Direct binding of Nur77/NAK-1 to the plasminogen activator inhibitor 1 (PAI-1) promoter regulates TNFα induced PAI-1 expression

Short Title: Nur77 regulates PAI-1 expression by TNFα

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Abstract
Plasminogen activator inhibitor 1 (PAI-1) is the main fibrinolysis inhibitor and high plasma levels are associated with an increased risk for vascular diseases. Inflammatory cytokines regulate PAI-1 through a hitherto unclear mechanism. Using reporter gene analysis we could identify a region in the PAI-1 promoter that contributes to basal expression as well as to TNF\(\alpha\) induction of PAI-1 in endothelial cells. Using this region as bait in a genetic screen we could identify Nur77 (NAK-1, TR3, NR4A1) as an inducible DNA-binding protein that binds specifically to the PAI-1 promoter. Nur77 drives transcription of PAI-1 through direct binding to a NGFI-B responsive element (NBRE) indicating monomeric binding and a ligand independent mechanism. Nur77 itself is transcriptionally upregulated by TNF\(\alpha\). High expression levels of Nur77 and its co-localization with PAI-1 in atherosclerotic tissues indicate that the described mechanism for PAI-1 regulation may also be operative in vivo.

Introduction
Plasminogen activator inhibitor 1 (PAI-1) is the main inhibitor of tissue type (t-PA) and urokinase type (u-PA) plasminogen activators (reviewed in refs.\(^1,2\)). It functions as a serine protease inhibitor (SERPIN) by forming stable 1:1 stochiometric complexes\(^3\) with its target proteases that are removed by scavenger receptors\(^4\). PAI-1 regulates intravascular fibrinolysis and tissue proteolysis and thereby controls thrombus dissolution as well as invasion and migration of cells through the extracellular matrix. PAI-1 is secreted by several tissues including the liver, adipose tissue, smooth muscle cells (SMC) and endothelial cells (EC), the latter ones producing low amounts of PAI-1 under normal, resting conditions. PAI-1 is present only in low concentrations (6 to 80 ng/ml) in normal human plasma. Elevated levels of plasma PAI-1 found in several pathologic
conditions are thought to increase the risk for vascular complications such as thrombosis\(^5,6\) or myocardial infarction\(^7\). Indeed, PAI-1 mRNA levels are increased in atherosclerotic vessels\(^8,9,10\). Such local upregulation of PAI-1 in vascular tissues might be caused by several mechanisms including hypoxia\(^11\), lipid mediators\(^12\), growth factors\(^13,14\) as well as inflammatory cytokines. Atherosclerosis is indeed regarded as a disease with a significant inflammatory component (reviewed in\(^15\)) and PAI-1 expression is upregulated by inflammatory mediators such as TNF\(\alpha\)\(^16\), IL-1\(\beta\)\(^17,18\) or LPS\(^19\) in human endothelial cells and in the model cell line HepG2\(^17,18,20\). In fact, PAI-1 levels are increased during sepsis\(^21\) where endothelial cells are a major source of this protein. Thus, PAI-1 is regarded as an "inflammatory response gene" that can alter the fibrinolytic potential of the vessel\(^22\) and might lead to a thrombotic tendency.

The PAI-1 promoter has been analyzed for the presence of transcription factor consensus binding sites possibly responsible for its inducible expression: Best studied is the strong induction by TGF-\(\beta\) mediated by binding of SMAD 3 and 4 proteins to consensus sites in the PAI-1 promoter\(^23\). SP1 elements in the proximal promoter region have been shown to mediate the response of PAI-1 to glucose\(^24\) and angiotensin II\(^25\); an AP-1 like binding site mediating the PAI-1 response to PKC and PKA signals\(^26,27\); a HRE (hypoxia response element) the binding of HIF-1 and the response to hypoxia\(^11\). The regulation of PAI-1 by inflammatory mediators is, however, still unclear. NF-\(\kappa\)B is the major transcription factor translocated to the nucleus in response to inflammatory stimuli where it drives directly transcription of responsive genes such as E-Selectin, VCAM-1, IL-8, tissue factor and others\(^18\). However, no NF-\(\kappa\)B binding site could be identified in the PAI-1 promoter. Others and we have shown that the early growth response gene 1 (EGR-1) is another
important transcription factor within the TNFα signaling cascade\textsuperscript{28,29} leading to increased PAI-1 synthesis\textsuperscript{30}. However, as in case of NF-κB, no EGR-1 consensus site is present in the PAI-1 promoter and it therefore remains unclear how PAI-1 is regulated during inflammation.

Materials and Methods

**Cell culture**: Human umbilical vein endothelial cells (HUVEC, different batches #91, 92, 181, 182, 183; the reactivity of the different batches of HUVECs towards stimulation by inflammatory cytokines varied as described previously\textsuperscript{31}) were cultured as described\textsuperscript{31, 32}. Cells were used for experiments up to passage five. MCF-7 cells and JURKAT were cultured as recommended by ATCC (Manassas, VA).

**Relative quantitative RT-PCR (Q PCR)**: RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) reagent. 900ng of total RNA were reverse transcribed with MuLV-reverse transcriptase using the Gene Amp RNA PCR kit (Applied Biosystems, Foster City, CA) and oligo dT (16) primers. The mRNA sequences of the investigated genes were obtained from GenBank. The primers for β-2 microglobulin were used as described by S. Wellman et al (ref. \textsuperscript{33}). The other primers were designed using the PRIMER3 software from the Whitehead Institute for Biomedical Research (Cambridge, MA). The following forward (F) and reverse (R) primers were used: PAI-1: F, 5'-CAGACCAAGAGCCTCTCCAC-3'; R, 5'-ATCACTTGGCCCATGAAAAG-3'; Nur77: F, 5'-CACCCACTTCTCCACACCTT-3'; R, 5'-ACTTGGCGTTTTTCTGCACT-3'. Relative Quantitative RT-PCR was performed by LightCycler technology (Roche Diagnostics,
Basel, Switzerland) using the Fast Start SYBR Green I kit for amplification and
detection. In all assays, cDNA was amplified using a standardized program (10'
denaturing step; 55 cycles of 5” at 95°C, 15” at 65°C and 15” at 72°C; melting point
analysis in 0.1°C steps; final cooling step). Each LightCycler capillary was loaded with
1.5 µl DNA Master Mix; 1.8 µl MgCl2 (25 mM); 10.1 µl H2O; 0.4 µl of each primer (10
µM). The final amount of cDNA per reaction corresponded to 2.5 ng of total RNA used
for reverse transcription. Relative quantification of target gene expression was performed
using a mathematical model by Pfaffl34. The expression of the target molecule was
normalized to the expression of β-2 microglobulin.

**Nuclear run-on assay:** Nuclear run-on assays were performed as described35. Nuclei
were isolated from HUVEC that were either untreated or treated for 4h with 100U/ml
TNFα. The run on reaction contained 1.5x10⁷ isolated nuclei and 250µCi of [α32P] UTP
for incorporation into the nascent pre-mRNA chains. 1µg of PAI-1 and GAPDH PCR
product was applied to Hybond-N (Amersham Biosciences, Piscataway, NJ) nylon
membranes using a slot blot apparatus followed by UV-crosslinking. The labeled RNA
was isolated from the nuclei using Trizol reagent, washed and re-suspended in DEPC
H₂O. 1.5 million cpm of each labeled RNA were used for hybridization and the
membranes were exposed on Phosphoimager plates (Amersham) for three days.

**Electrophoretic Mobility Shift Assay:** Electrophoretic mobility shift assays were
performed as described32. Confluent HUVECs were stimulated with 100U/ml TNFα for 2
hours. JURKAT were induced with PMA (10 ng/ml) and ionomycin (0.5 µg/ml) for 2
hours, controls with vehicle (1.1% DMSO). For the electrophoretic mobility shift assay 5
µg of nuclear extracts of HUVEC treated as indicated were incubated at room
temperature for 30 min, with $5 \times 10^6$ cpm/ml of the 32P (Amersham) of labeled oligonucleotides representing the nt $-270$ to $-250$ of the PAI-1 promoter, its single base mutations or the consensus NBRE. As a control, a competition assay was performed by adding a 100-fold molar excess of unlabeled oligonucleotide to the reaction prior to the addition of the labeled probe. The samples were separated on a 5% polyacrylamide gel. The gel was then dried and exposed to Phosphoimager plates (Amersham).

**One-hybrid screen:** Yeast one hybrid screening was performed with the Matchmaker 1-Hybrid system (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s recommendations. The 'bait' was prepared by synthesizing three tandem copies of the nt $-270$ to $-250$ region which was subcloned into pHISi (Clontech). The resultant plasmids were sequenced for verification, transformed into the yeast strain YM4271 and selected for integration in the his3 locus. 2 million transformants were obtained using a library from activated lymphocytes (Clontech). Five colonies were clearly HIS-positive and 3 of these contained inserts coding for Nur77, the other two were false positives.

**Transfection of HUVECs:** Fragments of the human PAI-1 promoter (deletion series from nt $-1520$ to $-80$ ending at $+20$ and a $-850$ construct with deletion of the bases from $-250$ to $-270$ [ΔNBRE] ) were cloned into a luciferase expression vector, pUBT-luc$^{36}$. Twenty-four hours before transfection, HUVEC were seeded in 6-well tissue-culture plates to reach 80% to 90% confluence the next morning. Transient transfections were performed by using the Lipofectamine Plus reagent (Invitrogen) according to the protocol. Cells were incubated with transfection mixture containing 1.5 µg DNA
(including a cytomegalovirus [CMV]-b-gal or CMV-renilla construct as internal control), 6µl Plus reagent and 4 µL Lipofectamine in a total volume of 1 ml medium M-199 per well for 130 minutes. Induction with TNFα (100 U/ml) was performed the next day for 6 hours. Luciferase and beta-galactosidase assays were performed with cellular lysates of transfected cells as previously described\textsuperscript{37}. All experimental values were determined from duplicate wells, and were performed at least twice.

The constructs for over-expression of Nur77 contained the coding sequence for amino acids 1 to 580 for the full length (wt) clone and 248 to 580 for the truncated (dominant negative) clone in the vector pCDNA3.1. The dominant negative construct for immunocytochemistry contained the coding sequence for amino acids 248 to 580 in the vector pEGFP-C1 (Clontech), resulting in a fusion protein with EGFP on its N-terminus. As transfection control we used the empty pEGFP C1.

**Immunohistochemistry:** For detection of PAI-1 mouse anti-PAI-1 (3PAI5, Technoclone GmbH, Vienna Austria) diluted 1:50 was used, for Nur77 rabbit anti-Nur77 (M-210, Santa Cruz Biotechnology, Santa Cruz, California), diluted 1:100 was used. Secondary antibodies rabbit anti mouse Alexa 488, goat anti rabbit Alexa 488 and goat anti mouse Alexa 568 all from Molecular Probes (Eugene, Oregon) were used diluted 1:2000 for immunocytochemistry and 1:300 for immunohistochemistry.

Tissues were snap frozen and embedded in O.C.T. (Optimal Cutting Temperature compound, Miles Laboratories, Elkhart IN). An anti CD31 antibody (WM59 mouse monoclonal, TCS,London, Great Britain) was used to identify endothelial cells. Cryosections were fixed in acetone at -20°C and used for standard immuno-detection immediately.
Results

TNFα induces PAI-1 expression

To verify that TNFα induces PAI-1 in endothelial cells, we followed PAI-1 expression in human umbilical vein endothelial cells (HUVECs) stimulated with TNFα using relative quantitative RT-PCR (Q-PCR). PAI-1 mRNA levels increased already after one hour and reached 10-fold higher levels after four hours (Fig. 1a). We performed nuclear run-on assays to assure that this induced mRNA increase is due to de novo synthesis. As shown in the insert to Fig. 1a, treatment with TNFα induced de novo synthesis of PAI-1 mRNA, consistent with results reported earlier. To analyze which part of the PAI-1 promoter might be responsible for TNFα dependent induction, we constructed a series of deletion mutants of the PAI-1 promoter (between nucleotides [nt] -1520 and +20 relative to the transcription start) driving transcription of a luciferase reporter gene. When HUVECs were transfected with these reporter gene constructs a 40bp stretch between nt -280 to -240 of the PAI-1 promoter could be identified as critical for the small but consistent TNFα induced transcriptional activation in EC (Fig. 1b).
**Fig.1 Expression of PAI-1 mRNA and PAI-1 reporter studies.**

**a,** Relative quantitative PCR (Q-PCR) analysis of PAI-1 mRNA expression. HUVEC were stimulated with TNFα for the indicated periods and harvested. Relative expression of PAI-1 was normalized to the expression of beta 2 microglobulin. The insert shows nuclear run on data. HUVEC were either untreated or treated for 4h with TNFα, and the RNA isolated after the run-on reaction hybridized to PAI-1 and GAPDH probes that were immobilized on membranes.

**b,** Reporter gene analysis. A series of deletions in the PAI-1 promoter were fused to a luciferase reporter gene, transfected into HUVEC and measured by luminometry. HUVECs expressing the reporter gene constructs were induced with TNFα;

**c,** EMSAs of overlapping parts of the PAI-1 promoter. Nuclear extracts were obtained from HUVEC and incubated with labeled double stranded oligonucleotides representing...
the nt –280 to –260, -270 to –250 and –260 to –240 of the PAI-1 promoter, respectively. Specific binding activity of nuclear protein complexes is indicated by black arrows.

Overview of the region of the PAI-1 promoter containing the NBRE. Oligonucleotide sequences that were used for EMSAs and for the yeast screens are shown.

A nuclear protein complex binds to nt -270 to -250 in the PAI-1 promoter

To define the binding site in this part of the PAI-1 promoter for the putative nuclear proteins, we performed serial electrophoretic mobility shift assays (EMSAs) with overlapping oligonucleotide probes spanning the region from nt -280 to -240. The central region (nt -270 to -250) showed specific binding of a protein complex(es) when incubated with nuclear extracts from HUVECs (Fig. 1c). We therefore used this 20 bp stretch (Fig. 1d) of the PAI-1 promoter as bait in a yeast one-hybrid screen employing an activated lymphocyte cDNA fusion library. We identified three positive clones, all of them containing the DNA binding domain of the orphan nuclear receptor Nur77.

Nur77 binds to a NBRE in the PAI-1 promoter

Upon analysis, we found that the PAI-1 promoter contains a putative NBRE (NGFI-B responsive element) from nt -261 to -254 also present in the oligonucleotides used for EMSA and in the bait sequence used for the yeast one-hybrid screen (Fig. 1d). The NBRE is a well-described binding site for Nur77 monomers, and binding of Nur77 to this element has been shown to strongly activate transcription of other genes.

To establish that Nur77 binds to that site in the PAI-1 promoter, we performed “supershift” assays after over-expressing a truncated Nur77 (aa 249-598, containing the DNA and ligand binding domains) in MCF-7 cells, which express low endogenous
amounts of Nur77. Lane 3 in Figure 2a shows increased specific binding to the labeled PAI-1 oligonucleotide (P) in transfected cells. This indicates that over-expression of Nur77 is sufficient to induce binding. Upon addition of an antibody recognizing Nur77, the specific bands were retarded in the gel, identifying the presence of Nur77 in the complex(es) bound to the labeled oligonucleotide (Fig. 2a, lane 5). The human T-cell leukemia cell line JURKAT is known to express significant levels of Nur77 only after stimulation\(^4\) and indeed nuclear extracts of PMA and ionomycin stimulated JURKAT cells exhibited specific binding to the PAI-1 oligonucleotide (P, Fig. 2a, lane 7) which was also “supershifted” by the anti Nur77 antibody (Fig. 2a, lanes 8). The differing positions of the antibody-supershifted bands in lane 5 as compared to lane 8 can be explained by the fact that in MCF-7 cells a shorter, truncated form of Nur77 was expressed, that still exhibits full DNA binding activity\(^42\), while JURKAT express a wildtype Nur77. Binding of JURKAT nuclear extracts to the PAI-1 oligonucleotide was comparable to that of an oligonucleotide containing a canonical NBRE consensus site (5’-AAAAGGTCAAG-3’ [Con. NBRE, N], Fig. 2a, lanes 11-14). Mutation of the NBRE in the PAI-1 oligonucleotide from 5’-AGGTCA-3’ (PAI-NBRE) to 5’-AGGACA-3’ (PAI-T15A, T, see Fig. 1d) thereby destroying the most conserved base of the consensus sequence resulted in loss of binding of the nuclear protein complex to the oligonucleotide (Fig. 2b; lane 6). A part of the CTE (C terminal extension) of Nur77, called the A box, confers specificity for binding of Nur77 monomer to the NBRE. This interaction is mediated by a stretch of 2 or more adenines located 5’ from the NBRE \(^43; 44\). In order to analyze whether binding of Nur77 to the NBRE in the PAI-1 promoter involves interaction with the 5’ adenines, we designed an oligonucleotide where the
original site in the PAI-1 promoter was changed from 5'-GAAAGGTCA-3’ (PAI-NBRE) to 5'-GACAGGTCA-3’ (PAI-A11C, A, see Fig.1d). This mutation strongly reduced binding of Nur77 to the oligonucleotide (Fig.2b; lane 8). Taken together these data indicate that the PAI-1 promoter contains a NBRE site that is a target for monomeric binding of Nur77 and that Nur77 by itself is sufficient to induce specific binding.
Fig. 2 PAI-1 promoter: EMSAs and "supershifts" of Nur77

a, Overexpression of a truncated clone of Nur77 (aa 248 to 580 that has full DNA binding activity) in MCF-7 cells (lanes 1-4) and addition of the E20 antibody, which recognizes the C-terminus of the Nur77 family of proteins (lane 5). JURKAT cells were induced with ionomycin and PMA and incubated with a radio-labeled NBRE found in the PAI-1 promoter (P, lanes 6-10) and a canonical consensus NBRE (N, lanes 11-14). The extracts were also incubated with E20 antibody (lanes 8 and 13). Binding was competed with a 100-fold molar excess of unlabeled oligonucleotides as indicated (PAI - NBRE, P; Con. NBRE, N). Specific binding activity of nuclear protein complexes is indicated by black arrows. A supershift resulting from binding of the Nur77 antibody is indicated by white arrows.

b, HUVECs were stimulated with TNFα and nuclear extracts were incubated with radioactively labeled oligonucleotides containing the NBRE of the PAI-1 promoter (P). The same extracts were incubated with radioactively labeled oligonucleotides containing the NBRE of the PAI-1 promoter that were mutated in the positions indicated in Fig 1d (PAI-T15A, T; PAI-A11C, A). Specific binding activity of nuclear protein complexes is indicated by black arrows

TNFα, LPS and IL-1 induce expression of Nur77

The specific binding activity to the PAI-1 oligonucleotide was strongly increased when nuclear extracts from TNFα stimulated cells were used, indicating regulation of Nur77 by TNFα (Fig. 2b; lane3); such increased binding activity resembles the results seen in Fig. 2 a(lane3), when Nur77 was overexpressed.
To assess whether in fact TNFα regulates Nur77 at the level of expression, we followed Nur77 mRNA levels by Q-PCR. TNFα induced a rapid burst of Nur77 detectable already after 30 minutes that peaked after one hour of stimulation and declined to unstimulated levels after four hours (Fig. 3a); this change in Nur77 mRNA was followed by an increase in Nur77 protein (insert Fig. 3a). PAI-1 mRNA levels increased with a delay and then continued to rise steadily until four hours after stimulation as already shown in Fig. 1a. Immunofluorescence data of corresponding experiments in cultured cells are shown in Fig. 3b: Upon stimulation of endothelial cells by TNFα, Nur77 protein increased after one hour and accumulates in the nucleus after two hours; PAI-1 protein synthesis starts in the cells after four hours. These data indicate that TNFα leads to induction of Nur77 transcription; Nur77 protein then accumulates in the nucleus followed by PAI-1 mRNA and protein expression suggesting that TNFα regulates PAI-1 via Nur77.

To test whether other inflammatory mediators also regulate Nur77 and PAI-1 in a similar way, we performed experiments as shown in Fig. 3a using LPS (Lipopolysaccharide) or IL-1β (Interleukin 1). As shown in Fig. 3c, IL-1β induced upregulation of Nur77 and PAI-1 mRNA in a comparable way. This indicates that Nur77-mediated PAI-1 regulation might not be restricted to TNFα. Also LPS induced upregulation of Nur77 and PAI-1; however, the different time course seen in response to LPS indicates overall differences in the signal transduction pathway between LPS and IL-1β or TNFα, respectively. Such different kinetics of the induction of inflammatory response genes have been described already previously\textsuperscript{45}. The early increase in PAI-1 mRNA further indicates addition regulatory mechanism including changes in mRNA stability as indicated by previous studies\textsuperscript{46}.

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Fig. 3 Expression of Nur77 and PAI-1 in HUVECs induced with TNFα.

**a**, Q-PCR analysis of Nur77 and PAI-1 mRNA expression. HUVEC were induced with TNFα for the indicated periods, relative expression was normalized to beta-2-microglobulin. The insert shows a western blot of HUVECs stimulated for the indicated periods with TNFα and stained using an antibody recognizing the N-terminus of Nur77.

**b**, Immunocytochemistry. HUVEC were stimulated by TNFα for indicated periods and stained with antibodies recognizing Nur77 or PAI-1.

**c**, Q-PCR analysis of Nur77 and PAI-1 mRNA expression. HUVEC were induced with LPS and IL-1 for the indicated time periods.
TNFα induction of PAI-1 depends on Nur77 and the presence of the NBRE in the PAI-1 promoter

To prove that increased expression of Nur77 directly activates the PAI-1 promoter, we performed reporter gene assays. PAI-1 reporter constructs containing the NBRE of the PAI-1 promoter were strongly induced when Nur77 was co-expressed (Fig. 4a), whereas PAI-1 promoter constructs with a NBRE deletion did not respond to Nur77 over-expression (not shown) or to TNFα (Fig. 4a). Consistently, over-expression of Nur77 as well as TNFα stimulation strongly induced a 4xNBRE luciferase reporter gene construct (Fig. 4b). Co-expression of a dominant negative mutant of Nur77 (dnNur77) reduced basal reporter gene activity and completely abolished the upregulation of the reporter gene by TNFα (Fig. 4a). When a dnNur77 fused to EGFP (dnNur77-EGFP) was transfected into HUVEC followed by stimulation with TNFα for 4h, no PAI-1 expression was seen in the transfected cells expressing the construct. Cells transfected with EGFP alone or untransfected cells responded normally with PAI-1 expression to TNFα stimulation (Fig. 4c)

These data further indicate that the regulation of the PAI-1 promoter as well as of PAI-1 protein by TNFα is Nur77 dependent and requires the NBRE.
Fig. 4 Analysis of PAI-1 reporter gene activity and protein expression upon co-expression of full length and dominant negative Nur77 constructs.

PAI-1 reporter gene activity. Bars 1-3 of (a) represent overexpression of Nur77 as well as TNFα induction in HUVEC; lanes 4 and 5 of (a) represent overexpression of a dominant negative mutant of Nur77 lacking its transactivation domain in untreated or TNFα.
stimulated HUVECs; lanes 6 and 7 of (a) represent the activity of a PAI-1 reporter gene construct lacking the 20 bp containing the NBRE with and without TNFα stimulation.

b, 4x NBRE reporter gene activity. Effect of TNFα stimulation and overexpression of Nur77 on activity of a reporter gene construct containing a 4x tandem repeat of a NBRE fused to a minimal promoter.

c, Immunocytochemistry. HUVEC were transiently transfected to express EGFP or dnNur77-EGFP. After treatment with TNFα for 4h cells expressing EGFP alone (green nuclear and cytoplasmatic signal) as well as untransfected cells produced large amounts of PAI-1 protein (red), whereas cells expressing dnNur77 (green, nuclear localization) showed no expression of PAI-1.

**Nur77 is present in atherosclerotic vessels and co-localizes with PAI-1**

Having shown that TNFα upregulates Nur77 and in turn PAI-1 expression in ECs, we were interested to see whether Nur77 is also upregulated in atherosclerotic vessels in vivo. When normal or atherosclerotic coronary arteries were stained for Nur77 and PAI-1, a strong Nur77 signal was seen only in the plaque area (in SMCs, macrophages and ECs – stained with antibodies against CD-31, Fig. 5g, of the neointima and vessels of the adventitia). In these areas, PAI-1 staining co-localized with Nur77 staining (Fig. 5a-j). PAI-1 was additionally seen in the extracellular matrix especially of the plaque (Fig. 5d and 5f). Nur77 was also strongly upregulated in the neointima (Fig.5l) while in the media only a scattered staining in SMCs was seen (Fig. 5m). This pattern of Nur77 staining is consistent with a model in which inflammatory cells in the neointima release inflammatory cytokines, which in turn induces Nur77 in SMC of the neointima and the
media. Our finding that inflammatory cytokines induce Nur77 mRNA in cultured SMC (not shown) supports this model.
Fig.5 Nur77 and PAI-1 expression in normal and atherosclerotic vascular tissues

*a-d*, Minimal expression for Nur77 (*a*) and PAI-1 (*b*) in normal vessels; high expression of both proteins in the plaque area in endothelial cells and smooth muscle cells in an atherosclerotic coronary artery (*c, d*).

*e-g*, Co-immunostaining of Nur77 and PAI-1 in a human atherosclerotic lesion. The vessel was stained for Nur77 (green, *e*) or PAI-1 (red, *f*) or CD31 (blue, *g*).

*h-j*, Endothelial cells of vasa vasora in human atherosclerotic coronary arteries express both Nur77 (*h*) and PAI-1 (*j*) which co-localize (*i*).

*k-m*, Expression of Nur77 in a human atherosclerotic vessel (*k*) with higher expression in the neointima (*l*) and lower expression in the media (*m*).

Discussion

Upregulation of PAI-1 during inflammation is known since long and in fact, the deleterious outcome of sepsis was for some time thought to be caused by the extremely high levels of PAI-1 found under these conditions\(^{48}\). However, the PAI-1 promoter is lacking consensus sequences for the inflammatory transcription factor NF-κB\(^ {49}\), and even binding sites for other transcription factors implicated in the inflammatory pathways such as EGR-1\(^ {28,29}\) are missing. Therefore, several indirect mechanisms have been implicated in PAI-1 induction during inflammation\(^ {38,50,51}\). TNFα efficiently induced de novo synthesis of PAI-1 mRNA in endothelial cells and induced an approximately 10-fold increase in PAI-1 mRNA levels after 4h of treatment. Stimulation of the PAI-1 promoter reporter gene construct used here by the same stimulus was only weak. A similar weak stimulation of PAI-1 promoter constructs was also reported by others\(^ {38}\) earlier, and the
differences between transcriptional activity on the PAI-1 promoter construct and induced PAI-1 mRNA levels might be inherent to the methodologies used. However, a contribution of a regulatory element present upstream from the promoter constructs used as well as additional PAI-1 mRNA stability induced by the inflammatory mediators cannot be excluded. Independent of such additional mechanisms, we here present a novel regulatory element by which TNFα and other inflammatory stimuli can activate PAI-1 expression in endothelial cells.

We could define a consensus sequence in the proximal part of the PAI-1 promoter for the orphan nuclear receptor Nur77 that drives basal transcription and is crucial for upregulation of PAI-1 in EC in response to the inflammatory cytokine TNFα. This consensus sequence mediates monomeric binding of Nur77. Such monomeric binding is compatible with a ligand independent mechanism and consistent with regulation of PAI-1 expression by changes in the levels of Nur77 expression, which then functions as a "constitutive orphan steroid receptor". Consistent with such a mechanism, we found that TNFα and other inflammatory stimuli induce transcriptional upregulation of Nur77: Upon stimulation with TNFα, Nur77 mRNA and in turn protein is induced within 30 and 60 minutes, respectively; Nur77 is then translocated to the nucleus followed by PAI-1 mRNA and protein expression. Our data, showing that the PAI-1 response to TNFα is abolished in ECs transfected with a dominant negative form of Nur77, point toward a pivotal role of Nur77 for the PAI-1 response to TNFα and possibly other inflammatory stimuli which concomitantly upregulate Nur77 and PAI-1.

Our data demonstrating that Nur77 expression is increased in atherosclerotic vessels and co-localizes there with PAI-1 indicate the importance of this mechanism also in vivo.
Recent work by deVries et al.\textsuperscript{53} who showed that Nur77 is found induced in a screen for genes upregulated by pro-atherogenic cytokines in smooth muscle cells, supports our finding, as well as a report by Arkenbout et al.\textsuperscript{54}, demonstrating the involvement of the nuclear receptor subfamily 4 in neointima formation in a mouse model. Nur77 (NAK-1, TR3) is a member of this nuclear receptor subfamily and the human homolog\textsuperscript{55, 56} of mouse Nur77 and rat NGFI-B. Nur77 was shown to be involved in T-cell receptor induced apoptosis\textsuperscript{42, 57}, and to be induced during B-cell differentiation\textsuperscript{58}, in response to growth factors\textsuperscript{59}, to mechanical stress\textsuperscript{60} and to dietary fatty acids\textsuperscript{61}. We here show for the first time that the transcription factor Nur77 is part of the TNF\(\alpha\) response and mediates TNF\(\alpha\) induced PAI-1 upregulation.

In conclusion, we have delineated the mechanism by which TNF\(\alpha\) induces PAI-1 expression in endothelial cells and identified Nur77 as the responsible transcription factor. Increased expression of Nur77 and PAI-1 in vascular cells of atherosclerotic tissues indicates that Nur77 might be a pivotal response gene for inflamed vascular cells and a possible target for therapeutic intervention.

References


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