRNA in HepG2 cells (Muegge et al., 1993; Arts et al., submitted). This would suggest that all three agents stimulate the expression of the CAT reporter gene in HepG2 cells when this reporter gene is under control of the c-Jun binding region of the PAI-1 promoter. However, the results depicted in Figure 1A show that only PMA and, to a somewhat lesser extent, serum effectively stimulate the accumulation of CAT mRNA in HepG2 cells, but that IL-1α poorly induces CAT mRNA accumulation. We therefore further evaluated the induction of c-Jun by PMA (100 nM) and IL-1α (300 U/ml) in HepG2 cells. As shown in Figure 3A, the mRNA for c-*jun* is strongly induced by PMA and IL-1α after 1 h. At this time-point, the mRNA for c-*fos*, the heterodimeric partner of c-*jun*, is also substantially elevated in the PMA-treated HepG2 cells. These stimulatory effects of PMA and IL-1α on c-*jun* and c-*fos* mRNA accumulation are completely suppressed in the presence of genistein (60 μg/ml) (Fig. 3A), but not by tyrphostin A47 (data not shown).

To determine whether the increase in c-*jun* and c-*fos* mRNA is reflected at the protein level, we analyzed c-Jun and c-Fos protein induction by PMA and IL-1α by performing immunoprecipitation of radiolabelled cell extracts using anti-c-Jun and anti-c-Fos polyclonal antibodies (Fig. 3B). To distinguish between complexes consisting of c-Jun/c-Fos heterodimers and c-Jun/c-Jun homodimers, cell extracts were first incubated with anti-c-Fos and subsequently with anti-c-Jun polyclonal antiserum. Figure 3 shows that radiolabelled c-Fos (a broad band around 55 kD) and coprecipitated c-Jun (39 kD) levels were strongly increased in PMA-treated HepG2 cells after 1.5 h. Levels of radiolabelled c-Jun not complexed to c-Fos also strongly increased after PMA treatment. These results thus confirm that the PMA-induced

increases in c-*jun* and c-*fos* mRNA are reflected at the protein level. In contrast to PMA, no significant amount of c-Jun/c-Fos protein was observed in IL-1α-treated HepG2 cells, and the induction of -homodimeric- c-Jun protein by IL-1α was relatively poor (Fig. 3B).

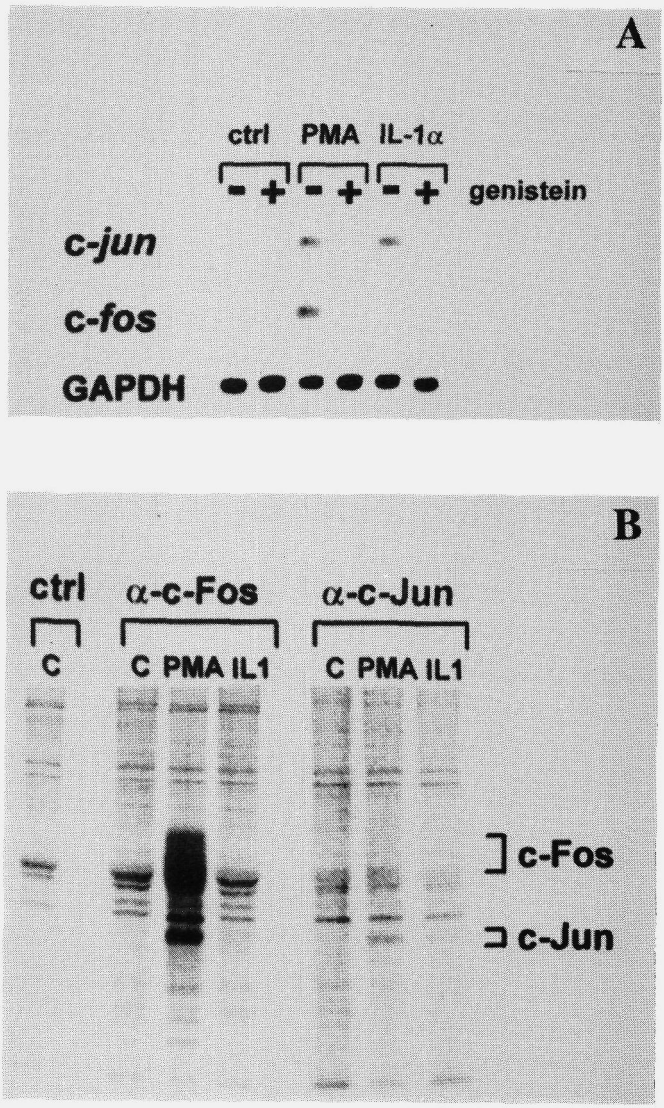

Fig. 3. Effect of PMA, IL-1α and genistein on c-*jun* and c-*fos* expression. HepG2 cells were preincubated with serum-free medium for 16 h, and then refreshed with serum-free medium containing PMA (100 nM), IL-1α (300 U/ml) or stock solvent (ctrl). 1 h prior to the start of the experiment, serum-free medium with (+) or without (-) genistein (60 μg/ml) was added. (A) Total RNA was isolated after 1 h, and analyzed by Northern blotting for c-*jun* and c-*fos* mRNA expression. Blots were exposed to Amersham hyperfilm for 7 days. The experiment shown is representative for 3 independent experiments. (B) [^{35}S]methionine was added 30 min after the addition of PMA, IL-1α (IL1) or stock solvent (C), and cell extracts were harvested after 1.5 h. Immunoprecipitations were performed successively with pre-immune serum (ctrl), anti-c-Fos polyclonal antiserum (α-c-Fos) and anti-c-Jun polyclonal antiserum (α-c-Jun). The immunoprecipitates were subjected to polyacrylamide gel electrophoresis and radiolabelled proteins were visualised by autoradiography for 2 days on a Fujix Bas-1000 phosphoimager screen. Brackets indicate the positions of c-Jun and c-Fos proteins on the basis of their molecular weights reported in literature (Oehler and Angel, 1992).

To analyze whether the induced c-Jun complexes are functionally active, we performed electromobility shift assays. As shown in Figure 4, two specific DNA-protein complexes can be observed upon incubation of the -66 to -43 region of the PAI-1 promoter with nuclear extracts from HepG2 cells. By pre-incubation of the nuclear extracts with anti-c-Jun or anti-c-Fos polyclonal antibodies it was deduced that the lower complex (complex 2) predominantly consists of DNA-bound c-Jun homodimers while the upper complex (complex 1) contains c-Jun protein heterodimerized with an unidentified protein (data not shown). We noted that PMA very strongly but IL-1α only weakly induced the formation of c-Jun homodimer binding complexes (complex 2). No significant induction of complex formation was observed with nuclear extracts of HepG2 cells that had been stimulated by PMA or IL-1α in the presence of genistein (Fig. 4).

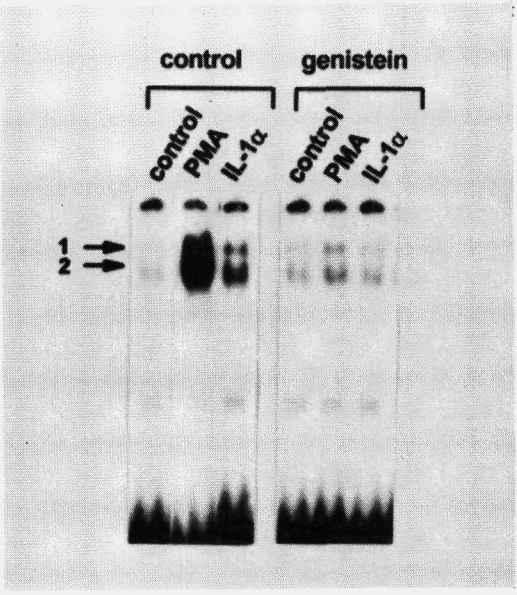

Fig. 4. HepG2 nuclear protein binding to the PAI-1 promoter AP-1 binding site as assayed by electromobility shift assay. HepG2 cells were preincubated with serum-free medium for 16 h, and then refreshed with serum-free medium with (+) or without (-) genistein (60 μg/ml). After a 1 h incubation, PMA (100 nM), IL-1α (300 U/ml) or stock solvent (control) was added. After 2.5 h, nuclear extracts were prepared as described in the Methods section. Extracts were incubated for 30 min with a radiolabelled oligodeoxynucleotide representing the -66 to -43 region of the PAI-1 promoter. DNA/protein complexes were separated on a 5% (w/v) polyacrylamide gel and radiolabelled complexes were visualised by auto-radiography for 5 days. Arrows mark specific DNA/protein complexes.

DISCUSSION

Regulation of PAI-1 gene transcription is responsive to a large variety of hormones, cytokines and growth factors, thus reflecting the important role of the inhibitor in several physiological and pathophysiological conditions (see reviews by Andreasen et al., 1990; Loskutoff, 1991). Although the induction of PAI-1 expression by the various compounds often shows very similar characteristics, the present studies demonstrate that precisely how PAI-1 transcription is regulated is, at least in part, a function of the stimulus involved.

Early experiments showed that the protein kinase C activating phorbol ester PMA, serum and IL-1α induce PAI-1 expression in HepG2 cells at the transcriptional level with a comparable time-profile and to a similar extent; these inductions were found to be dependent on ongoing protein synthesis (Bosma and Kooistra, 1991; Arts and Kooistra, unpublished results). Furthermore, the potent, dose-dependent inhibition of PMA-, serum- and IL-1α-induced PAI-1 expression by genistein, as demonstrated in this paper, is strongly suggestive of tyrosine phosphorylation being an intermediary step in the action of all three agents. Previous experiments showed that the PMA response required the PAI-1 AP-1 binding site at position -58 to -50, and DNA-protein binding studies showed an interaction between this promoter region and c-Jun homodimers (Descheemaeker et al., 1991; Arts et al., submitted). Here, we demonstrated in experiments with HepG2 cells that were stably transfected with a CAT expression vector under control of the -489 to +75 region of the PAI-1 promoter, that this promoter fragment is sufficient to mediate the PMA and, to a lesser extent, the serum response, but other regions of the PAI-1 promoter must mediate IL-1α induction of PAI-1 transcription. Gel shift experiments using the PAI-1 AP-1 binding promoter region showed that IL-1α, in contrast to PMA, hardly induced c-Jun binding activity. These results argue in favour of two separate PAI-1 inductory pathways for PMA and IL-1α, whereby the serum-mediated signal transduction pathway may (partially) overlap with the PMA-activated pathway.

In support of our finding that the IL-1α response mechanism in HepG2 cells differs from that of the PKC-activator PMA, Fandrey et al. (1994) found no translocation of PKC isoenzymes with IL-1α (or TNFα) in HepG2, strongly suggesting that these cytokines do not activate PKC in HepG2 cells. Furthermore, Daffada et al. (1994) reported a relatively poor IL-1α induction of an AP-1 responsive reporter construct in HepG2 cells. Also, IL-1 has been reported to be a weak inducer of the AP-1 responsive region of the collagenase and stromelysin-1 promoters in fibroblasts (Vincenti et al., 1994; Quinones et al., 1994). Finally, Bird et al. (1994) showed that, in HepG2 cells, IL-1α does induce the p54 stress activated

protein kinase (SAPK) which mediates c-*jun* transcriptional induction but, in contrast to PMA, is unable to activate the p42/44 mitogen activated protein kinase (ERK 1/2), which induces c-Jun DNA binding activity (Karin and Hunter, 1995). These latter findings may also explain the lack of c-Jun activation by IL-1α in our studies, even though c-*jun* mRNA levels were effectively induced.

Several studies implicate a role for nuclear factor kappa B (NF-κB) in IL-1 signal transduction in HepG2 cells. For example, Daffada et al. (1994) showed that IL-1α strongly increased NF-κB activity in HepG2 cells, using a CAT expression vector under control of an NF-κB region. Whether NF-κB plays a role in the IL-1α-induced PAI-1 gene transcription in HepG2 cells or through which site the IL-1α response is mediated, is not clear at present. One candidate region is the IL-1α inducible site between -675 and -669 reported by Dawson et al. (1993). This site has similarities to an NF-κB binding site and NF-κB has also been implicated in the PAI-1 transcriptional induction by TNFα in human endothelial cells (Ferran et al., 1995). In this context it is of interest that genistein has been reported to inhibit NF-κB activation by IL-1 (Iwasaki et al., 1992; Joshi-Barve et al., 1993).

We demonstrated that genistein effectively suppressed the induction of PAI-1 gene transcription by PMA, serum and IL-1α. Genistein probably acts by inhibiting a protein tyrosine kinase, because the structural genistein analogue daidzein, which has a low protein tyrosine kinase activity (Akiyama et al., 1987), did not inhibit stimulated PAI-1 synthesis. Consistent with an involvement of tyrosine kinases in the signal transduction pathways leading to stimulated PAI-1 gene transcription by PMA, serum and IL-1α are the data obtained with sodium orthovanadate, a potent inhibitor of protein tyrosine phosphatases (Evans et al., 1994; Fantus et al., 1995). We found that incubation of the stably transfected HepG2 cells with sodium orthovanadate resulted in the accumulation of the (3.2 kb form of) PAI-1 mRNA and CAT mRNA. Genistein was rather selective in its effect, because several other protein tyrosine kinase inhibitors, including tyrphostin A47 in this study with HepG2, and tyrphostin A47, the erbstatin analogue methyl-2,5-dihydroxy-cinnamate and the thiazolidine-dione compound 5 in human endothelial cells (Van Hinsbergh et al., 1994) were unable to interfere with PAI-1 gene induction. Similarly, we found that genistein but not tyrphostin effectively suppressed the induction of c-*jun* mRNA by PMA, serum and IL-1α. It is unlikely that the inhibition of c-*jun* mRNA induction by genistein is a direct effect on a PKC-dependent pathway, since genistein is a poor inhibitor of PKC activity with an apparent $IC_{50} > 100$ μg/ml (Akiyama et al., 1987). Several reports have suggested that mitogen-activated protein (MAP) kinases may be a target of genistein (Thorburn and Thorburn, 1994; Rzymkiewicz et al., 1995). MAP kinases have been identified as an integration point for multiple biochemical

signals (Karin and Hunter, 1995; Cobb and Goldsmith, 1995), and have also been implicated in signal transduction by both PMA and IL-1α in HepG2 cells (Bird et al., 1994). Whether distinct MAP kinase cascades are also part of the two separate PAI-1 inductory pathways for PMA and IL-α is not clear at present and requires further research.

An interesting observation during our studies on the role of protein tyrosine phosphorylation in PAI-1 transcriptional regulation was the fact that in the presence of sodium orthovanadate only the upper band of PAI-1 mRNA (the 3.2 kb form) accumulated in HepG2 cells, while in experiments with genistein predominantly the lower band (the 2.4 kb form) was detectable. How such a shift in the ratio between the two PAI-1 mRNAs is brought about, cannot be deduced from our work. One possibility is a shift in the use of the two alternative polyadenylation sites, as has been observed for the mouse dihydrofolate reductase gene during cell growth and for the rat GsαN1 signal transduction protein gene after dexamethasone treatment (Kaufman and Sharp, 1983; Crawford et al., 1993). The other possibility is a change in posttranscriptional regulation of PAI-1 gene expression depending on protein tyrosine phosphorylation. In this respect it might be significant that genistein has been found to decrease c-*myc* mRNA in NIH-3T3 cells at a similar concentration as it inhibits PAI-1 expression in HepG2 cells (Linassier et al., 1990): c-*myc* protein has been suggested to affect PAI-1 gene expression at the level of RNA processing, nuclear RNA turnover and RNA export (Prendergast and Cole, 1989; Prendergast et al., 1990).

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CHAPTER 7

Studies on the mechanism of fibrate-inhibited expression of plasminogen activator inhibitor 1 in cultured hepatocytes from cynomolgus monkey.

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ABSTRACT

Fibrates are widely used drugs in hyperlipidemic disorders. In addition to lowering serum triglyceride levels, fibrates have also been shown to reduce elevated plasma plasminogen activator inhibitor 1 (PAI-1) levels *in vivo*. We demonstrate that fibrates suppress PAI-1 synthesis in cultured cynomolgus monkey hepatocytes in a concentration-dependent way (0.1 - 1.0 mmol/L) and independent of their lipid-lowering effect. Different fibrates showed different potency in suppressing PAI-1 production: gemfibrozil and clofibric acid, at a concentration of 1 mmol/L, reduced PAI-1 synthesis over 24 h to 52±20% and 60±5%, while clofibrate and bezafibrate lowered PAI-1 synthesis only to 86±17% and 84±15% of control values, respectively. These changes in PAI-1 production by fibrates correlated with changes in PAI-1 mRNA levels and were also visible at the level of gene transcription. Fibrates did not lower basal PAI-1 synthesis, but attenuated an acceleration of PAI-1 production during culture. The suppressing effect of fibrates on PAI-1 synthesis could not be mimicked with activators or inhibitors of protein kinase C. Furthermore, fibrates did not inhibit the increase in PAI-1 synthesis induced by epidermal growth factor or transforming growth factor-ß. These results make mechanisms involving PKC modulation or growth factor receptor inactivation as a mode of action of fibrates unlikely. The suppressing effect of fibrates on PAI-1 synthesis could involve the nuclear receptor peroxisome proliferator-activated receptor (PPAR), and its heterodimeric partner, the retinoid X receptor (RXR). The alpha forms of PPAR and RXR were both found to be expressed in cynomolgus monkey hepatocytes. The ligand for RXRα, 9-*cis* retinoic acid, suppressed PAI-1 synthesis to the same extent as gemfibrozil, while a combination of gemfibrozil and 9-*cis* retinoic acid had no more effect on PAI-1 synthesis than any of these compounds alone at optimal concentrations. In conclusion, fibrates downregulate an induced PAI-1 production in cynomolgus monkey hepatocytes independent of a decrease in triglyceride levels. A possible involvement of PPARα/RXRα in this downregulation is discussed.

INTRODUCTION

Fibrates are a class of hypolipidemic drugs widely used in the treatment of diet-resistant hyperlipidemia. In humans, fibrates effectively lower elevated serum triglycerides and increase

high-density lipoprotein cholesterol. Fibrates also moderately lower low-density lipoprotein cholesterol levels in patients with hypercholesterolemia.[1] In addition to these lipoprotein profile-altering effects, some fibrates also exert a favourable influence on plasma levels of hemostatic risk factors, such as plasminogen activator inhibitor 1 (PAI-1). These combined actions of fibrates may be beneficial in reducing the risk of coronary heart disease.[1]

The mechanism by which fibrates reduce plasma PAI-1 levels is unknown. Several reports have documented a correlation between plasma triglyceride levels and PAI-1 levels.[2] Also, lowering triglyceride levels by diet or drugs has been shown to be associated with a decrease in PAI-1 levels[2-5], thus suggesting a relationship between triglyceride and PAI-1 levels.[2,6] On the other hand, several lines of evidence suggest a mechanism of action of fibrates independent of their triglyceride lowering effect. For example, the fibrate gemfibrozil was shown to significantly lower PAI-1 antigen levels, while in the same study no change in PAI-1 antigen levels by fenofibrate was observed, although both fibrates were equipotent in lowering triglyceride levels.[7] Furthermore, it was shown that gemfibrozil reduces PAI-1 secretion in vitro in the human hepatoma cell line Hep G2, suggesting a direct effect of this drug on PAI-1 expression.[8]

In the present report we demonstrate that fibrates directly, i.e. independent of lowering triglyceride levels, suppress PAI-1 expression in primary cultures of hepatocytes from cynomolgus monkey. We have further used this in vitro model of cultured monkey hepatocytes to compare the efficacy of various fibrates to lower PAI-1 production and to study the mechanism(s) by which fibrates exert their action. Studies were designed to evaluate the role of a number of signal transduction pathways reported to be affected by fibrates and/or to be involved in PAI-1 gene expression. First, we tested the role of protein kinase C (PKC), the activity of which has been shown to be modulated by fibrates[9,10] and to be important for PAI-1 expression in Hep G2.[11] Secondly, fibrates have been reported to affect the phosphorylation of growth factor receptors and thereby their signal transduction activity.[12] We have evaluated whether fibrates interfere with the response of hepatocytes to epidermal growth factor (EGF) and transforming growth factor-ß (TGF-ß), growth factors which have been found to be inducers of PAI-1 expression in Hep G2.[13,14] Thirdly, we examined a possible role of the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR), which is activated by fibrates.[15] PPAR has been reported to downregulate gene expression through squelching of c-Jun[16], a transcription factor which we found to be critical in PAI-1 gene expression in the human hepatoma cell line Hep G2 (Arts et al, unpublished data).

MATERIALS AND METHODS

Materials

Clofibric acid, clofibrate, dexamethasone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Gemfibrozil was a gift of Dr.B. Bierman, Warner-Lambert (Hoofddorp, The Netherlands). Bezafibrate was obtained from Boehringer Mannheim B.V. (Almere, The Netherlands). Stock solutions of fibrates (1 mol/L) and phorbol 12-myristate 13-acetate (PMA) (100 μmol/L) were prepared in dimethyl sulfoxide (DMSO) and ethanol, respectively and kept at -20°C. Before use, fibrate stocks were diluted in incubation medium and kept for 2 h at 37°C to ensure complete dissolution of the fibrates. Human epidermal growth factor (EGF) was obtained from Campro (Veenendaal, The Netherlands), transforming growth factor-ß (TGF-ß) from Harbor Bioproducts (Norwood, MA), tyrphostin from Brunschwig (Amsterdam, The Netherlands) and insulin (Actrapid Penfill 1.5) from Novo Nordisk Farma B.V. (Zoeterwoude, The Netherlands). 9-*cis* retinoic acid was a gift of Drs. M. Klaus and C. Apfel, Hoffmann-LaRoche Ltd. (Basle, Switzerland). The specific protein kinase C inhibitor Ro 31-8220 was a gift of Dr. G. Lawton, Hoffmann-LaRoche (Welwyn Garden City, U.K.). Deoxycytidine 5[α-^{32}P]triphosphate (3 Ci/mol), [^{35}S]methionine (>1000 Ci/mmol) and the Megaprime-kit were obtained from Amersham Nederland BV ('s Hertogenbosch, The Netherlands). The Tintelize enzyme immunoassay kit for determination of PAI-1 antigen was from Biopool (Umeå, Sweden). Bradford protein reagent was from Biorad (Veenendaal, The Netherlands). Other materials used in the methods described below have been specified in detail in relating references or were purchased from standard commercial sources.

Isolation and culture of cynomolgus monkey primary hepatocytes

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (*Macaca fascicularis*, 1.5-3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at that institute. The isolation procedure was essentially as described for human hepatocytes[17,18] with a few modifications as described by Kaptein et al.[19] Total cell yields varied from 0.5 to 1.5×10^9 viable cells. Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%, w/v), was at least 65%. The cells were seeded in culture dishes at a density of 2×10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 135 nmol/L insulin, 50 nmol/L dexamethasone, 2 mmol/L L-glutamine (Flow Laboratories, Irvine, U.K.), 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, at 37°C in a 5% CO_2/ 95% air atmosphere. After 16 hours, the non-adherent cells were washed from the plates and the remaining cells were refreshed with the same medium as described above. After 8 h, the medium was changed to incubation medium in which the amount of insulin was lowered to 10 nmol/L. Experiments were started 24 h after hepatocyte isolation.

Conditioned media were obtained by incubating cells at 37°C for various times with incubation medium containing the appropriate concentration of the test compound or stock solvent (DMSO or ethanol; final concentration 0.1% (v/v)) as control. For prolonged incubations, the media were refreshed every 24 h. Conditioned media were centrifuged for 4 min at 5000 g in a Beckman Microfuge centrifuge to remove cells and cellular debris, and the samples were kept at -20°C until use. The cells were washed twice with ice-cold phosphate buffered saline, and were used for isolation of RNA or nuclei.

Northern blot analysis

Total RNA was isolated from at least 2×10^6 simian hepatocytes according to Chomczynski and Sacchi.[20] RNA was fractionated by electrophoresis in a 1% (w/v) agarose gel under denaturing conditions using 1 mol/L formaldehyde[21], and blotted to Hybond-N filter according to the manufacturers instructions. The filters were hybridized overnight at 63°C (with the PAI-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), PPARα and RXRα probe) or at 42°C (with the acyl-CoA oxidase (ACO) probe) in hybridization mix, being: 7% (w/v) sodium dodecylsulfate (SDS), 0.5 mol/L Na_2HPO_4/NaH_2PO_4 buffer, pH 7.2, 1 mmol/L EDTA, containing 3 ng of [α-^{32}P]CTP-labeled

probe/ml. After hybridization with GAPDH or PAI-1 probe, the filters were washed twice with 2xSSC (1xSSC being: 0.15 mol/L NaCl, 0.015 mol/L Na_3citrate), 1% (w/v) SDS, and twice with 1xSSC, 1% (w/v) SDS for 20 min at 63°C. In the case of hybridizations with PPARα or RXRα probe the filters were washed four times for 20 min with 2xSSC, 1% (w/v) SDS at 63°C, while blots hybridized with ACO probe were washed three times for 15 min with 2xSSC, 1% (w/v) SDS and one time for 15 min with 0.5xSSC, 1% (w/v) SDS at 42°C. The filters were then exposed to Kodak XAR-5 X-ray film with an intensifying screen at -80°C. The relative intensities of the bands present were determined on a Fujix Bas 1000 phosphoimager.

cDNA probes

cDNA probes used are: a 2.5 kb EcoRI fragment of the human PAI-1 cDNA[22]; a 1.2 kb PstI fragment of the rat GAPDH cDNA provided by Dr.R. Offringa[23]; a 1.3 kb NruI/BamHI fragment of the human PPARα cDNA provided by Dr.F.J. Gonzalez[24]; a 1.4 kb EcoRI/BglII fragment of the human RXRα cDNA provided by Dr.J. Grippo (Hoffmann-LaRoche, Nutley, USA); a 2.0 kb SacI fragment of the rat ACO cDNA, provided by Dr.T. Osumi[25]; and a 1.2 kb PstI fragment of the hamster actin cDNA provided by Dr.W. Quax.[26]

Assays

PAI-1 antigen levels in conditioned media of cynomolgus monkey hepatocyte cultures were determined with an adapted Tintelize PAI-1 assay from Biopool (Umeå, Sweden). This assay normally does not recognize cynomolgus monkey PAI-1 antigen, but when the coating antibody is replaced by a goat anti-human PAI-1 polyclonal antiserum (5μg/ml) (Biopool, Umeå, Sweden), both monkey and human PAI-1 antigen can be determined. Monkey PAI-1 antigen values were calibrated using the human PAI-1 calibration sample present in the kit. Overall protein synthesis was determined by measuring the incorporation of [^{35}S]methionine into the 10% (w/v) trichloroacetic acid precipitable fraction of radiolabeled conditioned medium and cell extract.[27] Cell viability was assessed by the MTT assay[28] which is based on the cellular reduction of MTT by mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically at 545 nm. Protein was measured using the Bradford protein assay. Nuclear run on assays were performed according to Groudine et al[29] with some minor modifications as described by Twisk et al.[30]

Statistical analysis

Statistical significance of differences was calculated using Student's t-test for paired data with the level of significance selected to be $p < 0.05$ (*) and $p < 0.01$ (**). Values are expressed as means ± S.D.

RESULTS

Dose-dependency and time-course of the effect of different fibrates on PAI-1 synthesis

Cynomolgus monkey hepatocytes were incubated with three concentrations (0.1, 0.3 or 1 mmol/L) of four different fibrates (gemfibrozil, clofibric acid, clofibrate or bezafibrate) for two consecutive periods of 24 h. As shown in Figure 1, all four fibrates dose-dependently lowered PAI-1 levels, reaching 52±20% (gemfibrozil), 60±5% (clofibric acid), 86±17% (clofibrate) and 84±15% (bezafibrate) of control values after 24 h at a 1 mmol/L concentration. During the second 24 h incubation period, the fibrates did not lower PAI-1 levels markedly further than in the first 24 h incubation period (Fig. 1). Similar results were obtained when experiments were

performed with medium containing 10% (v/v) lipoprotein-depleted serum or 1% (v/v) human serum albumin instead of 10% (v/v) fetal calf serum, indicating that fibrates can suppress PAI-1 synthesis independent of changes in triglyceride levels (data not shown). The decreases in PAI-1 production by fibrates were not due to diminished cell viability (as tested with the MTT test) or changes in overall protein synthesis (as assessed by simultaneous measurement of the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable products). Fibrate concentrations higher than 1 mmol/L, however, were found to lower overall protein synthesis (data not shown). These results indicate that different fibrates possess different potency to reduce PAI-1 expression.

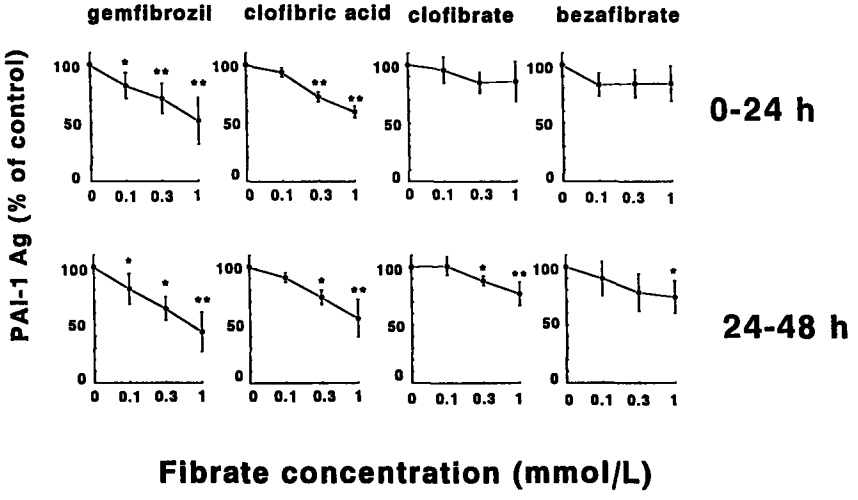

Fig. 1. Effect of fibrates on PAI-1 antigen production in cultured simian hepatocytes.
Cynomolgus monkey hepatocytes were incubated for two consecutive periods of 24 h with different concentrations of gemfibrozil, clofibric acid, clofibrate and bezafibrate, and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. Results are means ± S.D. of 3 to 7 independent experiments performed in duplicate; the data are expressed as percentage values of controls. Control values ranged between 171 and 1700 ng/ml over the first 24 h period and between 295 and 2501 ng/ml over the second 24 h period in the different experiments. Values significantly different from control values are indicated with asterisk (*:$p<0.05$ and **:$p<0.01$).

Figure 2 shows a representative time-course of the suppressive effect of gemfibrozil (1 mmol/L) on PAI-1 antigen accumulation. Both in the absence and presence of gemfibrozil, PAI-1 antigen levels increase linearly in time during the first 16 h. However, between 16 and 24 h of incubation, PAI-1 shows an accelerated increase under control conditions, whereas with gemfibrozil PAI-1 continues to accumulate at a constant rate. This results in an about 2-fold higher PAI-1 antigen level in conditioned medium of control cells than in the conditioned medium of gemfibrozil-treated cells after 24 h. The accelerated increase in PAI-1 synthesis varied between 32% and 75% in five independent experiments and was in magnitude comparable to the observed inhibition of PAI-1 synthesis by gemfibrozil. Similar results were obtained with clofibric acid, and analysis of the attenuating effect of fibrates on PAI-1 synthesis in Hep G2 cells learned that these effects could be attributed also to a diminishing effect of fibrates on the induction of PAI-1 synthesis during culture of Hep G2 cells (data not shown). Apparently, fibrates do not inhibit basal PAI-1 synthesis, but prevent the accelerated production of PAI-1, as occurring during control incubation conditions.

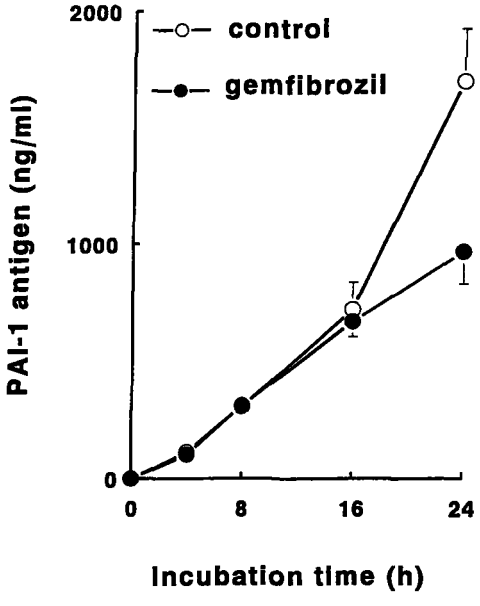

Fig. 2. Time course of PAI-1 antigen production by cultured simian hepatocytes incubated with or without gemfibrozil. Cynomolgus monkey hepatocytes were incubated for various times up to 24 h in the presence (●) or absence (○) of 1 mmol/L gemfibrozil, and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. The experiment shown is representative for five independent experiments in duplicate. Values shown are means ± range.

Effect of gemfibrozil on PAI-1 mRNA levels and transcription

To determine whether the accelerated increase in PAI-1 antigen levels and inhibition thereof by fibrates was reflected at the mRNA level, we performed Northern analysis. As shown in Figure 3, there is no marked difference in PAI-1 mRNA levels between control and 1 mmol/L gemfibrozil-treated hepatocytes at 8 h. At 16 h, however, PAI-1 mRNA levels are strongly elevated in control, but not in gemfibrozil-treated hepatocytes, and this difference is maintained at 24 h. These mRNA data explain the different PAI-1 protein production rates in control and gemfibrozil-treated hepatocytes between 16 and 24 h (Fig. 2). The transient, about two-fold induction of PAI-1 mRNA by gemfibrozil at 4 h was consistently found in three independent experiments, and could be suppressed by an inhibitor of protein kinase C (see below). No such induction was observed with the other three fibrates (data not shown).

To determine whether the differences in PAI-1 mRNA levels in control and gemfibrozil-treated hepatocytes were the result of different PAI-1 transcription rates, we performed a nuclear run-on assay. Figure 4 shows that hepatocytes incubated with gemfibrozil have a 2-fold lower PAI-1 transcription rate than control cells.

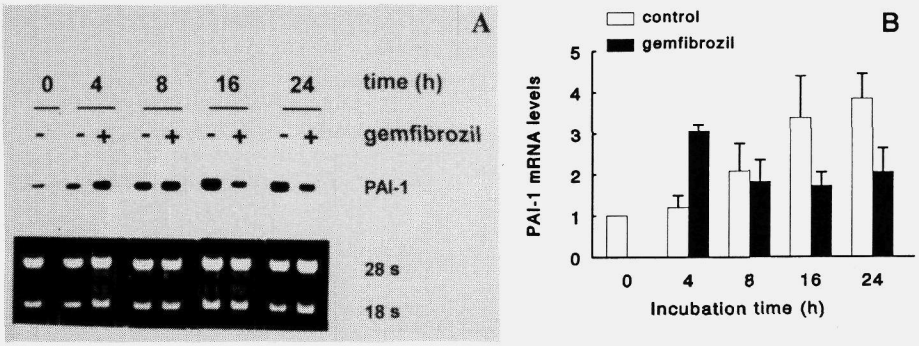

Fig. 3. Time course of the effect of gemfibrozil on PAI-1 mRNA levels in cultured simian hepatocytes. Cynomolgus monkey hepatocytes were incubated with (+) or without (-) 1 mmol/L gemfibrozil for various times up to 24 h. Total RNA was isolated, and 5 μg of RNA was analyzed by Northern blotting for PAI-1 mRNA. Equal loading was checked by ethidium bromide staining of 18S and 28S ribosomal RNA. The experiment shown in panel A is representative for three independent experiments. The signals for PAI-1 mRNA were quantified by densitometry and adjusted for the corresponding GAPDH mRNA signals. The amount of PAI-1 mRNA present at the different time points is expressed relative to that found at t=0. The results shown in panel B are means $\pm$ S.D. of three independent experiments.

Role of Protein Kinase C in PAI-1 expression

The transient increase in PAI-1 mRNA levels observed with gemfibrozil at 4 h (Fig. 3) is probably due to activation of PKC, since a similar rapid increase in PAI-1 mRNA levels was also seen with the specific PKC activator PMA, and both effects could be suppressed by the PKC inhibitor, Ro 31-8220 (Fig. 5). To assess whether modulation of PKC activity also plays a role in the suppression of the accelerated increase in PAI-1 synthesis between 16 and 24 h (see Fig. 2), we examined the effect of PMA and Ro 31-8220 on PAI-1 synthesis over a 24 h period (Table 1). Opposite to gemfibrozil, PMA increased PAI-1 levels, thus reflecting the strong induction of PAI-1 mRNA levels at 4 h (Fig. 3). The PKC inhibitor Ro 31-8220 suppressed the effect of PMA, but had no effect on PAI-1 production under control conditions. Together with the finding that clofibric acid, which did not transiently activate PKC after 4 h, was almost as effective as gemfibrozil in suppressing PAI-1 synthesis after a 24 h incubation period, these results indicate that fibrates suppress PAI-1 synthesis through a mechanism independent of PKC.

TABLE 1. Effect of PMA and Ro 31-8220 on PAI-1 synthesis in simian hepatocytes.

compound	PAI-1 synthesis (% of conrol)
PMA	169 ± 6
Ro 31-8220 (10 μmol/L)	107 ± 3
PMA (100 nmol/L) + Ro 31-8220 (10 μmol/L)	108 ± 3
Gemfibrozil (1 mmol/L)	25 ± 6

Cynomolgus monkey hepatocytes were incubated for 25 h with incubation medium containing gemfibrozil (1 mmol/L), the PKC inhibitor Ro 31-8220 (10 μmol/L) or solvent (control). 1 h after the start of the experiment, the PKC activator PMA (100 nmol/L) was added. At the end of the incubation, media were collected and analyzed for PAI-1 antigen as described in the Methods section. Values given are means ± range of two independent experiments performed in duplicate and expressed as a percentage of the control values (171 and 290 ng/ml, respectively).

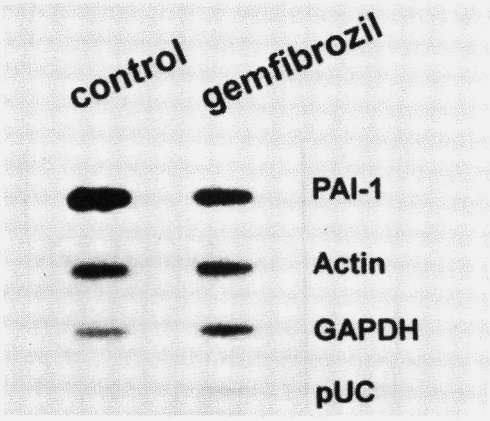

Fig. 4. Analysis of PAI-1 gene transcription rate after incubation of cultured simian hepatocytes with gemfibrozil. Cynomolgus monkey hepatocytes were incubated for 48 h in the absence (control) or presence of 0.6 mmol/L gemfibrozil. Nuclei were isolated and used for run-on assays as described in the Methods section. Actin and GAPDH are controls for variation in mRNA labeling and pUC served as a control for nonspecific hybridization.

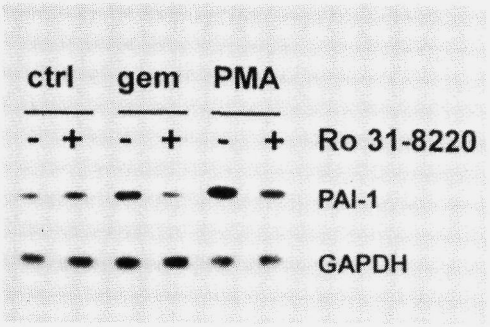

Fig. 5. Effect of gemfibrozil and PMA on PAI-1 mRNA levels in simian hepatocytes incubated in the presence or absence of Ro 31-8220. Cynomolgus monkey hepatocytes were incubated for 5 h with incubation medium with (+) or without (-) 10 μmol/L of the PKC inhibitor Ro 31-8220. 1 h after the start of the experiment, gemfibrozil (1 mmol/L), the PKC activator PMA (100 nmol/L) or solvent (ctrl) were added. At the end of the incubation, total RNA was isolated and 5 μg of RNA was analyzed by Northern blotting for PAI-1 mRNA and for GAPDH mRNA as a control for equal mRNA loading. The experiment shown is representative for three independent experiments.

Effect of fibrates on the induction of PAI-1 by EGF and TGF-ß

As shown in Figure 6, epidermal growth factor (EGF; 5 ng/ml) and transforming growth factor-ß (TGF-ß; 5 ng/ml) induce PAI-1 mRNA levels about 3-fold in primary hepatocytes after a 4 and 6 h incubation period, respectively. The EGF-mediated induction of PAI-1 mRNA levels was suppressed for over 50% by the tyrosine protein kinase inhibitor tyrphostin (30 μg/ml; data not shown), a compound known to interfere with EGF-receptor mediated signaling.[31] The induction of PAI-1 mRNA could not be inhibited, however, with 1 mmol/L gemfibrozil added to the cells 1 h prior to the addition of EGF or TGF-ß (Fig. 6). Similar negative results were obtained with 1 mmol/L clofibric acid, clofibrate and bezafibrate (data not shown). Because the effect of fibrates on PAI-1 synthesis became apparent after 16 h, we repeated the experiment after a 16 h preincubation with the fibrates. Again, no quenching effect on PAI-1 mRNA

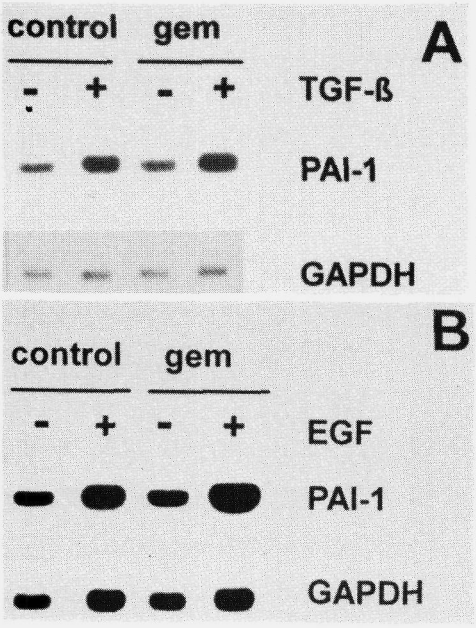

Fig. 6. Effect of gemfibrozil on PAI-1 mRNA levels in simian hepatocytes incubated in the presence or absence of TGF-ß or EGF. Cynomolgus monkey hepatocytes were incubated for 7 h (panel A) or 5 h (panel B) with incubation medium containing solvent (control) or 1 mmol/L of gemfibrozil (gem). 1 h after the start of the experiment, solvent (-), 5 ng/ml TGF-ß (+, panel A) or 5 ng/ml EGF (+, panel B) was added. At the end of the incubation, total RNA was isolated, and 5 μg of RNA was analyzed by Northern blotting for PAI-1 mRNA and for GAPDH mRNA as a control for equal mRNA loading. The experiment shown is representative for three independent experiments.

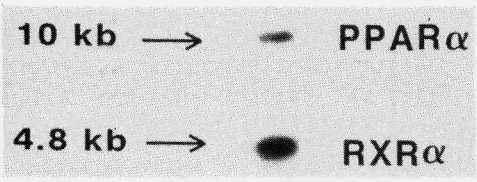

Fig. 7. Simian hepatocytes express PPARα and RXRα mRNA. Cynomolgus monkey hepatocytes were incubated for 24 h with incubation medium. At the end of the incubation, total RNA was isolated, and 5 μg of RNA was analyzed by Northern blotting for PPARα and RXRα mRNA expression. The experiment shown is representative for three independent experiments.

induction by EGF or TGF-ß was observed (data not shown). These data indicate that fibrates do not interfere with growth factor receptor mediated signaling.

Studies on a role of PPAR in the inhibition of PAI-1 synthesis by gemfibrozil

We next considered a possible role for the peroxisome proliferator-activated receptor (PPAR) in the inhibition of PAI-1 synthesis by gemfibrozil. As shown in Figure 7, cynomolgus monkey hepatocytes express the mRNAs of PPARα and retinoid-X-receptor α (RXRα), another steroid hormone receptor with which PPARα interacts to form heterodimers. Since the RXRα ligand, 9-*cis* retinoic acid, has been demonstrated to enhance PPAR action,[32] we tested the effect of gemfibrozil, 9-*cis* retinoic acid, and combinations thereof on PAI-1 synthesis. As shown in Figure 8, 10 μmol/L 9-*cis* retinoic acid was almost as effective as 1 mmol/L gemfibrozil in

inhibiting PAI-1 synthesis: in three independent experiments, 9-*cis* retinoic acid and gemfibrozil decreased PAI-1 synthesis to 65±4% and 59±6% of control values (mean ± SD), respectively. The inhibitions of PAI-1 synthesis by 9-*cis* retinoic acid and gemfibrozil in these experiments were of the same magnitude as the corresponding accelerated increases in PAI-1 synthesis under control conditions (data not shown). These data suggest that 9-*cis* retinoic acid, like gemfibrozil, only prevents the accelerated increase in PAI-1 production, but does not affect the uninduced PAI-1 synthesis rate. Combinations of suboptimal concentrations of the two ligands cooperatively decreased PAI-1 synthesis (data not shown), but the maximal effect never exceeded the inhibiting effect seen with the optimal concentration of gemfibrozil as illustrated in Figure 8 for 1 μmol/L 9-*cis* retinoic acid and 1 mmol/L gemfibrozil. Taken together, these data suggest that 9-*cis* retinoic acid and gemfibrozil interfere with the same PAI-1 stimulatory pathway. They do not necessarily imply, however, that 9-*cis* retinoic acid and gemfibrozil act via the same regulatory pathway or mechanism.

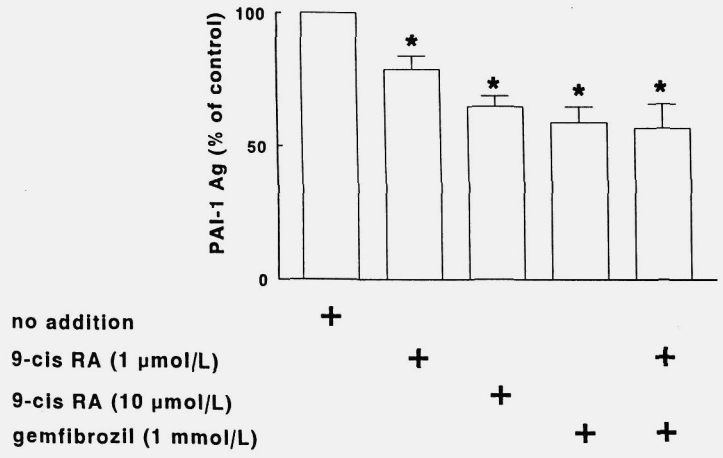

Fig. 8. Effect of gemfibrozil and 9-*cis* retinoic acid on PAI-1 synthesis in simian hepatocytes. Cynomolgus monkey hepatocytes were incubated for 24 h with incubation medium containing solvent (no addition), 9-*cis* retinoic acid (1 μmol/L or 10 μmol/L) and/or gemfibrozil (1 mmol/L), and the conditioned media were analyzed for PAI-1 antigen as described in the Methods section. Results presented are means ± S.D. of 3 independent experiments performed in duplicate; the data are expressed as percentage values of controls. Values significantly different from control values are indicated with an asterisk ($p < 0.05$).

DISCUSSION

In this study, we demonstrated a suppressive effect of fibrates on PAI-1 synthesis and PAI-1 mRNA levels in cultured cynomolgus monkey hepatocytes independent of changes in concentrations of triglycerides in the culture medium. We found that fibrates inhibit PAI-1 synthesis in a dose-dependent way and that different fibrates differ in their capacity to suppress PAI-1 production. Fibrates appeared to attenuate the accelerated increase in PAI-1 synthesis occuring under basal culture conditions. The changes in PAI-1 protein synthesis correlated closely with changes in PAI-1 mRNA and could also be demonstrated at the transcriptional level. Therefore, fibrates act selectively on a pathway that stimulates the activation of PAI-1 gene transcription. The regulatory mechanism by which this suppressive effect is brought about remained unknown, but could involve PPARα/RXRα.

Our finding that the suppression of PAI-1 synthesis by fibrates is a direct effect, i.e. independent of lowering triglyceride levels, is in agreement with *in vivo* studies which show that simply reducing triglycerides is not sufficient to lower PAI-1 in patient populations with elevated triglyceride and PAI-1 levels.[1,33,34] Furthermore, our *in vitro* observation that fibrates inhibit an induction of PAI-1 synthesis rather than basal PAI-1 expression, is comparable to findings reported for the human hepatoma cell line Hep G2[8] and is in agreement with the *in vivo* situation where fibrates lower only elevated PAI-1 levels in patients.[1] Similarly, our *in vitro* finding that different fibrates which are equipotent in lowering triglyceride levels can differ in their efficacy to lower PAI-1 synthesis, parallels *in vivo* results. For example, we found in our cultured cynomolgus monkey hepatocytes that gemfibrozil and clofibric acid were potent PAI-1 suppressors while clofibrate and bezafibrate were not. Similarly, gemfibrozil but not bezafibrate was found to decrease enhanced PAI-1 levels in type IV hypertriglyceridemic patients.[7,35,36]

Our observation that PAI-1 synthesis in the simian hepatocytes is induced during basal culture conditions resembles similar findings in Hep G2 cells.[37] PAI-1 synthesis in Hep G2 was found to be induced by an autocrine factor, secreted during cell culture. The nature of this factor has not been identified until now, but had no similarity to any steroid, retinoid, growth factor or cytokine, factors known to induce PAI-1.[38] Similarly, the factor(s) responsible for the accelerated production of PAI-1 in cynomolgus monkey hepatocytes remained elusive.

The obscurity of the PAI-1 inducing factor hampered a rational approach to understand the mechanism by which fibrates suppress PAI-1 expression. We found that gemfibrozil rapidly and transiently increased PAI-1 mRNA expression in a manner comparable to the PKC activator

PMA, albeit much weaker, and this could be inhibited with the PKC inhibitor, Ro 31-8220. Activation of PKC by fibrates has also been reported in rat hepatocytes.[9,10] However, specific activation or inhibiton of PKC did not prevent the accelerated increase in PAI-1 during culture. Also, clofibric acid, which did not activate PKC, was as effective as gemfibrozil in suppressing PAI-1 production. These results make it unlikely that fibrates exert their PAI-1 suppressing action by interference with a PKC-dependent pathway. We could also exclude an effect of fibrates on the signal transduction pathways activated by growth factors like EGF and TGF-ß. We found that EGF and TGF-ß did induce PAI-1 mRNA levels in simian hepatocytes, but this could not be prevented by fibrates. Apparently, not every PAI-1 induction is inhibited by fibrates. This is also true *in vivo*. For example, gemfibrozil did not lower elevated PAI-1 levels in patients with a history of venous thrombosis[33] or in men with combined hyperlipoproteinemia.[34]

The finding that a number of fibrates are potent activators of the nuclear receptor PPAR suggested the possibility that this receptor mediates the beneficial action of fibrates on PAI-1. Indeed, PPARα and its heterodimeric partner, RXRα, are both expressed in cultured cynomolgus monkey hepatocytes, and the ligand for RXRα, 9-*cis* retinoic acid, also suppressed PAI-1 production. Two mechanisms have been described how PPAR/RXR can interfere with gene transcription and which might be relevant for PAI-1 expression. PPAR/RXR can antagonize transcriptional activation by competing with another transcription factor for binding to the same *cis*-acting element, as described by Keller et al.[39] Secondly, inhibition can be due to mechanisms involving DNA-independent negative interferences such as squelching. Sakai et al[16] recently showed that PPARα downregulates transcription of the glutathione transferase-P gene in rat hepatocytes through squelching of the transcription factor c-Jun. Interference with c-Jun activity has also been reported for the RXRα receptor.[40] In this context it is of interest that c-Jun is an important factor in PAI-1 gene transcription in the human hepatoma cell line Hep G2 (Arts and Kooistra, unpublished data). If PPAR activation is important for inhibition of PAI-1 expression by fibrates, then gemfibrozil and clofibric acid should be much stronger activators of PPAR activity in our system than bezafibrate or clofibrate. As a measure for PPAR activity we examined the expression of the acyl-CoA oxidase (ACO) gene. ACO is a peroxisomal target of PPARs, and its level has been found to be elevated manyfold in the livers of fibrate-treated rodents.[41,42] In contrast to rodents, however, ACO mRNA levels in cynomolgus monkey hepatocytes were only slightly increased upon treatment with fibrates (maximal increases of 150% were found after 24 h incubation with 1 mmol/L gemfibrozil). This is in agreement with

previous reports showing that fibrates poorly induce peroxisome proliferation and peroxisomal ß-oxidation enzymes like ACO, in both human and cynomolgus monkey hepatocytes.[43,44] Ultimately, transfection experiments with dominant negative PPAR/RXR mutants, antisense technology or the use of PPAR/RXR knock out mice are needed to answer the question whether PPAR and RXR are indeed involved in mediating the fibrate-induced decrease in PAI-1 production.

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CHAPTER 8

General Discussion

8.1 INTRODUCTION

The studies reported in this thesis were aimed at elucidating some aspects of the regulation of human tissue-type plasminogen activator (t-PA) and its main physiological inhibitor, plasminogen activator inhibitor 1 (PAI-1) gene expression in cultured human endothelial cells and hepatocytes, respectively.

Previous investigations on the regulation of human t-PA gene transcription have been limited and were mainly restricted to the epithelial cervix adenocarcinoma cell line HeLa (Medcalf et al., 1990). The question remains whether these findings are also true for other cell types, including human vascular endothelial cells. Vascular endothelial cells are the main source of t-PA in the bloodstream and modulation of t-PA synthesis in these cells has been the subject of many studies (see Kooistra et al., 1994 and references therein). Attempts to confirm the findings of Medcalf et al. (1990) in cultured human endothelial cells failed, however, as a result of poor transfection efficiencies and a lack of regulation of transiently transfected constructs (Hanemaaijer et al., 1994). As an alternative approach to compare t-PA promoter regions and the transcription factors involved in basal and phorbol ester-activated t-PA gene transcription, we have performed *in vivo* footprinting and bandshift assays. Such studies reveal the t-PA promoter regions bound by nuclear protein in intact cells and the identity of the transcription factors bound. The significance of these findings for t-PA gene regulation in human vascular endothelial cells is discussed below in a broader context. Furthermore, attention will be paid to the role of histone-DNA interactions in t-PA transcriptional regulation, since we found that an increase in histone H4 acetylation was associated with a strong induction of t-PA gene expression.

Many studies have described the regulation of PAI-1 synthesis by components like cytokines, growth factors and protein kinase C (PKC)-activating phorbol esters such as PMA in a variety of cell types (Saksela et al., 1987; Van Hinsbergh et al., 1988; Schleef et al., 1988; Van den Berg, 1988; Alessi et al., 1988; Lucore et al., 1988; Santell and Levin, 1988; Pertovaara et al., 1989; Kooistra et al., 1989; Grulich-Henn and Müller-Berghaus, 1990; Schneider and Sobel, 1991; De Boer et al., 1991; Westerhausen et al., 1991; Bosma and Kooistra, 1991; Healy and Gelehrter, 1994). Changes in PAI-1 synthesis are, in most cases, preceded by changes in PAI-1 gene transcription and mRNA level (Bosma and Kooistra, 1991; Loskutoff, 1991; Healy and Gelehrter 1994). The picture emerged from these studies that, dependent on the cell type and stimulus used, different PAI-1 promoter regions and transcription factors are involved in PAI-1 gene

expression. Because many of the PAI-1 inducing compounds are able to increase the (activity of) the transcription factor activator protein-1 (AP-1) and the PAI-1 gene promoter contains four putative AP-1 binding sites, the question arises why AP-1 is not a common denominator in all these PAI-1 inductory processes. The studies in this thesis on the role of AP-1 in the regulation of PAI-1 gene transcription in the human hepatoma cell line HepG2 under basal conditions and after stimulation with PMA, serum or IL-1α indicate that the composition of the dimeric AP-1 molecule is a discriminating factor: whereas the c-Jun homodimeric form of AP-1 showed a high affinity for the AP-1 binding site involved, the c-Jun/c-Fos heterodimeric form of AP-1 hardly bound to this DNA sequence. To which extent the critical role of the composition of the AP-1 molecule, as demonstrated in the present studies, may also explain some other seemingly contradictory studies on PAI-1 regulation, is discussed. In the same context, we will briefly elaborate the possibility that fibrates, which are hypolipidemic drugs that also suppress PAI-1 levels, may interfere with c-Jun activation or action.

8.2 DNA-PROTEIN INTERACTIONS AND t-PA GENE TRANSCRIPTION

Transient transfection experiments in HeLa cells using deletion mutants of the t-PA gene promoter, combined with mutational analysis studies, revealed two regions to be crucial for basal and PMA-stimulated t-PA promoter activity: an AP-1 binding site (TRE) between position -115 and -102 and a GC-box between +60 and +74 (Medcalf et al., 1990). An important finding of the present work is that these two regions in the t-PA promoter are bound by the same nuclear proteins in human endothelial cells as well as in HeLa cells, as shown by *in vivo* footprinting analysis and gel mobility shift assays. This would imply that the t-PA promoter activity studies previously performed in HeLa cells are also relevant for human endothelial cells. In support of this, Bulens et al. (1995) recently showed that transient expression of a 2 kb retinoic acid responsive element-containing t-PA promoter-CAT construct responded similarly to retinoic acid in HeLa cells and the hybrid endothelial cell line EA.hy926 (Bulens et al., 1995). HeLa cells thus seem a good model and alternative for endothelial cells to perform t-PA promoter regulatory studies and to circumvent the cumbersome transfection studies in cultured human endothelial cells.

In addition to the TRE-like sequence and the GC-box, *in vivo* footprinting analysis of the -135 to +100 region of the t-PA promoter demonstrated three other binding sites,

including a CTF/NF-1-like consensus and two additional GC-boxes which were not previously detected with transfection or *in vitro* footprinting techniques (Fisher et al., 1985; Medcalf et al., 1990; Holmberg et al., 1995). Whether these three additional protein binding sites identified by *in vivo* footprinting analysis are indeed important for t-PA transcription remains to be established (for example, by mutational analysis and transfection experiments). However, since deletion and/or mutation of these sites in the very homologous murine t-PA promoter hampered t-PA transcription (Rickles et al., 1989; Darrow et al., 1990; Pecorino et al., 1991; Ohlsson et al., 1993), they are likely to be so. The existence of DNA-protein interactions *in vivo* which are not detected in (previous) *in vitro* studies is puzzling. One explanation could be that binding of these transcription factors is dependent on the presence of secondary chromatin structures, which are lacking in the DNA fragments used for *in vitro* promoter studies. Secondary chromatin structures may enhance transcription factor binding through correct positioning of transcription factors bound to distant binding sites. When brought in each others proximity, transcription factors may stabilize their own binding and fix the promoter structure by protein-protein interactions. For example, the transcription factor SP-1 stabilizes distant binding of the bovine papillomavirus type 1 enhancer protein E2 to the thymidine kinase promoter (Li et al., 1991). Similarly, the upstream binding of c-Jun to a class II promoter construct was shown to be essential for the formation of pre-initiation complexes (PIC), presumably due to stabilization of PIC formation through a direct interaction with c-Jun (Becker et al., 1995).

An important role of chromatin structure and nucleosome positioning in the regulation of t-PA transcription in human endothelial cells is suggested by our finding that an increase in histone H4 acetylation is associated with an enhanced t-PA gene expression. An increased histone acetylation is believed to relieve transcriptional repression by the histones, due to a relaxation in the chromatin structure which facilitates access of the DNA template for transcription factors (Workman and Roeder, 1987; Buckle et al., 1991; Lee et al., 1993). It has been reported that acetylated histones regulate major changes in structure and function of chromatin during the cell cycle. In this context it is of interest to note that t-PA synthesis can also be induced by cultivating cells at high density or by inhibitors of DNA synthesis (Nishimune et al., 1983). At which sites in the t-PA promoter histones interfere with transcription factor binding is unknown at present. The proximal region of the t-PA promoter seems to be highly accessible for nuclear proteins, since all consensus sites for transcription factor binding were actually occupied by nuclear protein *in vivo* (Chapter 2). No protein binding was

observed at the site of transcription initiation, but the dimethyl sulphate (DMS) treatment as used in the *in vivo* footprinting procedure has been reported to disrupt interaction of a transcription initiation complex protein, transcription factor IIIA (TFIIIA), with the DNA (Wang and Becker, 1988), indicating that the *in vivo* DMS footprinting technique is not suitable to detect binding of the transcription initiation complex. Which upstream t-PA promoter elements are associated with positioned nucleosomes remains to be established by micrococcal nuclease footprinting (Englander and Howard, 1995). Several candidate regions have been reported. For example, the t-PA promoter region contains Alu repeats (Friezner Degen et al., 1986; Bulens, 1995) which are frequently associated with rotationally positioned nucleosomes and influence chromatin structure (Wolffe, 1994). Also, a 160 bp long enhancer has been identified which has the characteristic that it needs its entire length to be functional, a length far exceeding that of a regular enhancer or a "normal" transcription factor binding site (Fujiware et al., 1994).

8.3 REGULATION OF t-PA GENE TRANSCRIPTION BY PMA

Our studies on the PMA-stimulated gene expression in human endothelial cells and HeLa cells by *in vivo* footprinting analysis and gel mobility shift assays confirmed and extended previous studies by Medcalf et al. (1990) using *in vitro* transfection studies. Medcalf et al. (1990) identified two sites in the proximal t-PA promoter region (a TRE and GC-box III) that were involved in PMA induction of t-PA gene transcription in HeLa cells. The present work shows that, in both human endothelial cells and HeLa cells, PMA induces protein binding to the TRE (AP-1) and to GC-box III (SP-1). PMA, however, also induced nuclear protein binding to two of the newly identified protein binding sites, namely binding of SP-1 to GC-box II and of an (unidentified) protein to GC-box I, suggesting that these sites may also contribute to the t-PA transcriptional induction by PMA. In this context, it is important to note that in HeLa cells mutation of either the TRE or GC-box III alone did not interfere with PMA induction of t-PA, but only mutation of both these sites effectively abolished induction. On the basis of these results, Medcalf et al. (1990) concluded that both sites independently mediate the t-PA induction by PMA. However, since the mutation of both sites abolished basal expression, it cannot be excluded that actually other site(s) in the promoter also contribute to the PMA induction of t-PA in HeLa cells.

The PMA induction of nuclear protein binding to the TRE in the t-PA promoter in human endothelial cells was relatively poor as compared to that in HeLa cells. Weak induction of AP-1 binding activity by PMA in HUVEC has previously also been reported by Montgomery et al. (1991) and by Parry and Mackman (1995). The relatively low PMA induction of both AP-1 and SP-1 binding to the t-PA promoter in HUVEC raises the question to what extent these sites contribute to t-PA transcriptional induction by PMA in human endothelial cells. In this context it is of interest that a tissue factor promoter region containing two c-Jun/c-Fos binding sites was not inducible by PMA (or IL-1α, LPS or TNFα) in human endothelial cells, although mutation of the AP-1 binding sites abolished induction (Parry and Mackman, 1995). Apparently, in human endothelial cells AP-1 is a constitutively bound protein which supports gene induction, but in itself does not strongly confer PMA-responsiveness.

8.4 INITIATION OF t-PA TRANSCRIPTION FROM A TATA-LESS PROMOTER

Although initial studies on the human t-PA gene by Fisher et al. (1985) and Friezner Degen et al. (1986) suggested a classical TATA box approximately 25 bp upstream from a site which they demonstrated to be a start site for gene transcription in human cells, a more recent study showed that only a minor portion of the t-PA mRNA transcripts initiate at the TATA-proximal location (Henderson and Sleigh, 1992). A 10-fold greater proportion of the t-PA mRNA transcripts initiated 110 bp downstream from a conserved sequence, also utilized as a TATA-independent transcription start site in the rodent t-PA genes (Henderson and Sleigh, 1992).

It has been noted that TATA-less or TATA-independent promoters often contain GC-rich areas, that bind the transcription factor SP-1 (Dynan, 1986). SP-1 or functionally equivalent factors are necessary to recruit the transcription factor IID (TFIID) which facilitates gene transcription initiation by interacting with RNA polymerase II and other general transcription factors (Dynan, 1986; Pugh and Tjian, 1990). Our observation that GC-boxes II (position +39 to +45) and III (position +62 to +68), which are situated near the transcription initiation site of human t-PA at position +110, bind SP-1 protein is in line with the above concept. Additional evidence for an important role of SP-1 in t-PA transcription is provided by the study of Medcalf et al. (1990) who reported a strict correlation between nuclear protein binding to GC-box III (i.e. SP-1) and t-PA expression in different cell-types: a high nuclear protein binding and t-PA expression in Bowes

melanoma cells, intermediate protein binding and t-PA expression in HeLa cells and hardly detectable nuclear protein binding and no t-PA expression in HepG2 cells. These data suggest that, at least in cell lines, SP-1 is a limiting factor in t-PA transcription. Further evidence could be obtained by analyzing the effect of SP-1 overexpression on t-PA transcription both *in vitro* and *in vivo*.

8.5 THE ROLE OF AP-1 IN PAI-1 TRANSCRIPTIONAL REGULATION

Several studies have demonstrated that two TREs at positions -58 to -50 and -79 to -72 in the proximal region of the PAI-1 promoter are important for basal and PMA-stimulated PAI-1 gene transcription in a variety of cell types, including HepG2 (Descheemaeker et al., 1992; Knudsen et al., 1994; Arts et al., submitted). We found that the activity of the TRE at position -58 to -50 (box A) is dependent on the binding of the c-Jun homodimeric form of AP-1. The c-Jun/c-Fos heterodimeric form of AP-1, which is present in excess to c-Jun/c-Jun homodimers in PMA-treated HepG2 cells, showed a poor affinity for this TRE, and is apparently not involved in PAI-1 gene regulation. This selective affinity of a crucial TRE for the c-Jun homodimeric form of AP-1 may explain some results from studies in other cell types in which a role for AP-1 in PAI-1 transcription was questioned. For example, Knudsen et al. (1994) showed in MCF-7 cells that, although AP-1 (i.e. c-Jun/c-Fos heterodimers) was present, a different (unidentified) protein bound to the PAI-1 TRE at -58 to -50. The poor affinity of the PAI-1 promoter for c-Jun/c-Fos heterodimers may also explain why PMA hardly induces PAI-1 transcription in cultured human endothelial cells (Scarpati and Sadler, 1989), since these cells only contain the c-Jun/c-Fos heterodimeric form of AP-1 (this thesis).

Our observation that PAI-1 transcription depends on c-Jun homodimers rather than c-Jun/c-Fos heterodimers may also explain the inhibitory effect of activators of protein kinase A (PKA) on PAI-1 gene expression (Santell and Levin, 1988; Kooistra et al., 1991; Bergonzelli et al., 1992). It has been shown that activation of PKA enhances AP-1 activity by inducing c-Fos, but not c-Jun expression (Angel and Karin, 1991). This will result in a decrease in the amount of c-Jun homodimers because of the preferential formation of c-Jun/c-Fos heterodimers (Angel and Karin, 1991). Since the PAI-1 promoter is far more effectively bound by c-Jun homodimers than by c-Jun/c-Fos heterodimers, this may result in a decrease in PAI-1 transcription. Such a mechanism indeed has been suggested for u-PA expression in HepG2 cells, which expression is dependent on c-Jun

homodimer binding to an octameric TRE in the u-PA promoter: overexpression of c-Fos resulted in transcriptional inhibition of u-PA expression (DeCesare et al., 1995). However, we cannot exclude alternative mechanisms for the inhibition of PAI-1 transcription by PKA. For example, activation of PKA has been reported to result in an inhibition of c-Jun binding to octameric TREs through activation of CREB which then competes for binding with AP-1 (Lamph et al., 1990).

c-Jun-dependent gene transcription in human hepatoma cells has been reported to be downregulated in a ligand-dependent manner by nuclear hormone receptors, such as the retinoic acid receptor, the retinoid X receptor (RXRα), the thyroid hormone receptor and, to a lesser extent, by the glucocorticoid receptor (Salbert et al., 1993). This inhibition of Jun-dependent transcription is generally attributed to DNA-independent negative interferences between the nuclear hormone receptors and c-Jun, so-called squelching. We found that fibrates, which activate the nuclear hormone receptor peroxisome proliferator activated receptor (PPAR), inhibited an increase in PAI-1 transcription during culture of primary cynomolgus monkey hepatocytes. PPAR has recently been reported to downregulate the c-Jun dependent transcription of glutathione transferase P in rat fibroblasts (Sakai et al., 1995). Whether PPARα is involved in the inhibition of PAI-1 synthesis by fibrates, and whether this is due to squelching of c-Jun remains to be established. Preliminary bandshift assays showed a relative decrease in protein binding to the c-Jun binding site of the human PAI-1 promoter with nuclear extracts from fibrate-incubated cynomolgus monkey hepatocytes, but no correlation was observed between the potency of a fibrate to inhibit PAI-1 synthesis and its effect on nuclear protein binding to the c-Jun binding TRE (Arts et al., unpublished results). Also, Sakai et al. (1995) were unable to show a decrease in c-Jun binding to the glutathione transferase P promoter AP-1 binding site after PPAR-activation. An alternative role for nuclear receptors in downregulation of AP-1-dependent gene transcription was recently proposed by Kamei et al. (1996). These investigators showed that multiple nuclear hormone receptors compete with AP-1 for binding of CREB-binding protein (CBP), a factor essential for the transactivation capacity (but not binding activity) of c-Jun.

PAI-1 transcriptional activity *in vivo* may also be supported by c-Jun homodimers rather than by c-Jun/c-Fos heterodimers. It is striking that the expression of PAI-1 mRNA *in vivo* coincides with the expression of c-*jun* rather than c-*fos*. For example, both PAI-1 and c-*jun* mRNA expression is high in murine lung and heart tissue and absent in murine brain, liver and testis (Hirai et al., 1989; Sakai et al., 1989, Sawdey and Loskutoff, 1991), while c-*fos* is predominantly expressed in bone marrow, growing bone and the

developing central nervous system, but can hardly be detected in murine lung tissue (Müller et al., 1983; Caubet, 1989). Also, correlations between the regulation of c-*jun* and PAI-1 have been observed. For example, a coordinate induction of c-*jun* and PAI-1 transcription has been reported in pig myocytes after short coronary occlusions (Knöll et al., 1994).

8.6 REGULATION OF PAI-1 GENE TRANSCRIPTION IN DIFFERENT CELL TYPES AND BY VARIOUS STIMULI

Although in all cell types studied the TREs at -58 to -50 and -79 to -72 proved to be important for basal as well as PMA-induced PAI-1 gene expression (Descheemaeker et al., 1992; Knudsen et al., 1994; Keeton et al., 1994), the nuclear factors found to bind to these elements differed considerably, depending on the cell type studied. Whereas we demonstrated binding of c-Jun homodimers to the -58 to -50 TRE, Knudsen et al. (1994) showed that, in MCF-7 cells, a non-AP-1-related protein mediates the PMA induction of PAI-1 through this proximal TRE. Similarly, the -79 to -72 promoter element was demonstrated to bind SP-1 in human endothelial cells (this thesis), SP-1 and a 72 kD protein in HeLa cells (Descheemaeker et al., 1992), and 5 other (unidentified) proteins in HepG2 cells (this thesis).

Besides the two proximal TREs, Westerhausen et al. (1991) showed that two distal TRE-like sequences (at positions -721 to -714 and -662 to -656) mediated TGF-ß induction of PAI-1. Furthermore, promoter elements other than TREs may be involved in PAI-1 transcriptional regulation. Dawson et al. (1993) reported an IL-1α inducible promoter element in HepG2 cells between -675 and -669. Our studies show that the cytokine IL-1α induces PAI-1 gene transcription in HepG2 cells through a mechanism independent of PKC and through a PAI-1 promoter region upstream of the first 489 bp promoter region. In conclusion, from the studies on the regulation of PAI-1 in cultured cells it can be concluded that, depending on the stimulus used and the various intracellular signal transducing pathways present, different cell types may respond through different promoter regions and a variety of specific transcription factors to increase PAI-1 gene expression. While such studies provide insights into the mechanisms and factors involved in PAI-1 gene regulation in cultured cells *in vitro*, their relevance to the regulation of PAI-1 in normal physiological and pathological conditions *in vivo* remains to be determined.

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SUMMARY

In Chapter 1, tissue-type plasminogen activator (t-PA) and its main physiological inhibitor, plasminogen activator inhibitor 1 (PAI-1) are introduced. t-PA converts the inactive proenzyme plasminogen into the broad spectrum protease plasmin which plays a key role in a number of proteolytic processess, including the dissolution of fibrin, the matrix structure of a blood clot. Fibrin degradation (fibrinolysis) needs to be extensively regulated: whereas inadequate dissolution of fibrin may result in the obstruction of a blood vessel, excessive premature fibrin degradation can lead to bleeding. The activity of t-PA is regulated at several levels, including its synthesis and the presence of PAI-1. Given the importancy of t-PA and PAI-1 in maintaining the intravascular fibrinolytic balance, the regulation of their synthesis is a critical feature. In this thesis, the expression of t-PA and PAI-1 was studied in human endothelial cells and hepatocytes, cell types which are considered to be major contribuants of plasma t-PA and PAI-1. Among the compounds reported to modulate t-PA and/or PAI-1 synthesis in cultured endothelial cells and hepatocytes are the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), sodium butyrate, interleukin-1, genistein, and fibrates. The work presented in this thesis was mainly directed at gaining more insight into the regulatory mechanisms by which these changes in t-PA and PAI-1 gene expression were brought about.

In Chapter 2, the -135 to +100 region of the human t-PA promoter was analyzed for persistent and PMA-inducible DNA-protein interactions in intact cultured human vascular endothelial cells and HeLa cells, using a dimethyl sulphate *in vivo* footprinting approach and gel mobility shift assays. Five distinct promoter elements sites were bound by nuclear proteins in both cell-types, including a PMA-responsive element (TRE), a CTF/NF-1 binding site and three GC-boxes. The proteins bound were identified as AP-1 (TRE), CTF/NF-1 and SP-1 (GC-boxes II and III). Human endothelial cells showed a relatively high c-Jun/c-Fos binding to the TRE as compared to HeLa cells, which was at least partially due to a higher binding affinity of AP-1 in endothelial cells. After incubation of the cells with PMA the binding of AP-1, SP-1 and an unidentified protein to the t-PA promoter was increased 2-fold in cultured human endothelial cells and over 20-fold in HeLa cells. These differences parallel the difference in transcriptional induction of t-PA by PMA in the two cell types.

In Chapters 3 and 4, we provide evidence for a role of histone acetylation in the strong increase in t-PA gene transcription by sodium butyrate in cultured human endothelial cells. First, the effect of the short-chain fatty acid butyrate can be mimicked

by a structurally unrelated, specific histone deacetylase inhibitor, trichostatin A (TSA). Secondly, transcriptional induction of t-PA by sodium butyrate and TSA was preceded by histone H4 acetylation, and the extent of t-PA induction correlated with the degree of histone H4 acetylation. We have excluded other mechanisms previously implicated in gene induction by sodium butyrate, including a putative induction of c-*jun* and a role for glucose.

In Chapter 5 we applied antisense technology and performed bandshift experiments to study the role of c-Jun and c-Fos in basal and PMA-induced PAI-1 gene expression in the human hepatoma cell line, HepG2. Previous studies had shown two AP-1 like binding sites in the PAI-1 promoter to be required for basal and PMA-stimulated PAI-1 gene transcription: a TRE between positions -58 and -50 and a TRE between -79 and -72. Our results demonstrate that binding of c-Jun homodimers rather than c-Jun/c-Fos heterodimers to the TRE at position -58 to -50 is important for both basal and PMA-induced PAI-1 synthesis. PMA treatment of the cells increased c-Jun homodimer binding about 30-fold and also slightly induced binding of a second c-Jun-containing complex to this TRE. In addition, PMA induced a very strong increase in the binding of an unknown factor to the TRE between -79 and -72. This unidentified protein is not related to c-Jun and c-Fos or to any of the transcription factors previously shown to bind this TRE in HeLa cells (i.e. SP-1 and a novel 72 kD transcription factor).

In Chapter 6 we describe that PMA, serum and IL-1α induce PAI-1 gene transcription in HepG2 cells in a comparable way, but that only PMA and, to a lesser extent, serum, induce the expression of a stably integrated reporter gene under control of the -489 to +75 region of the PAI-1 promoter in these cells. This promoter region contains the c-Jun homodimer binding site found to be essential for PMA-induction of PAI-1 transcription (Chapter 5). Although IL-1α effectively induced c-*jun* mRNA expression, this was not reflected in an increase in c-Jun protein or c-Jun binding activity. These results point to two separate PAI-1 inductory pathways for PMA and IL-1α. The protein tyrosine kinase inhibitor genistein inhibited PAI-1 transcriptional induction by PMA through an inhibition of AP-1 induction, but also prevented the (AP-1-independent) PAI-1 induction by IL-1α.

In Chapter 7 we demonstrate that fibrates dose-dependently suppress PAI-1 synthesis in primary hepatocyte cultures from cynomolgus monkey (*Macaca fascicularis*), independent of their lipid-lowering effect. Different fibrates showed different potency to lower PAI-1 synthesis (gemfibrozil and clofibric acid > clofibrate and bezafibrate) and the changes in PAI-1 synthesis were reflected at the PAI-1 mRNA level and were also

visible at the level of transcription. Fibrates did not lower basal PAI-1 synthesis, but attenuated an acceleration of PAI-1 production during culture. The nature of this fibrate-inhibitable PAI-1 induction during culture remained unknown, but was not related to PKC activation or growth factor mediated signalling. The cultured cynomolgus monkey hepatocytes expressed the fibrate-activated nuclear hormone receptor PPARα and its heterodimeric partner, RXRα, and the ligand for RXRα, 9-*cis* retinoic acid, also suppressed PAI-1 production. Whether fibrates (and 9-*cis* retinoic acid) exert their PAI-1 quenching effect via the nuclear hormone receptors remained elusive.

In Chapter 8, findings in this study are discussed in a broader context.

SAMENVATTING

Trombose, het ontstaan van bloedstolsels in aderen en slagaderen, is een ernstige complicatie van vaatziekten, en is vaak de uitlokkende factor van een acuut hartinfart. Twee manieren om trombose te voorkomen zijn ofwel te verhinderen dat het stollingssysteem geactiveerd wordt ofwel het zogenoemde fibrinolyse systeem zodanig te activeren, dat het zich vormende stolsel tenminste even snel wordt afgebroken als dat het zich vormt. Het fibrinolytisch systeem is in principe ook in staat een gevormd stolsel weer op te lossen. Een bekend voorbeeld van deze laatste situatie is de behandeling van het acute myocard infarct met het fibrinolytische enzym weefsel-type plasminogeen activator (t-PA). Het in dit proefschrift beschreven onderzoek was erop gericht uit te zoeken hoe het eigen fibrinolytisch systeem van het lichaam gereguleerd wordt. Inzicht in deze regulatie kan uiteindelijk leiden tot rationele beïnvloeding van de fibrinolytische activiteit in het bloed.

Het belangrijkste enzym uit het fibrinolytische systeem om het fibrinolyse proces (het afbreken van fibrine, de matrix structuur van een bloedstolsel) op gang te brengen, is t-PA. t-PA in de bloedbaan is voornamelijk afkomstig uit endotheelcellen, welke de binnenkant van de vaatwand bekleden. De activiteit van t-PA in het bloed wordt echter niet alleen bepaald door de hoeveelheid t-PA, maar ook door het meer of minder aanwezig zijn van een specifieke remmer van t-PA activiteit, de zogeheten plasminogeen activator remmer type 1 (PAI-1). PAI-1 in bloed is waarschijnlijk ook voor een deel afkomstig van het endotheel en van bepaalde levercellen, de hepatocyten. Als een endotheelcel of een hepatocyt geprikkeld wordt, kan dit leiden tot een verandering in de produktie van t-PA en/of PAI-1. Er zijn verscheidene stoffen beschreven, die zo'n verandering in t-PA en/of PAI-1 synthese teweeg kunnen brengen, maar de intracellulaire regulatiemechanismen die hieraan ten grondslag liggen, zijn vaak nog onbekend. In dit proefschrift is van een aantal van zulke verbindingen nader uitgezocht hoe ze de expressie van t-PA en PAI-1 beïnvloeden. Hierbij is gebruik gemaakt van gekweekte cellen, voornamelijk humane endotheelcellen (geïsoleerd uit navelstrengvene), HepG2 cellen (een humane hepatocyten cellijn) en primaire apehepatocyten (*Macaca fascicularis*).

De synthese van t-PA, kan gestimuleerd worden door activatie van het signaaltransductie molecuul proteïne kinase C (PKC) met de stabiele PKC activator phorbol 12-myristaat 13-acetaat (PMA). Deze inductie van t-PA expressie vindt in ieder geval gedeeltelijk plaats op het niveau van t-PA gentranscriptie (d.w.z. mRNA synthese). Eerder onderzoek in HeLa cellen (een humane cervica carcinoma cellijn) heeft aangetoond

dat twee gebieden in de proximale t-PA promoter van belang zijn voor basale en PMA-geïnduceerde t-PA gentranscriptie. Om verder te onderzoeken of de humane t-PA promoter zich in endotheelcellen vergelijkbaar gedraagt als in de cellijn HeLa, werd het -135 tot +100 gebied van de t-PA promoter in deze twee celtypen onderzocht op DNA-eiwit interacties m.b.v. *in vivo* footprinting (dimethylsulfaat-methode) en bandshift assays (Hoofdstuk 2). Met de *in vivo* footprinting techniek is het mogelijk om in de intacte cel te kijken waar transcriptiefactoren aan het DNA gebonden zijn. De resultaten laten zien, dat in beide celtypen dezelfde vijf t-PA promoterelementen gebonden waren door eiwitten, waaronder de twee elementen eerder geïdentificeerd in HeLa cellen d.m.v. transfectie- en mutatie- studies, een GC-box waaraan SP-1 bond, en een activator protein 1 (AP-1) bindingselement waaraan Jun/Fos heterodimeren gebonden waren. Verder werden ook nog een CTF/NF-1 bindingselement, een tweede GC-box waaraan de transcriptiefactor SP-1 bond, en een GC-box waaraan een onbekend eiwit bond, geïdentificeerd. PMA induceerde eiwitbinding aan zowel de GC-boxen als aan het AP-1 responsieve element, waarbij de toename in de transcriptiefactor binding in de twee celtypen goed overeen kwam met de inductie van t-PA gentranscriptie. De bijdrage van de drie nieuw geïdentificeerde eiwit-bindingselementen aan de regulatie van t-PA transcriptie zal nog moeten worden vastgesteld d.m.v. transfectie en mutatie studies.

De transcriptionele activiteit van het t-PA gen in humane endotheelcellen bleek niet alleen gereguleerd te worden door de binding van transcriptiefactoren aan specifieke DNA elementen, maar waarschijnlijk ook door de chromatine-structuur. Remmers van histon deacetylase, zoals boterzuur en (het meer specifieke) trichostatine A, induceerden namelijk een zeer sterke toename in t-PA transcriptie (Hoofdstukken 3 en 4). Deze inductie werd voorafgegaan door een toename in histon H4 acetylatie, en de mate van t-PA inductie correleerde met de toename in histon H4 acetylatie, zowel in de tijd als met verschillende boterzuur en trichostatine A concentraties. Als gevolg van de toegenomen histon H4 acetylatie onstaat mogelijk een verminderde interactie van het histon eiwit met het DNA, waardoor DNA elementen beter toegankelijk worden voor transcriptiefactoren. De effecten van boterzuur en trichostatine A bleken vrij specifiek te zijn voor t-PA en endotheelcellen: geen verandering werd waargenomen t.a.v. een aantal andere genen, waaronder PAI-1 en c-*jun*/c-*fos*, en geen significant effect werd gezien op t-PA expressie in Bowes cellen. De reden van deze specificiteit is nog onduidelijk.

De PKC activator PMA induceerde een sterke toename van PAI-1 transcriptie in HepG2 cellen, maar niet in endotheelcellen, hoewel in beide celtypen een sterke inductie plaatsvond van c-*jun* en c-*fos*, en de PAI-1 promoter potentiële AP-1 bindingselementen

bevat. In Hoofdstuk 5 is d.m.v. antisense experimenten gecombineerd met transfectie-studies en bandshift assays aangetoond, dat voor PAI-1 transcriptie in HepG2 cellen c-Jun homodimeren, maar niet c-Jun/c-Fos heterodimeren essentieel zijn. Deze c-Jun homodimeren komen voor en worden door PMA geïnduceerd in HepG2, maar niet in endotheelcellen, hetgeen de celspecifieke verschillen in PAI-1 genregulatie kan verklaren. c-Jun/c-Jun bleek te binden aan de het PMA-responsieve element (TRE) op positie -58 tot -50 van de PAI-1 promoter. Verder induceerde PMA in HepG2 cellen de binding van een onbekende transcriptiefactor aan de TRE op positie -79 tot -72.

Een andere component die in HepG2 cellen zowel PAI-1 als c-*jun* mRNA induceert, is interleukine-1α (IL-1α). Uit de transfectiestudies beschreven in Hoofdstuk 6 blijkt echter, dat IL-1α de transcriptie van PAI-1 niet verhoogt via de c-Jun-bindende proximale TRE op positie -58 tot -50 van de PAI-1 promoter, maar waarschijnlijk via een gebied upstream van positie -489. Immunoprecipitatie- en bandshift-experimenten lieten zien, dat de sterke c-*jun* mRNA inductie door IL-1α niet gevolgd wordt door een toename in c-Jun eiwit of c-Jun binding aan de TRE op positie -58 tot -50. Opvallend was verder dat het effect van zowel PMA als IL-1α op PAI-1 genexpressie te onderdrukken was met de tyrosine kinase remmer, genisteïne. Het is nog onbekend waar genisteïne interfereert met de verschillende signaaltransductie-routes betrokken bij de inductie van PAI-1 expressie door PMA en IL-1α.

Van een bepaalde groep geneesmiddelen, de zogenaamde fibraten, is bekend dat ze, naast hun hypolipidemische werking, ook de plasma niveaus van PAI-1 kunnen verlagen. Uit het werk beschreven in Hoofdstuk 7 blijkt, dat fibraten direct de synthese van PAI-1 kunnen verlagen in primaire cultures van apehepatocyten (*Macaca fascicularis*). De remming van PAI-1 synthese was ook zichtbaar op het niveau van PAI-1 mRNA expressie en PAI-1 gentranscriptie. Op welke wijze fibraten PAI-1 expressie verlagen is nog onduidelijk, maar bleek onafhankelijk te zijn van het lipide-verlagende effect. Verder was de remming van PAI-1 synthese niet gerelateerd aan een (remmend) effect op PKC activiteit of op groeifactor-gemediëerde PAI-1 inductie. Wel zijn aanwijzingen gevonden voor een rol van de door fibraten geactiveerde nucleaire hormoon receptor peroxisoom proliferator geactiveerde receptor α (PPARα). Mogelijk voorkomt PPARα de binding van c-Jun aan de PAI-1 promoter. Dit vraagt echter nog verder onderzoek.

Samenvattend kan gesteld worden dat het werk beschreven in dit proefschrift inzicht heeft gegeven in de rol proteïne kinase C en de transcriptie factoren c-Jun/c-Fos in de regulatie van t-PA en PAI-1 genexpressie in humane endotheelcellen en hepatocyten.

De promoterelementen en een aantal transcriptiefactoren betrokken bij deze regulatie zijn geïdentificeerd. Daarnaast zijn enkele factoren onderzocht (chromatine structuur, fibraten) die modulerend op bovengenoemde regulatieprocessen kunnen inwerken.

ABBREVIATIONS

AP-1	activator protein 1
AP-2	activator protein 2
bp	basepair(s)
CAT	chloramphenicol acetyltransferase
DMEM	Dulbecco's modified Eagle's medium
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cells
IL	interleukin
kb	kilo basepair(s)
kD	kilodalton
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
PAI-1	plasminogen activator inhibitor 1
PKA	protein kinase A
PKC	protein kinase C
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator activated receptor
RXR	retinoid X receptor
SD	standard deviation
TGF-ß	transforming growth factor-ß
t-PA	tissue-type plasminogen activator
TRE	PMA responsive element
TSA	trichostatin A
WE	Williams E medium

NAWOORD

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 23 juni 1968 te 's Gravenhage. Nadat zij bij het doorlopen van het Atheneum te Oosterhout geinteresseerd raakte in "DNA", ging zij Biologie studeren aan de Katholieke Universiteit Nijmegen. Deze studie werd afgerond met een doctoraal examen in januari 1992. De stage bestond uit een studie naar de weefselspecifieke en soortspecifieke promoterregulatie van het ϵ-crystalline bij de vakgroep Moleculaire Biologie te Nijmegen, o.l.v. Dr.N. Lubsen en Prof.J. Schoenmakers. Verder werd als stage onderzoek gedaan naar de interactie van polymorfe humane IgG2 subklassen met de lage affiniteit FcγRIIa (CD32) op humane monocyten, neutrofielen en plaatjes bij de vakgroep Experimentele Immunologie van het Academisch Ziekenhuis Utrecht, o.l.v. Dr. J.G.J. van de Winkel en Prof. P.J.A. Capel. Van november 1991 tot 1995 was zij als AIO werkzaam op een door de Nederlandse Hartstichting gesubsidieerd project aan de Rijksuniversiteit Leiden en gedetacheerd bij het Gaubius Laboratorium TNO-PG te Leiden. Het aldaar, onder begeleiding van Dr.T. Kooistra en Prof.P. Brakman verrichte onderzoek, staat beschreven in dit proefschrift. Vanaf 1 april 1996 is zij werkzaam als onderzoeker bij de Faculteit geneeskunde van de Erasmus Universiteit Rotterdam (onderzoeksschool PGD) en werkt aan de rol van vitamine D en oestrogenen in botontwikkeling en afbraak.

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