Plasminogen activator inhibitor-1 promoter activity in adipocytes is not influenced by the 4 G/5 G promoter polymorphism and is regulated by a USF-1/2 binding site immediately preceding the polymorphic region

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Abstract

Plasminogen activator inhibitor-1 (PAI-1) levels were found to be associated with obesity indicating that adipocytes influence PAI-1 plasma levels. In addition, the 4 G/5 G promoter polymorphism of the PAI-1 gene may modulate PAI-1 transcription. We investigated the transcriptional regulation of the human PAI-1 gene in adipocytes and analyzed the genetic contribution of the 4 G/5 G polymorphism. The PAI-1 promoter was analyzed using electrophoretic mobility shift assays (EMSAs) and luciferase reporter gene assays. A putative binding site for the upstream stimulatory factor-1/2 (USF-1/2) at the polymorphic region of the PAI-1 promoter was identified. The binding of USF-1/2 was studied using nuclear extracts prepared from adipocytes and was similar in all the promoter variants as analyzed by EMSA. A 257 bp PAI-1 promoter fragment including the 4 G/5 G site was transcriptionally active in adipocytes and was not influenced by the polymorphism. The present data indicate for the first time that USF-1/2 is transcriptionally active in differentiated adipocytes. However, USF-1/2 binding activity and PAI-1 transcription are not influenced by the 4 G/5 G-allele. These data possibly explain the observation that PAI-1 secretion from adipose tissue is not influenced by the PAI-1 promoter polymorphism.

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Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the primary physiological inhibitor of the endogenous fibrinolytic system and elevated plasma PAI-1 was found associated with thrombotic diseases. Low plasma fibrinolytic activity due to increased PAI-1 levels precedes coronary artery disease (Francis *et al.* 1988, Meade *et al.* 1993) and even predicts the occurrence of first acute myocardial infarction (Held *et al.* 1997, Thogersen *et al.* 1998) and reinfarction as well (Hamsten *et al.* 1987). Insulin resistance is associated with higher PAI-1 concentrations and increased PAI-1 has been detected in atheromatous material of patients with type 2 diabetes as compared with non-diabetic patients (Schneiderman *et al.* 1992). Elevated PAI-1 plasma levels were discussed to be related to a 1 bp

guanine deletion/insertion (4 G/5 G) polymorphism within the promoter of the PAI-1 gene (Dawson *et al.* 1993, Eriksson *et al.* 1995). However, PAI-1 plasma concentration was neither significantly different in PAI-1 genotype subgroups of type 2 diabetic patients (Mansfield *et al.* 1995a,b) nor in healthy Japanese males (Matsurbara *et al.* 1999) while associations between plasma levels of PAI-1 and parameters of insulin resistance such as total cholesterol, triglycerides (Mansfield *et al.* 1995a,b, Matsurbara *et al.* 1999), fasting glucose and insulin as well as body mass index (Mansfield *et al.* 1995a,b, Hoffstedt *et al.* 2002) were described. In women, the 4 G-allele predicted a slightly increased risk of myocardial infarction (Leander *et al.* 2003). In contrast, the 5 G/5 G-genotype of PAI-1 has been found associated with myocardial infarction in women (Yamada *et al.*

2002) whereas in 171 type 2 diabetic Pima Indians, subjects with the 4 G/4 G and the 4 G/5 G genotype had a higher prevalence of retinopathy (Nagi *et al.* 1997). In 519 Caucasian diabetic patients (192 with type 1 and 327 with type 2 diabetes) (Mansfield *et al.* 1995b) and, in another study, in 204 type 2 diabetic patients no association between PAI-1 polymorphism and the prevalence of retinopathy could be identified (Globocnik-Petrovic *et al.* 2003).

Recent studies suggest that the adipose tissue itself is a main contributor to elevated PAI-1 plasma levels under obese conditions (Samad *et al.* 1996, 1997, Shimomura *et al.* 1996; for review see Alessi *et al.* 2000, Juhan-Vague *et al.* 2000). PAI-1-mRNA is expressed in visceral and subcutaneous adipose tissue of human subjects, mice and the 3T3-L1 preadipocyte cell line (Lundgren *et al.* 1996, Samad *et al.* 1996, 1997, Shimomura *et al.* 1996, Alessi *et al.* 1997, Halleux *et al.* 1999). During the development of obesity, PAI-1 expression is enhanced in visceral (Alessi *et al.* 1997, Halleux *et al.* 1999) and subcutaneous (Eriksson *et al.* 1998) adipose tissue. However, it remains unclear what kind of tissue represents the major site of PAI-1 expression in the physiological and the pathophysiological state. Additionally, the transcriptional regulation of the PAI-1 gene in adipocytes and the potential influence of the common 4 G/5 G insertion/deletion promoter polymorphism on adipocytic gene transcription have not been investigated in detail.

The aim of the present study was to characterize the human PAI-1 promoter at the polymorphic site in the context of adipocyte differentiation and to study the possible influence of the promoter polymorphism on the transcriptional activation of the PAI-1 promoter in adipocytes.

Materials and methods

Adipocyte cell culture

3T3-L1-preadipocytes were cultured at a 10% CO₂ atmosphere at 37 °C in DMEM-medium (Biowittaker, Verviers, Belgium) supplemented with 10% newborn calf serum (Sigma Biosciences) and penicillin/streptomycin (GIBCO BRL, Berlin, Germany). At confluence, cells were differentiated into adipocytes by treating them with DMEM/F12/glutamate-medium supplemented with

0.5 mM 3-isobutyl-methyl-xanthine, 10⁻⁷ M corticosterone, 10⁻⁶ M insulin, 200 M ascorbate, 2 µg/ml transferrin, 1 M biotin, 17 M panthothenate and 300 mg/l Pedersen-fetuin (Zaitzu & Serrero 1990, Bachmeier & Löffler 1994) for 5 days. Thereafter, the cells were exposed to DMEM/F12/glutamate-medium with insulin (10⁻⁹ M) until they reached the fully differentiated phenotype, which was confirmed by light microscopy for the existence of a more rounded cell shape and the typical appearance of extensive lipid droplet accumulation (Green & Meuth 1974, Green & Kehinde 1975, 1976, 1979, Cornelius *et al.* 1994, MacDougald & Lane 1995).

Preparation of nuclear extracts

3T3-L1-adipocytes differentiated for 7 days were harvested by centrifugation, washed once with ice-cold PBS and twice with wash buffer composed of 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After resuspension in 1 ml ice-cold wash buffer, cells were centrifuged for 1 min at 120 g. Hypotonic buffer containing 0.1% NP40 was added to lyse the cell pellet. Following 5 min incubation on ice, nuclei were pelleted by centrifugation for 15 min (15 000 r.p.m.) at 4 °C. Nuclei were resuspended in lysis buffer containing 20 mM Hepes (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 10% v/v glycerol, and incubated at 4 °C for 15 min with gentle vortexing. Subsequently, the nuclear debris were pelleted by centrifugation at 4 °C for 15 min and the supernatant was diluted 1:6 with storage buffer composed of 20 mM Hepes (pH 7.9), 0.05 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 20% v/v glycerol. The extracts were aliquoted and stored at -80 °C. Protein concentration was determined using the Bradford (1976) method.

Electrophoretic mobility shift assays (EMSAs)

Ten micrograms of nuclear extracts were incubated with 2 µg polydeoxyinosinic-deoxycytidylic acid in a volume of 20 µl binding buffer containing 50 mM Hepes (pH 7.9), 6 mM MgCl₂, 50 mM KCl, 5 mM DTT, 100 g/ml BSA and 0.01% NP40. ³²P-end-labeled synthetic oligonucleotides (30 × 000 c.p.m.)

Table 1 Oligonucleotides used for PCR analysis and EMSAs. The polymorphic site (4 G/5 G) is italicised and the USF-1/2 binding site is printed in bold

Oligonucleotide	Nucleotide sequence
5G up	5'-GTCTGGACACGTGGGGG-3'
4G up	5'-GTCTGGACACGTGGGGA-3'
PAI down	5'-TGCAGCCAGCCACGTGATTGTCTAG-3'
PAI up	5'-AAGCTTTTACCATGGTAACCCCTGGT-3'
PAI-4G sense	5'-AGTGGAC CACGTG GGGGAGTCAGCC-3'
PAI-4G antisense	5'-AGGGCTGACTCCCCACGTGTCCA-3'
PAI-5G sense	5'-AGTGGAC CACGTG GGGGAGTCAGCC-3'
PAI-5G antisense	5'-AGGGCTGACTCCCCACGTGTCCA-3'
USF consensus	5'-CACCCGGT CACGTG GCCTACACC-3'

were added and the reaction mixture was incubated for 20 min at room temperature. In competition experiments nuclear extracts were preincubated for 10 min with a 50- to 100-fold molar excess of non-labeled competitor oligonucleotides. Supershift assays were performed with antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against USF-1 (upstream stimulatory factor-1), USF-2 (upstream stimulatory factor-2), c-Myc and c-Fos were used. The DNA-protein complexes were analyzed on a 8% polyacrylamide gel at 80 V using 0.25 × tris-boric-acid-EDTA as electrophoresis buffer. Gels were dried and exposed to Kodak X-Omat AR films overnight at -80 °C. For the electrophoretic mobility shift experiments the following oligonucleotides with 5'-AG-ends annealed to their respective complementary oligonucleotides were used: PAI-4 G-sense, PAI-4 G-antisense, PAI-5 G-sense and PAI-5 G-antisense, USF consensus (Table 1).

Transient transfection of adipocytes and luciferase reporter gene assays (LRAs)

A 257 bp genomic PCR product (primer PAI-up and PAI-down; Table 1) encompassing the polymorphic promoter site was generated from individuals with both the 4 G/4 G and the 5 G/5 G-allele, isolated and cloned in the luciferase expression vector pGL3-enhancer (Promega). The PCR products were purified, digested with SacI and BglII and cloned into the luciferase vector that had also been cut with SacI and BglII. All chimeric plasmids were sequenced prior to transfection.

After the cells were grown to a density of 80–90%, cells were transiently transfected with 10 µg plasmid DNA using the Superfect-reagent based on activated dendrimer technology (Qiagen, Hilden, Germany) as described by the manufacturer. After 3 h of incubation, cells were differentiated as described above. Measurement of luciferase activity was performed 48 h after the induction of differentiation. In each experiment, cells were transfected with pGL-3 enhancer and pGL-3 control plasmids that served as negative and positive controls respectively. The pGL-3 enhancer vector has no eukaryotic promoter upstream of the luciferase gene. The pGL-3 control vector contains the SV40 early promoter driving the expression of the luciferase mRNA transcripts. To standardize the transfection efficiency, 7.5 µg pSV-galactosidase plasmid (Promega) were always cotransfected. In addition, the amount of measured relative light units was normalized to total protein content of each cell lysate.

For luciferase assays, the transfected cells were harvested 48 h after differentiation and lysed in 1 ml reporter lysis buffer (Promega). Aliquots of 20 µl lysates were mixed with 100 µl luciferase assay reagent containing luciferyl-CoA. The luciferase activity was measured in a Lumat LB9501 (Berthold, Munich, Germany).

Statistics

For statistical analysis, the SPSS/PC+ statistical software package was used (SPSS 11.0). Means were compared by the MannWhitney test. A *P* value below 0.05 (two tailed) was considered to be statistically significant.

Results

Analysis of the polymorphic promoter site encompassing nucleotides –672 bp to –676 bp for putative transcription binding sites

Since the significance of the polymorphic promoter site on PAI-1 gene transcription is unclear, we investigated the nucleotide sequence for the presence of putative transcription factor binding sites. A detailed computer-based analysis using the professional software program TESS (transcription element search software) based on the TRANSFAC database (Wingender *et al.* 1996) resulted in the location of putative transcription factor binding sites for USF-1, USF-2, c-Myc and c-Fos. The location of the USF-1/2 binding site relatively to the polymorphic promoter site is given in Table 1.

Transcription factors USF-1/2 are expressed in adipocytes and induced during differentiation from 3T3-L1 preadipocytes to mature adipocytes.

Although USF-1 and USF-2 have been considered as widely distributed nuclear proteins (Gregor *et al.* 1990, Shieh *et al.* 1993, Henrion *et al.* 1995, Groenen *et al.* 1996), precise data on their protein expression and regulation in adipocytes is not available. Therefore, it was necessary to demonstrate sufficient and transcriptionally active USF-1/2 protein expression in adipocytes. Transcriptionally active USF-1/2 protein expression was investigated by EMSA using a radiolabeled USF consensus oligonucleotide (Fig. 1, lane 1) and nuclear extracts from preadipocytes and fully differentiated adipocytes at day 7 of differentiation. Incubation of the radiolabeled USF consensus oligonucleotide with nuclear extracts from 3T3-L1-preadipocytes produces a weak, but reproducible band shift (Fig. 1, lane 2) that it is strongly induced during adipocyte differentiation (Fig. 1, lane 3). The specificity of the binding activity was shown by competition experiments with unlabeled oligonucleotides, which eliminates this complex completely (Fig. 1, lanes 6, 7). Preincubation of the radiolabeled USF consensus oligonucleotides with nuclear extracts from mature adipocytes and antibodies against USF-1 (Fig. 1, lane 4) and USF-2 (Fig. 1, lane 5) produces specific supershift bands. As a control, nuclear

Figure 1 EMSA. The binding of nuclear extracts (NE) from preadipocytes, fully differentiated adipocytes (day 7 of differentiation) and Hep-G2 cells to a USF consensus oligonucleotide is investigated. Lane 1, free probe (radiolabeled USF consensus oligonucleotide); lane 2, NE preadipocytes; lane 3, NE adipocytes (7 days of differentiation); lane 4, NE adipocytes+USF-1 antibody; lane 5, NE adipocytes+USF-2 antibody; lanes 6 and 7, NE adipocytes preincubated with a molar excess of unlabeled probe (50- and 100-fold molar excess respectively) as specific competitor; lane 8, NE HEP-G2 cells; lane 9, NE HEP-G2 cells+USF-1 antibody; lane 10, NE HEP-G2 cells+USF-2 antibody. Shifted/supershifted complexes are marked with brackets. Free probe is indicated by an arrow.

extracts from the hepatocyte cell line HEP-G2 were shown to shift the USF-consensus oligonucleotide (Fig. 1, lane 8) identically. Preincubation of the hepatocyte nuclear extracts with antibodies against USF-1 (Fig. 1, lane 9) and USF-2 (Fig. 1, lane 10) produces specific supershift bands.

Adipocytic USF-1 and USF-2 nuclear proteins bind specifically to the PAI-1 promoter

The radiolabeled 5 G-allele promoter fragment (Fig. 2, lane 1) specifically binds nuclear extracts from mature adipocytes (day 7 of differentiation) (Fig. 2, lane 2) and binding is effectively competed with increasing amounts of unlabeled oligonucleotides (Fig. 2, lanes 6–8). For further characterization of the binding complex, supershift assays

using specific antibodies against USF-1 and USF-2 were performed. Preincubation of the radiolabeled promoter construct with nuclear extracts from adipocytes and antibodies against USF-1 (Fig. 2, lane 3), USF-2 (Fig. 2, lane 4) or USF-1 and USF-2 (Fig. 2, lane 5) together leads to the appearance of specific supershift bands.

The 4 G/5 G insertion/deletion polymorphism does not influence the transcriptional binding activity of USF-1 and USF-2 from adipocytic nuclear extracts

In order to investigate the influence of the single base pair polymorphism on the binding activity of USF-1/2 from adipocytic nuclear extracts, we repeated the experiments using both a 4 G-allele and a 5 G-allele promoter fragment. Incubation of the radiolabeled 4 G promoter fragment (Fig. 3, lane 1) and the 5 G promoter fragment (Fig. 3, lane 2) with nuclear extracts from adipocytes (Fig. 3, lanes 3 and 4) produces the identical strong band shifts without any differences in binding activity. Thus, USF-1 and USF-2 in nuclear extracts from adipocytes bind to the polymorphic PAI-1 promoter site independently from the genotype of the polymorphic promoter site. As already demonstrated for the 5 G-allele, incubation of the radiolabeled 5 G promoter fragment with antibodies against USF-1 (Fig. 3, lane 5), USF-2 (Fig. 3, lane 6) or USF-1 and USF-2 together (Fig. 3, lane 7) produces specific supershift bands. The specificity of binding was demonstrated by competition experiments with increasing amounts of unlabeled oligonucleotides, which eliminates this complex completely (Fig. 3, lanes 8, 9).

Since the polymorphic promoter site does also carry putative binding sites for c-Myc and c-Fos, we tested for the appearance of supershift bands using antibodies against both factors. Preincubation of the radiolabeled 5 G-allele promoter fragment with antibodies against c-Fos (Fig. 3, lane 10) and c-Myc (Fig. 3, lane 11) did not generate any specific supershift bands.

The low molecular mass complexes in Figs 2 and 3 could not yet be identified. However, since these complexes are specific (competition experiments), they might be of interest in identifying additional nuclear proteins regulating PAI-1 gene expression.

Figure 2 EMSA. The binding of nuclear extracts (NE) from fully differentiated adipocytes (day 7 of differentiation) to the PAI-1 promoter site (5 G-genotype) is shown. Lane 1, free probe (radiolabeled promoter fragment); lane 2, NE adipocytes; lane 3, NE adipocytes+USF-1 antibody; lane 4, NE adipocytes+USF-2 antibody; lane 5, NE adipocytes+USF-1 antibody+USF-2 antibody; lanes 6–8, NE adipocytes preincubated with 25-, 50- and 100-fold molar excess of unlabeled probe as specific competitor. Shifted/supershifted complexes are marked with brackets. Free probe is indicated by an arrow. The bands marked with a cross are specific low molecular mass complexes that have not been identified.

Functional assessment of the 4 G and the 5 G chimeric promoter constructs using LRAs did not reveal any differences in transcriptional activation between genotypes

Transient transfection of adipocytes (Fig. 4) with the pGL-basic vector resulted in a mean value of $180 \pm 70 \times 10^3$ RLU (relative light units). This activity was set as 1 and the activation of the reporter gene constructs was calculated as the x-fold induction of the activity of the pGL-basic vector. The SV40 control vector generated $15.0 \pm 4.5 \times 10^6$ RLU (84-fold induction of the pGL-basic vector, $P < 0.001$). Transient transfection of adipocytes with the 4 G-genotype promoter construct (Fig. 4) led to a significant, 24-fold induction of the promoter activity ($4.3 \pm 1.7 \times 10^6$

Figure 3 EMSA. The binding of nuclear extracts (NE) from fully differentiated adipocytes (day 7 of differentiation) to the polymorphic PAI-1 promoter site (5 G-genotype and 4 G-genotype) is investigated. Lane 1, free probe (radiolabeled promoter fragment, 4 G-genotype); lane 2, free probe (radiolabeled promoter fragment, 5 G-genotype); lane 3, NE adipocytes and 4 G promoter fragment; lane 4, NE adipocytes and 5 G promoter fragment; lane 5, NE adipocytes+5G promoter fragment+USF-1 antibody; lane 6, NE adipocytes+5G promoter fragment+USF-2 antibody; lane 7, NE adipocytes+5G promoter fragment+USF-1 antibody+USF-2 antibody; lane 8, NE adipocytes preincubated with 50-fold molar excess of unlabeled probe as specific competitor; lane 9, NE adipocytes preincubated with 50-fold molar excess of unlabeled probe as specific competitor; lane 10, NE adipocytes+5G promoter fragment+c-Fos antibody; lane 11, NE adipocytes+5G promoter fragment+c-Myc antibody. Shifted/supershifted complexes are marked with brackets. Free probe is indicated by an arrow. The bands marked with a cross are specific low molecular mass complexes that have not been identified.

RLU, $P=0.01$). Similarly, transfection with the 5 G-genotype promoter construct resulted in a significant, 17-fold induction of promoter activity ($3.1 \pm 1.1 \times 10^6$ RLU, $P=0.001$). However, the difference in promoter activity between the 4 G- and the 5 G-allele (24-fold vs 17-fold induction) did not reach any statistical significance ($P=0.318$). For a more detailed analysis of PAI-1 promoter regulation additional luciferase experiments using mutants and USF-1/2 coexpression would be helpful.

Discussion

The cellular USF, also known (Gregor *et al.* 1990) as major late transcription factor, is ubiquitously expressed and consists of 43 kDa (USF-1) and 44 kDa (USF-2) polypeptides (Gregor *et al.* 1990, Shieh *et al.* 1993, Henrion *et al.* 1995, Groenen *et al.* 1996) that independently exhibit site-specific DNA binding activity. USF-1/2 belong to the c-Myc-related family of regulatory factors containing helix-loop-helix domains and leucine repeats.

Figure 4 LRA. Promoter activity of human PAI-1 luciferase gene chimeras (4 G- and 5 G-genotype) transiently transfected in 3T3-L1 adipocytes. Data are normalized to total protein content. Relative promoter activity (measured by relative light units) is given as x-fold induction of the pGL-basic vector. The SV40-vector was used as control vector. Significance was set at $P < 0.05$.

USF-1/2 bind to a symmetrical hexanucleotide (CACGTG) DNA sequence named E-box motif. Up to now, no data are available concerning USF-1/2 expression or regulation in adipocytes. Our data obtained by EMSA with nuclear extracts from preadipocytes and mature adipocytes clearly demonstrate that USF-1/2 are expressed at very low levels in preadipocytes, but increase during adipocyte differentiation with a maximum level in late adipocyte differentiation. These data provide the basis for a putative role of USF in regulating genes during adipocyte differentiation. The binding of USF-2 was demonstrated to inhibit PAI-1 expression in hepatocytes (Samoylenko *et al.* 2001) whereas USF-1 increased PAI-1 transcription in epithelial cells (Providence *et al.* 2002). We were able to demonstrate by competition experiments, and by the appearance of specific supershift bands in EMSA, that both USF-1 and USF-2 from adipocytic nuclear extracts can bind specifically to the PAI-1 promoter site. Additionally, PAI-1

promoter constructs encompassing the USF binding site were shown to transactivate the luciferase reporter gene in adipocytes. Therefore we conclude that USF-1/2 stimulate PAI-1 expression in human adipocytes.

The E-box motif for binding of USF-1/2 immediately precedes the well-described 4 G/5 G promoter polymorphism of the PAI-1 gene. However, neither the binding of the transcription factors nor the luciferase activity was significantly altered when the 4 G-(deletion allele)- or 5 G-(insertion allele) promoter constructs were analyzed. These data indicate that – at least in adipocytes – the promoter polymorphism next to the USF binding site affects neither transcriptional USF binding activity nor functional promoter transactivation. These data are in contrast to previous findings in different cell types (Dawson *et al.* 1993).

A functional promoter mutation should result in an increase or a decrease in PAI-1 plasma

concentrations. Our results are comparable with those of others who found no significant association between the 4 G/5 G polymorphism and plasma levels of PAI-1 in diabetic or non-diabetic patients (Mansfield *et al.* 1995b, Matsubara *et al.* 1999, Festa *et al.* 2003). In addition, Van Harmelen *et al.* (2000) reported that PAI-1 secretion from abdominal subcutaneous adipose tissue is not influenced by the polymorphism.

The present data indicate for the first time that USF-1/2 is transcriptionally active in differentiated adipocytes. The binding activity of USF-1/2 and the PAI-1 transcription are not affected by the 4 G/5 G promoter polymorphism overlapping the E-box motif. These results might explain the observation that the 4 G/5 G polymorphism does not influence PAI-1 secretion from adipose tissue.

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