Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1α, and C/EBPα

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ABSTRACT **Hypoxia, as occurs during tissue ischemia, tips the natural anticoagulant/procoagulant balance of the endovascular wall to favor activation of coagulation. Plasminogen activator inhibitor-1 (PAI-1) is an important factor suppressing fibrinolysis under conditions of low oxygen tension. We previously reported that hypoxia induced PAI-1 mRNA and antigen expression in murine macrophages secondary to increased *de novo* transcription as well as increased mRNA stability. We now show in RAW264.7 murine macrophages that the transcription factors early growth response gene-1 (Egr-1), hypoxia-inducible factor-1α (HIF-1α), and CCAAT/enhancer binding protein α (C/EBPα) are quickly activated in hypoxia and are responsible for transcription and expression of PAI-1. Murine PAI-1 promoter constructs, including Egr, HIF-1α, and/or C/EBPα binding sites, were transfected into RAW 264.7 murine macrophages. To identify the relative importance of each of these putative hypoxia-responsive elements, cells were exposed to normobaric hypoxia, and transcriptional activity was recorded. At 16 h of hypoxic exposure, murine PAI-1 promoter deletion constructs that included Egr, HIF-1α, and/or C/EBPα binding sites demonstrated increased transcriptional activity. Mutation of each of these three murine PAI-1 promoter sites (or a combination of them) resulted in a marked reduction in hypoxia sensitivity as detected by transcriptional analysis. Functional data obtained using ^{32}P-labeled Egr, HIF-1α response element (HRE), and C/EBPα oligonucleotides revealed induction of DNA binding activity in nuclear extracts from hypoxic RAW cells, with supershift analysis confirming activation of Egr-1, HIF-1α, or C/EBPα. ChIP analysis confirmed the authenticity of these interactions as each of these transcription factors binds to chromatin under hypoxic conditions. Further, the induction of PAI-1 by Egr-1, HIF-1α, or C/EBPα was replicated in primary peritoneal macrophages. These data suggest that although HIF-1α appears to dominate the PAI-1 transcriptional response in hypoxia, Egr-1 and C/EBPα greatly augment this response and can do so independent of HIF-1α or each other. These studies are relevant to ischemic up-regulation of the PAI-1 gene and consequent accrual of microvascular thrombus under ischemic conditions.—Liao, H., Hyman, M. C., Lawrence, D. A., Pinsky, D. J. Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1α, and C/EBPα. *FASEB J.* 21, 935–949 (2007)**

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When the steady flow of blood to a vital tissue is interrupted, even transiently, tissue oxygen concentrations plummet. The lack of oxygen triggers a characteristic pattern of responses that occur at either the tissue level (such as vasomotion) or at the cellular level. When oxygen is scarce, the phenotype of the endovascular wall changes markedly. Normally, an anticoagulant surface, new proteins are expressed by cells of the endovascular wall that fundamentally alter its properties, resulting in a prothrombotic phenotype (1–3). A number of different cell types participate in this phenotypic shift, including endothelial cells (in which NO bioavailability and thrombomodulin levels decline), vascular smooth muscle cells (which are driven to express tissue factor), and mononuclear phagocytes (MPs) (3, 4), which express both tissue factor and plasminogen activator inhibitor-1 (PAI-1), thereby simultaneously triggering formation of fibrin and retarding its degradation. Because oxygen scarcity also drives expression of chemotactic factors and expression of leukocyte adhesion receptors, MPs accumulate in ischemic vessels and tissue, thereby amplifying the cycle leading to thrombus accretion. Given their key roles in hypoxia-driven thrombosis, it is essential to understand operative mechanisms in MPs that contribute to their prothrombtic phenotypic shift (5).

Plasminogen activator/inhibitor-1 (PAI-1), a 50 kDa protein that is the main circulating inhibitor of plasminogen activation (and therefore the chief inhibitor of fibrinolysis), is strongly expressed by MPs exposed *in vitro* to pO_2s of 14–16 Torr. The importance of PAI-1 induction by hypoxia in the development of intravascular thrombosis is underscored by the observation that fibrin accrual increases markedly in mice placed in a

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normobaric hypoxic environment and that fibrin accrual is substantially diminished in hypoxia-exposed PAI-1 gene null mice (5). It appears that the predominant mechanism by which hypoxia leads to PAI-1 induction is transcriptionally driven, as nuclear run-on analysis of hypoxic MPs reveals a *de novo* increase in nascent PAI-1 mRNA (5). Although the PAI-1 promoter has a recognized HIF-1α motif (6–9), the importance of this motif in driving hypoxia-induced expression of PAI-1 in hypoxic MPs is not known (10, 11). Furthermore, the relative contribution of other HIF-1α-independent, hypoxia-responsive elements in the PAI-1 promoter, such as early growth response-1 (Egr-1), is unknown. Egr-1 is an immediate-early gene that encodes a nuclear phosphoprotein containing three zinc finger elements; these bind target GC-rich elements with a consensus sequence of 5′-GCG(T/G)GGGCG-3′ in the promoter of many different genes, thereby regulating transcription (12–16). PAI-1 is one such gene that has an Egr-1 site in its promoter region. Others included the hypoxia-responsive genes tissue factor, various growth factors, cytokines/chemokines, and adhesion receptors. Even vascular endothelial growth factor (VEGF), a classical HIF-1α responsive gene, has hypoxia-responsive Egr-1 elements in its promoter region (17). Egr-1 is rapidly activated and expressed in hypoxia, both *in vitro* and *in vivo*, and is responsible for transcription and expression of a diverse array of effector genes in hypoxic/ischemic lungs (18–25).

Hypoxia-inducible factor 1 (HIF-1) is another DNA binding protein comprised of a heterodimer between two basic helix-loop-helix transcription factors: HIF-1α and the ARNT (aryl hydrocarbon receptor nuclear translocator) (26). When cells are exposed to oxygen concentration considered to be normal (normoxia), newly synthesized HIF-1α undergoes rapid degradation. This degradation is mediated by prolyl hydroxylases (27, 28), a family of dioxygenase enzymes that catalyze hydroxylation of conserved proline residues (Pro^{402} and Pro^{564}) in the critical oxygen-dependent degradation domain of HIF-1α (29). Hydroxylated HIF-1α is then recognized by von Hippel-Lindau protein (a component of the E3 ubiquitin ligase complex), which targets it for subsequent proteasomal degradation (30–32). In this manner, normal oxygen concentrations ensure tonic degradation of this key component of the active HIF-1 heterodimer, ensuring nonactivity at multiple gene promoter sites. As the activity of the prolyl hydroxylases depend on O_2 concentration, under prevailing hypoxic conditions, proline hydroxylation is inhibited, eliminating binding between HIF-1α and the von Hippel–Lindau protein. This causes HIF-1α to stabilize, reflected by increased half-life, accumulation, and migration to the nucleus, where the HIF-1α-ARNT complex interacts with hypoxia-responsive elements (HREs) in transcriptional regulatory regions of multiple target genes (33). Many of these HIF-1α-induced genes, such as those encoding for glycolytic enzymes, growth factors, and vasoactive peptides, share the common feature of blunting tissue damage in situations of oxygen scarcity.

CCAAT /enhancer binding proteins (C/EBPs) comprise a family of transcription factors that each contain a highly conserved, basic C-terminal leucine zipper that mediates dimerization and DNA binding. There are at least six known members of the C/EBP family that have been isolated and characterized so far, including C/EBP-α, -β, -γ, -δ, -ε, and -ζ (34). C/EBP is a basic region leucine zipper (bZIP) DNA binding protein that recognizes the symmetric sequences ATTGCGCAAT and ATGAGTCAT, respectively (35). Binding occurs between target gene promoter DNA and either C/EBP homodimers or heterodimers. Although each family member stimulates different portfolios of genes, the focus in the current work is on C/EBPα, which has been regarded mainly as a regulator of cell growth and proliferation (36–40), and perhaps of inflammation (41).

To investigate the mechanism by which hypoxia elicits PAI-1 induction, deletion constructs fused to the luciferase reporter gene were transiently expressed in RAW cells. Measurement of luciferase activities, as well as EMSA analysis of transcription factor binding sites and the relative effect of site-specific mutations, enable us to identify three functional regulatory elements—Egr-1, HIF-1α, and C/EBPα—in the murine PAI-1 promoter region (**Fig. 1*A***) that regulate the response to hypoxia.

MATERIALS AND METHODS

Cell culture and hypoxic treatment

RAW cells (a transformed murine macrophage cell line) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and grown in RPMI 1640 medium with 10% FCS (Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin (50 U/ml and 5 μg/ml, respectively). When RAW cells achieved confluence, experiments were performed by washing cells three times with serum-free medium, then placing them in either a standard cell culture incubator (37°C, 5% CO_2) or a similar incubator within an hypoxia chamber. This environmental chamber (BioSpherix, Redfield, NY, USA) provides a controlled temperature (37°C) and a humidified anoxic atmosphere, resulting in an oxygen tension in the culture medium of 14–18 Torr.

Isolation of a mouse PAI-1 genomic clone

A 1278 bp fragment containing 1155 bp of 5′-flanking (–1155) and 123 bp of the untranslated first exon (+123) of mouse PAI-1 was isolated using a polymerase chain reaction (PCR) on RAW cell's total genomic DNA. The primers (5′ primer, 5′-CCTAACTTCCATTCCCAACACCCACGAC-3′; 3′ primer, 5′-GCCTTGTGATTGGCTCTTGTTGGCTGTC-3′) were designed by analysis of the sequence of the mouse PAI-1 gene, 5′ flank (GenBank accession #M33961) (42). The 1279 bp insert was subcloned into pGEM-T vector (Promega, Madison, WI, USA) using the pGEM-T Easy Vector System (Promega), and sequenced in both directions to confirm identity.

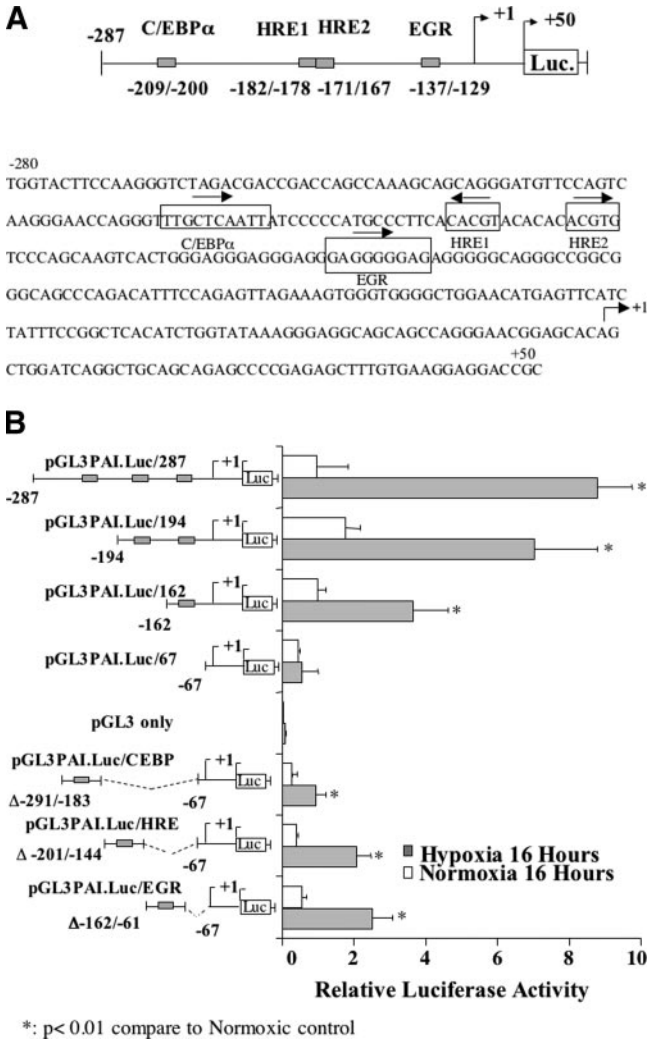

Figure 1. Hypoxia-inducible PAI-1 expression results from transcriptional activation at EGR, HIF-1α, and C/EBPα site-delete analysis of the PAI-1 promoter. (GenBank accession # M33961) (42). *A)* The 5′-flanking region of the mouse PAI-1 gene with its Egr, HRE, and C/EBPα binding sites. Transient cotransfection of RAW cells was performed using either pGL3PAI.Luc/287, pGL3PAI.Luc/201, pGL3PAI.Luc/166, or pGL3PAI.Luc/112, pGL3PAI.Luc/CEBP, pGL3PAI.Luc/HRE, pGL3PAI.Luc/EGR, and pCMV/β-galactosidase (Invitrogen). Cultures were transfected with each of the indicated constructs using the SuperFect procedure (Qiagen), then cells were exposed to normoxia or hypoxia (*B*). Luciferase activities were then determined with a luciferase reporter assay system (Promega). Relative firefly luciferase activity is normalized to control pCMV/β-galactosidase activity.

Construction of mouse PAI-1 promoter-luciferase reporter plasmids and site-directed mutagenesis

PCR was used to amplify mouse PAI-1 promoter-luciferase reporter constructs representing 5′-deleted PAI-1 upstream sequences, and these PCR products were subcloned into the pGL3 basic vector (Promega). In these PCRs, the PAI-1 genomic clone (described above) was used as a template. The 5′-deletion constructs of the PAI-1 promoter represent –287 to + 50 (pGLPAI.Luc/287), –194 to + 50 (pGLPAI.Luc/194), –162 to + 50 (pGLPAI.Luc/162), –67 to + 50 (pGLPAI.Luc/67), Δ-291 to –183 (pGLPAI.Luc/CEBP, containing C/EBPα binding sites only), Δ-201 to –144 (pGLPAI.Luc/HRE, containing HIF-1α binding sites only), and Δ-162 to –61 (pGLPAI.Luc/EGR, containing Egr binding sites only) (Fig. 1*B*). For mutants, oligonucleotides used for priming were synthesized based on sequences of the 5′-flanking region of the mouse PAI-1 gene. The site-directed mutants of the pGLPAI.Luc/Emut, pGLPAI.Luc/Hmut, pGLPAI.Luc/Cmut, GLPAI.Luc/EHmut, pGLPAI.Luc/ECmut, pGLPAI.Luc/HCmut, and pGLPAI.Luc/EHCmut constructs (**Fig. 2**) were generated using QuikChange site-directed mutagenesis kits (Stratagene, La Jolla, CA, USA). In these PCRs, the 5′-deletion constructs, representing –287/+50 (337 bp) of the PAI-1 gene, were used as a template. The mutated priming oligonucleotides represented overlapping sense and antisense sequences of the mutant site that amplified the entire pGL3 plasmid and insert. The mutated primer for each transcription factor binding site in the –287/+50 fragment are shown as follows (target regions are underlined and alterations are indicated by boldface):

EGR mutant primer for pGLPAI.Luc/Emut, GLPAI.Luc/EHmut GLPAI.Luc/ECmut, and pGLPAI.Luc/EHCmut
5′-GAGGGAGGG*AGGGATAGGGAG*AGGGGGCAGGG-3′;

HRE mutant primer for pGLPAI.Luc/Hmut, GLPAI.Luc/Ehmut GLPAI.Luc/Hcmut and pGLPAI.Luc/EHCmut 5′-CATGCCCTTCA*CAC**TAT**CA*CA*CAC**TAT**TCCC*AGCAAGTCA-3′;

C/EBP mutant primer for pGLPAI.Luc/Cmut, GLPAI.Luc/ECmut GLPAI.Luc/HCmut and pGLPAI.Luc/EHCmut 5′-GGGAACCAGAG*T**GACTAGTC**T*TATCCCCCATGCCCTT-CACACG-3′. Forward and reverse DNA sequencing of inserts

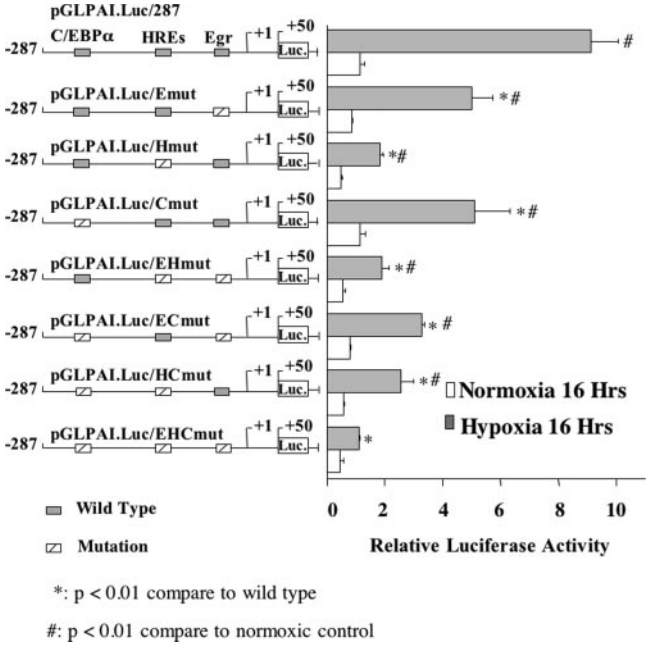

Figure 2. Mutational analysis of hypoxia-responsive elements of the PAI-1 promoter. Nucleotides sequence of the 5′-flanking region and 50 nucleotides of the first exon of the mouse PAI-1 gene. The transcription start site is denoted as + 1. A potential Egr-1, two HRE, and one C/EBPα binding sites are underlined. Transient transfection of RAW cells using pGL3PAI.Luc/287 (consensus Egr, HRE, and C/EBPα WT sequence) or mutationally inactivated sequence, pGLPAI.Luc/Emut (Egr), pGLPAI.Luc/Hmut (HIF-1α), pGLPAI.Luc/Cmut (C/EBPα), pGLPAI.Luc/Ehmut (Egr+HIF-1α), pGLPAI.Luc/Ecmut (Egr+C/EBPα), pGLPAI.Luc/Hcmut (HIF-1α, C/EBPα), and pGLPAI.Luc/EHCmut (Egr+HIF-1α+C/EBPα), and pCMV/β-galactosidase (Invitrogen) by the same procedure described in Fig. 1.

confirmed the sequences of all constructs employed for transfection assays.

Construction of mouse Egr-1, HIF-1α, and C/EBPα overexpression plasmids

The cDNA clones of mouse Egr-1, HIF-1α, and C/EBPα were ordered from ATCC (Manassas, VA, USA). Egr-1 expression vector (pEgr-1), HIF-1α expression vector (pHIF-1α), and C/EBPα expression vector (pC/EBPα) were constructed by first using PCR to amplify the open reading frame (ORF) of the Egr-1, HIF-1α, and C/EBPα cDNAs. The ORF of Egr-1 and HIF-1α cDNA was cloned into pcDNA3.1 (Invitrogen) expression vectors. The ORF of C/EBPα cDNA was cloned into pMT/V5-His-TOPO (Invitrogen) heavy metal (zinc)-inducible expression vector.

Transient transfections

RAW cells were cotransfected using SuperFect Transfection Reagent (Qiagen, Valencia, CA, USA). The day before transfection, RAW cells were plated on 12-well plates (Costar, Corning, NY, USA) with 1×10^5 cells per well and grown in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FCS (Invitrogen, Carlsbad, CA, USA). Cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until they reached 50–80% confluence. The cells were cotransfected using 0.75 μg of PAI-1 promoter-pGL3 reporter plasmid and 0.75 μg of control plasmid, pCMV/β-galactosidase, which served as an internal control to normalize transfection efficiency, as well as 7.5 μl of SuperFect Transfection Reagent in 75 μl of serum-free RPMI 1640 medium. This was followed by incubation at room temperature for 10 min, after which the complexes were mixed with 0.4 ml of RPMI 1640 medium with 10% FBS. After incubating at 37°C and 5% CO_2 for 3 h, the cells were washed three times with HBSS (Invitrogen), then incubated with RPMI with 10% FBS. After 30 h incubation, the cell medium was changed to RPMI 1640, then the cells were placed in either a standard cell culture incubator (37°C, 5% CO_2) or a similar incubator within the hypoxia chamber for 16 h. Luciferase reporter assays were performed using a Luciferase reporter assay system (Promega). Transfected cells were washed twice with cold PBS, then harvested, lysed, and assayed for luciferase activity by a VICTOR LIGHT luminometer (Perkin-Elmer, Boston, MA, USA). β-Galactosidase enzyme activity was measured using β-galactosidase enzyme assay system (Promega). Fifty microliters of the sample was mixed with 50 μl of 2× buffer containing 200 mM of sodium phosphate pH 7.3, 2 mM of $MgCl_2$, 100 mM of β-mercaptoethanol, and 1.33 mg/ml of *o*-nitrophenyl-β-galactopyranoside in a 96-well plate. After incubation for 1 h at 37°C, the absorbances of the samples were read at 420 nm in the VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

For the overexpression experiments, RAW cells were cotransfected with 0.25 μg of luciferase reporter plasmid (pGL3PAI.Luc/287 or pGL3 basic control vector), 0.5 μg of pCMV/β-galactosidase vector, and one, two, or all three overexpression constructs (pEgr-1, pHIF-1α, and/or pC/EBPα, 0.25 μg/each construct). In these experiments the total transfected DNA was 1.5 μg/well consisting of the overexpression vectors (0.25 μg/vector), the balance being comprised of control vectors (pcDNA3.1 for pEgr-1 and pHIF-1α, pMT for pC/EBPα) as needed (see Fig. 10). The method of transfection and report assay is described above.

Murine peritoneal macrophage isolation and transfection

Peritoneal macrophages were isolated as described previously (43). In brief, 10-wk-old male C57Bl6/J mice were injected with 1.0 ml of a 5% thioglycollate solution (BD, Franklin Lakes, NJ, USA) i.p.. Four days later macrophages were isolated by peritoneal lavage in ice-cold PBS. Red blood cells were then lysed using a 0.15 M NH^4Cl lysing solution before plating at a concentration of 1×10^6 cells per well in 12-well plates. After 24 h, the cells were transfected with 0.5 μg/well of overexpression plasmid (pEgr-1, pHIF-1α, or pCEBPα) and 1.5 μl/well of Fugene 6 Transfection Reagent (Roche, Indianapolis, IN, USA). While incubating the transfected cells in hypoxia or normoxia, cells transfected with pC/EBPα or pMT were simultaneously incubated with 50 μM of $ZnCl_2$ to induce C/EBPα protein expression. Total RNA was then isolated from the transfected murine peritoneal macrophage cells using Total RNA Easy Kits (Qiagen); RT-qPCR was used to assay levels of PAI-1, Egr-1, HIF-1α, and C/EBPα mRNA.

Quantitative RT-PCR

RAW cell were transfected with the overexpression constructs (pEgr-1, pHIF-1α, or pCEBPα) to induce Egr-1, HIF-1α, or C/EBPα protein expression, respectively. The empty vectors (pCDNA3.1 for pEgr-1 and pHIF-1α or pMT vector for pC/EBPα) were used as control. RAW cells were plated onto 6-well plates (Costar, Corning, NY, USA). After RAW cells reached 80% confluence, the cells were transfected using 2 μg of expression or control vectors and 10 μl of Superfect Transfection Reagent (Qiagen). After 24 h of transfection, the cells were incubated in hypoxic or normoxic conditions for the indicated durations. Total RNA was then isolated from the transfected RAW cells by using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and RT-qPCR was used to assay level of PAI-1, Egr-1, HIF-1α, and C/EBPα mRNA. Real-time PCR assay was carried out with the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using One-Step RT-polymerase chain reaction (RT-PCR) Master Mix (Applied Biosystems) according to the manufacturer's instruction. The primers and probes sets were purchased from Applied Biosystems. All RT-qPCRs were done in triplicate. Hypoxic up-regulation of PAI-1, Egr-1, HIF-1α, C/EBPα mRNA was normalized to β-actin levels and reported as relative fold increase compared with normoxia, determined by the $2^{-\Delta\Delta Ct}$ method (44, 45).

Gel shift assay

Nuclear extracts were prepared by the procedure of Dignam *et al.* (46) from RAW cells after exposure to hypoxic or normoxic (control) conditions. Complementary reverse orientation oligonucleotide probes were synthesized by Invitrogen and annealed by suspending in TE buffer, heating to 90°C for 5 min, followed by cooling at room temperature for 1 h. Strand sequences were as follows:

EGR: (–151/–127) 5′-GGGAGGGAGGGAGGGAGGGGGAGAG-3,

5′-CTCTCCCCCTCCCTCCCTCCCTCCC-3′

HRE 1&2:(–189/–151) 5′-CCCTTCACACGTACACACACGTGTCCCAGCAAGTCACTG-3′

5′-CAGTGACTTGCTGGGACACGTGTGTGTACGTGTGAAGGG-3′

C/EBPα: (–220/–193), 5′-GGAACCAGGGTTTGCTCAATTATCCCCC-3′,

5′-GGGGGATAATTGAGCAAACCCTGGTTCC-3′.

In each case, double-stranded oligonucleotide probes were 5′ end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham, Arlington Heights, IL, USA) by using T4 polynucleotide kinase (Promega). The labeled probe was incubated with 10 μg of RAW cell nuclear extract at room temperature for 30 min (for Egr-1 and C/EBPα) and at 4°C for Hif-1α. For

competition studies, a 100-fold molar excess of one of the above unlabeled double-stranded probes was employed. Samples were loaded onto nondenaturing (6%) polyacrylamide gels that had been subjected to an electrophoretic voltage (100 V) for 20 min prior to loading. Electrophoresis was performed for 1.5–2 h at 200V at room temperature (for Egr-1 and C/EBPα) and at 4°C for Hif-1α (because of the lability of Hif-1α). The gels were dried and subjected to autoradiography overnight at −80°C. Gel supershifts were performed as described above, except that before incubation of oligonucleotide probe with nuclear extracts, 3 μl of anti-EGR-1, C/EBPα SuperShift antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-HIF-1α IgG (Novus, Littleton, CO, USA), was added to the nuclear extract and incubated for 30 min at room temperature (for Egr-1 and C/EBPα) and at 4°C for HIF-1α.

Western blot assay

Fifty micrograms of nuclear protein (prepared as described above) was added to 4× sample buffer and 10× reducing agent (Invitrogen, Carlsbad, CA, USA), boiled for 3 min, separated by 10% SDS-PAGE, and electrophoretically transferred onto nitrocellulose membranes (Invitrogen). The membranes were incubated with anti-Egr-1 antibody, anti-C/EBPα (Santa Cruz Biotechnology), or anti-HIF-1α (Novus, Littleton, CO, USA) and autoradiographed using the enhanced chemiluminescence (ECL) method (ECL detection system, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Chromatin imunoprecipitation (ChIP) assay

The ChIP assay was performed using an EZ ChIP™ Chromatin Immunoprecipitation Kit (Upstate Biotechnology, Charlottesville, VA, USA). RAW cells were cross-linked at 37°C for 10 min in 1% formaldehyde. Cells were then sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) 10 times for 15 pulses at 30% output at 4°C. The supernatant was divided into three tubes for subsequent immunoprecipitation. Samples were precleared in i.p. buffer (0.01% SDS, 1.1% Triton, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) and added to protein A Sepharose beads. The mixture was placed on a rotator for 1 h at 4°C, after which beads were spun out and discarded (this step was to clear the mixture of excess IgG). To the supernatant, 5 μg of anti-Egr-1, HIF1α, C/EBPα antibody (Santa Cruz), or rabbit IgG was then added and the mixture was placed on a rotator for 16 h at 4°C. Immunoprecipitates were recovered by adding protein A Sepharose beads that were washed for 5 min with low salt immune complex wash buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 8.1, and 150 mM NaCl) for 5 min with high-salt immune complex wash buffer (0.1% SDS, 1% Triton, 2 mM EDTA, 20 mM Tris pH 8.1, and 500 mM NaCl), 5 min with LiCl immune complex wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% nadeoxycholate, 1 mM EDTA, and 10 mM Tris pH 8.1), and twice for 3 min with TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA pH8.0). Immune complexes were eluted during rotation with 1% SDS and 0.1 M $NaHCO_3$ for a total of 30 min at room temperature. Immunoprecipitates and inputs were reverse cross-linked with 200 mM NaCl at 65°C for 4 h. Samples were then incubated 1 h at 45°C with 10 mM EDTA, 40 mM Tris pH 6.5, and 40 μg/ml proteinase K. DNA was extracted by phenol/chloroform extraction and ethanol precipitation. DNA was amplified by PCR with a temperature cycle of 30 s at 94°C, 30 s at 52°C, and 1 min at 68°C for 35 cycles. Primers used for PCR correspond to the mouse PAI-1 promoter region (–280 to +123, 5′ primer: 5′-CCAGGGTTTGCTCAATTATC-3′; 3′ primer: 5′-GCCTTGTGATTGGCTCTTGTTGGCTGTC-3′).

Statistics

Statistical analysis was performed using ANOVA to detect differences between groups, using Statview® software. Differences were considered significant if $P < 0.05$.

RESULTS

When mononuclear phagocytes are placed in an environment in which oxygen is scarce, a number of gene programs are activated that result in the expression of new proteins that are either pathological or adapative, depending on the context. One such protein of critical importance to hemostasis and wound healing is plasminogen activator/inhibitor-1 (PAI-1), whose expression increases 10-fold in RAW macrophages placed in an oxygen-scarce environment (~16 Torr).

We previously reported (5, 47) that hypoxia induces mouse PAI-1 mRNA and protein expression in the RAW macrophage cell line. Hypoxia induces an 11-fold induction of PAI-1 transcripts due to both increased *de novo* transcription and increased mRNA stability compared with normoxic PAI-$1^{+/+}$ controls.

To characterize the mechanism by which PAI-1 transcription in RAW cells is up-regulated by hypoxia, we cloned a 337 bp genomic fragment that contains 287 bp of 5′-flanking region upstream of the mouse PAI-1 gene (Fig. 1*A*). To delineate promoter elements that regulate PAI-1 transcription and may be responsive to hypoxia, a series of luciferase reporter gene constructs was generated in a pGL3 basic vector that represents a 5′ deletion of the 5′-flanking region of the PAI-1 gene (Fig. 1*B*). The resulting chimeric constructs (termed pGL3PAI.Luc/287, pGL3PAI.Luc/194, pGL3PAI.Luc/162, and pGL3PAI.Luc/67) were transfected into RAW264.7 cells to measure their transcriptional activities. When exposed to hypoxia for 16 h, RAW cells transfected with pGL3PAI.Luc/162, pGL3PAI.Luc/194 and pGL3PAI.Luc/287 plasmids demonstrated increased transcriptional activity of the PAI-1 promoter (3.64-fold, $P<0.001$; 7.04-fold, $P<0.001$; and 9.54-fold, $P<0.001$, respectively, compared with normoxic controls) (Fig. 1*B*). In contrast, hypoxia did not induce luciferase activity in RAW cells transfected with pGL3PAI.Luc/67 plasmids compared with RAW cells transfected with the same plasmid but incubated in normoxic conditions. These data indicate that hypoxia controls PAI-1 transcription in RAW cells by its effects on a segment of the 5′-flanking DNA located between –287 and –67 upstream of the transcription start site of PAI-1.

To predict the hypoxia-activated transcription factors binding sites between –287 and –67 upstream of the transcription start site of the mouse PAI-1 gene, we searched for sequences similar to known transcription

factor binding sequences. This search revealed multiple potential transcription factor binding sequences, including an early growth response (Egr) binding site in the –137/–129 region and a CCAAT enhancer binding protein (C/EBP alpha) binding sites in the –209/–200 region of the PAI-1 promoter (Fig. 1*A*). Two potential HREs that might bind HIF-1 are present in the –182/–178 and –171/–167 region of the PAI-1 promoter. The first potential HIF-1 binding element (which we designated HRE-1) –182/–178 5′-*CACGT*ACA-3′matches the core HIF-1 binding sequence 5′-RCGTG-3′ (48) (with *R*=A/G) in 5 of 5 base pairs. The second potential HIF-1 binding element (designated HRE-2; –171/–167) 5′-C*ACGTG*TC-3′ also matches the HIF-1 consensus site in five of five base pairs (Fig. 1*A*). Each hypoxia-responsive motif has been implicated in hypoxic upregulation of other target genes under different conditions.

To determine whether the Egr, HRE1, HRE2, and C/EBPα binding sites are functionally important for inducing PAI-1 transcription by hypoxia, we generated reporter constructs that contained only the C/EBPα (pGLPAI.Luc/CEBP), HER1, and HER2 (pGLPAI.Luc/HRE) or EGR (pGLPAI.Luc/EGR) binding site (Fig. 1*B*). After exposure to hypoxia for 16 h the luciferase activity was increased in RAW cells transfected with pGLPAI.Luc/CEBP (3.39-fold, $P=0.0019$), pGLPAI.Luc/HRE (5.29-fold, $P<0.001$), and pGLPAI.Luc/EGR (4.44-fold, $P<0.001$) compared with normoxic controls (Fig. 1*B*). We also generated reporter constructs from the –287/+50 DNA segment that contained mutated Egr, HRE1, HRE2, and/or C/EBPα binding sites (Fig. 2). As a control, a wild-type (WT) 345 bp construct (pGLPAI.Luc/287) was used. After exposure to hypoxia for 16 h, the RAW cells transfected with mutant Egr site (pGLPAI.Luc/Emut), mutant HRE-1 and-2 sites (pGLPAI.Luc/Hmut), or mutant C/EBPα site (pGLPAI.Luc/Cmut) constructs elicited a 48% ($P<0.001$), 80% ($P<0.001$), or 44% ($P<0.001$) decrease in transcriptional activity, respectively, compared with WT constructs. RAW cells transfected with the double mutant Egr and C/EBPα [construct designated pGLPAI.Luc/ECmut], triple mutant Egr, HRE-1, and HRE-2 [construct designated pGLPAI.Luc/EHmut], or triple mutant C/EBPα and, HRE-1 and HRE-2 [construct designated pGLPAI.Luc/CHmut] reduced luciferase activity 64% ($P<0.001$), 79% ($P<0.001$), or 72% ($P<0.001$) compared with WT controls. RAW cells transfected with mutant constructs at each of the four sites (Egr, C/EBPα, HRE-1, and HRE-2) [construct designated pGLPAI.Luc/EHCmut] demonstrated an 88% reduction of luciferase activity ($P<0.001$ compared with WT controls; Fig. 2). Note that when all of these sites were mutated, the hypoxic response was completely lost. These data suggest that all of the Egr, HRE-1, HRE-2, and C/EBPα binding sites within the PAI-1 promoter play important roles in the transcriptional activation of PAI-1 expression by hypoxia.

To further characterize the functional significance of the Egr, HRE-1, HRE-2, and C/EBPα binding sites and the transcription factors that bind to them, EMSAs were performed on nuclear extracts from RAW cells exposed to hypoxia or normoxia. For these studies, synthetic 26-mer Egr (–151/–127), 39-mer Hif-1α (–189/–151), and 27-mer C/EBPα (–220/–193) probes were constructed based on the nucleotide sequence from the –287/+50 construct we had cloned from the 5′-flanking region of the murine PAI-1 gene (sequences for these probes are found in Materials and Methods). Binding of nuclear proteins to the various promoter elements was evaluated under hypoxic and normoxic conditions.

Egr-1 binds to the mPAI-1 promoter

Nuclear proteins extracted from RAW cells cultured under hypoxia or normoxia for 30 min, 45 min, or 1 h were incubated with ^{32}P-labeled Egr probe for 30 min at room temperature; 45 min hypoxic exposure induced at least a 5-fold increase in the intensity of a band corresponding to retardation of the labeled Egr probe compared with normoxic control. The intensity of the bands corresponding to retardation of Egr probe was less strongly induced in RAW cell nuclear extracts exposed to hypoxia for 1 or 2 h (**Fig. 3*A***, lanes 3–7). Competition experiments performed by preincubation with excess unlabeled probe confirmed the specificity of the radiolabeled Egr probe because the signal was completely lost. Addition of antibody against murine Egr-1 to the nuclear extract resulted in further retarded migration of the radiolabeled band. This supershifting confirms identity of the protein that interacts with the DNA as being Egr-1 (Fig. 3*A*). Western blot analysis of nuclear Egr-1 protein levels reveals a time course that closely matches that of the DNA binding activity seen with EMSA (Fig. 3*B*).

HIF-1α binds to the mPAI-1 promoter

Nuclear proteins were extracted from RAW cells cultured under 1% O_2 or normoxia for 1, 2, 6, or 16 h. The extracts were incubated for 30 min at 4°C with ^{32}P-labeled HRE-1/2 probe, which encompassed both the HRE-1 and HRE-2 sites (**Fig. 4*A***). The onset of HIF-1α HRE-1/2 probe binding is readily apparent 1 h after placing cells in 1% oxygen. In this time course experiment, binding of the HRE-1/2 probe is greatest after 6 h of 1% oxygen exposure (Fig. 4*A*). Competition experiments performed by preincubation with excess unlabeled probe confirmed the specificity of the radiolabeled HRE-1/2 probe, because the signal was completely lost. Addition of antibody against murine HIF-1α to the nuclear extract resulted in further retarded migration of the radiolabeled band. This supershifting confirms identity of the protein that interacts with the DNA as being HIF-1α (Fig. 4*A*). Western blot analysis of nuclear HIF-1α protein levels reveals a time course that closely matches that of the DNA binding activity seen with EMSA (Fig. 4*B*).

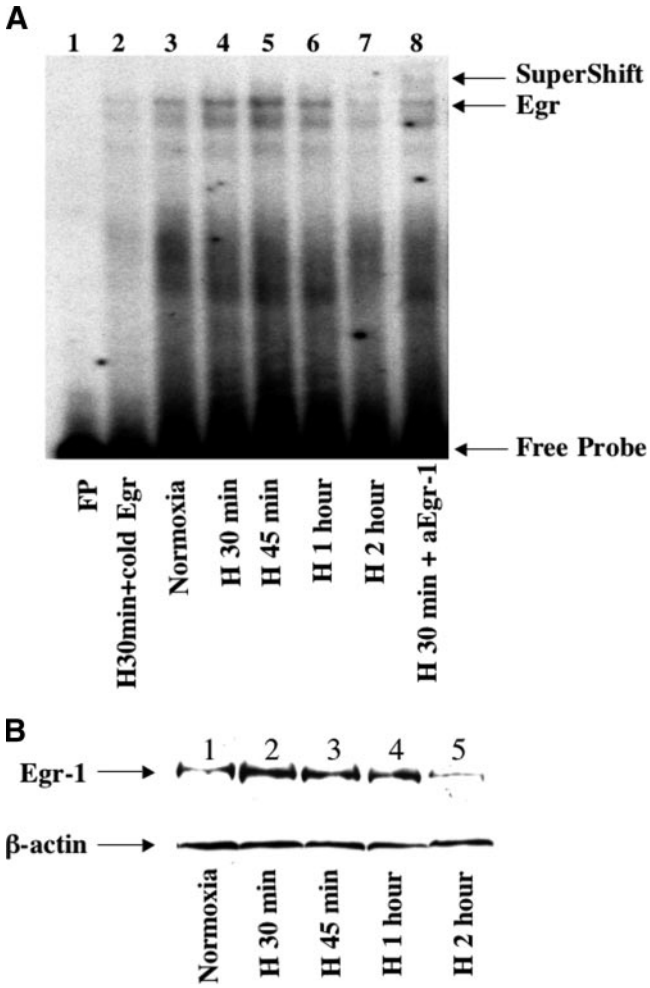

Figure 3. Effect of hypoxia on Egr-1 DNA binding and protein expression[b]. *A)* EMSA of the Egr-1 binding to the PAI-1 promoter. RAW cells were exposed to normoxia (lane 3), hypoxia (H) for 30 min, 45 min, 1 h, or 2 h (lanes 4, 5, 6, or 7 respectively). Nuclear extracts were prepared and EMSA was performed using ^{32}P-labeled Egr-1 probe (–151/–127 of PAI-1 promoter). Each lane received 10 μg/lane of total nuclear extract protein. A 100-fold excess of the indicated unlabeled (cold) Egr-1 probe was added (lane 2). For supershift analysis, anti-Egr-1 IgG (lane 8) was added. *B)* Western blot analysis was performed on nuclear extracts from RAW cells exposed to normoxia (lane 1) or hypoxia for 30 min, 45 min, 1 h, or 2 h (lanes 2, 3, 4, or 5, respectively).

C/EBPα binds to the mPAI-1 promoter

To demonstrate a functional effect of C/EBPα on mPAI-1 promoter activity, we performed EMSA using RAW cell nuclear extracts incubated with ^{32}P-labeled double-stranded oligonucleotide consisting of a C/EBP probe, whose sequence encompasses a putative C/EBP binding site in the 5′-flanking region of the mouse PAI-1 gene (see Materials and Methods for sequence). Compared with normoxia, higher C/EBP binding activity was detected by EMSA in RAW cell nuclear extracts (**Fig. 5*A***). Labeled C/EBP probe showed a higher level of binding activity with nuclear extracts from RAW cells that had been subjected to a hypoxic environment for 1, 2, 6, or 16 h compared with comparable nuclear extracts from RAW cell kept in a normoxic environment for the same period. The DNA binding was specific since it was efficiently suppressed by the addition of 100-fold molar excess of the unlabeled WT C/EBP probe. Preincubation of nuclear extracts with specific antibodies to C/EBPα resulted in partial supershifting of binding complexes (Fig. 5*A*). These data suggest that the C/EBP probe binds specifically to activated C/EBPα. Western blot analysis of nuclear C/EBPα protein levels reveals similar hypoxia-driven increases C/EBPα as seen in the EMSA experiments (Fig. 5*B*).

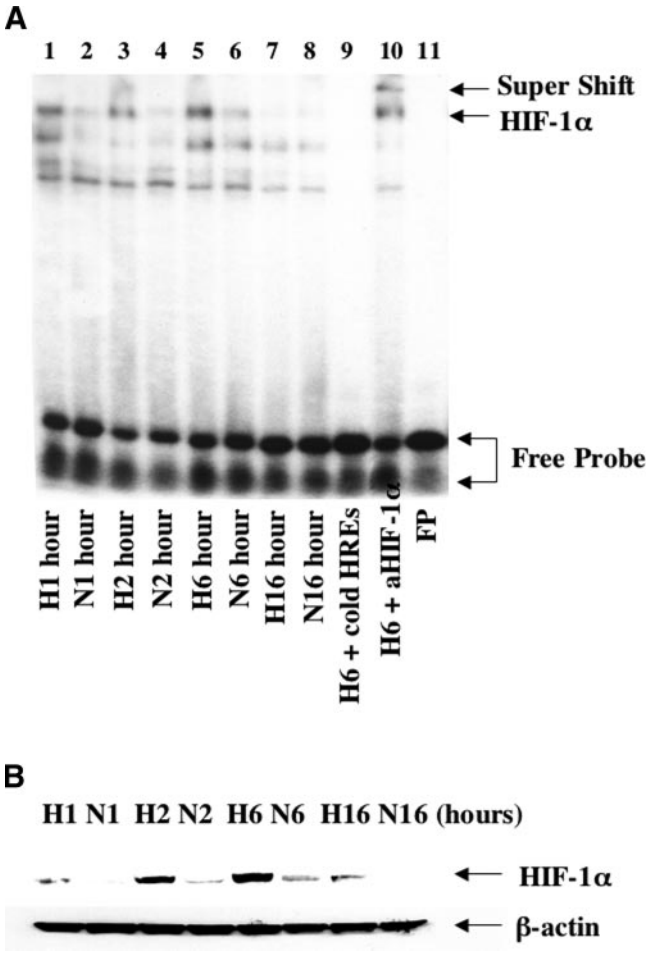

Figure 4. Effect of hypoxia on HIF-1α DNA binding and protein expression. *A)* Effect of hypoxia on the PAI-1 promoter HRE binding motifs. RAW cells were exposed to normoxia (N) or hypoxia (H) for the indicated times (lanes 1–8). Nuclear extracts were prepared and EMSA was performed using ^{32}P-labeled HIF-1α probe (–189/–151 of PAI-1 promoter). Each lane received 10 μg/lane of total nuclear extract protein. A 100-fold excess of the indicated unlabeled (cold) HIF-1α probe was added (lane 9). For supershift analysis, anti-HIF-1α IgG (lane 10) was added. *B)* Western blot analyses were performed on nuclear extracts from RAW cells exposed to hypoxia or normoxia using the same time course as for the EMSA of HIF-1α.

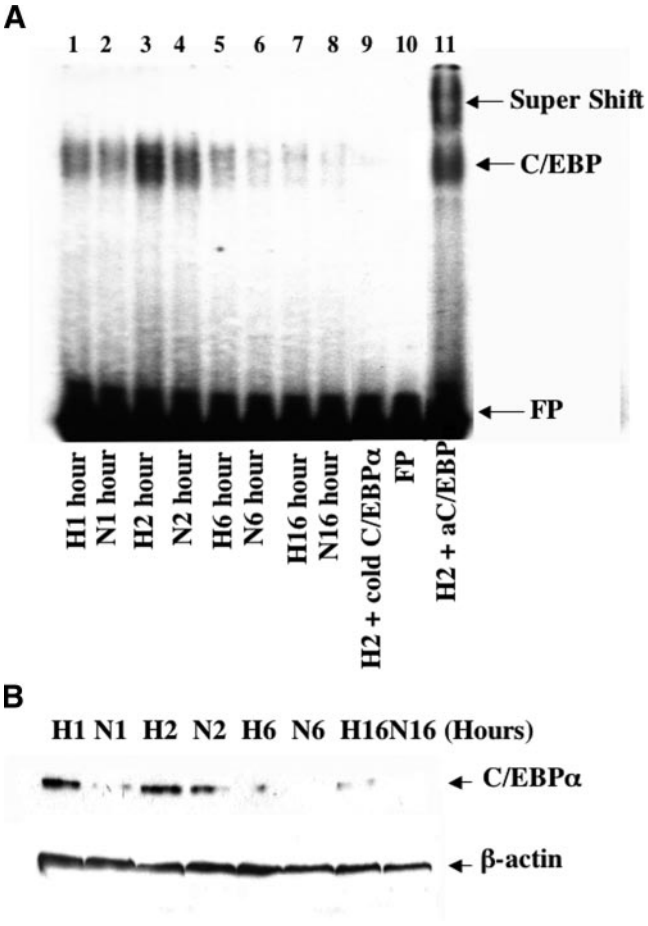

Figure 5. Effect of hypoxia on C/EBPα DNA binding and protein expression. *A)* Effect of hypoxia on the C/EBPα binding motif in the PAI-1 promoter. RAW cells were exposed to normoxia (N) or hypoxia (H) for the times indicated (lanes 1–8). Nuclear extracts were prepared and EMSA was performed using ^{32}P-labeled C/EBPα probe (–220/–193 of PAI-1 promoter). Each lane received 10 μg/lane of total nuclear extract protein. A 100-fold excess of the indicated unlabeled (cold) C/EBPα probe was added (lane 9). For supershift analysis, anti-C/EBPα IgG (lane 11) was added. *B)* Western blot analyses were performed on nuclear extracts of RAW cells exposed to hypoxia or normoxia for the same time course as for the EMSA of C/EBPα.

Egr-1, HIF-1α, and C/EBPα bind to the mPAI-1 promoter in chromatin

To demonstrate that Egr-1, HIF-1α, and C/EBPα can bind to the mouse PAI-1 promoter in a macrophage chromatin environment, chromatin immunoprecipitation assays were performed using RAW cells. RAW cells were incubated in hypoxia or normoxia for 40 min (for Egr-1), 2 h (for C/EBPα), or 6 h (for HIF-1α). Cross-linked RAW cell lysates were immunoprecipitated with anti-Egr-1, anti-HIF-1α, or anti-C/EBPα antibodies and PCR was used to amplify a 333 bp fragment of mouse PAI-1 promoter (–220 to +123). These data show that Egr-1, HIF-1α, and C/EBPα bind to the mouse PAI-1 promoter only in hypoxic RAW cell chromatin (**Fig. 6**, lane 7) but not that of normoxic cells (Fig. 6, lane 4). These results confirm the gel shift assay results and indicate that the transcriptional increase in PAI-1 seen in hypoxic RAW cells is associated with the interaction of Egr-1, HIF-1α, and C/EBPα with the PAI-1 promoter.

Effect of overexpression of Egr-1, HIF-1α, and C/EBPα on mPAI-1 expression in macrophages

Transient overexpression of Egr-1, HIF-1α, and C/EBPα was used to increase PAI-1 transcription in hypoxic RAW cells. The cells were transiently transfected with pEgr-1, pHIF-1α, pC/EBPα overexpression constructs or control vectors. While incubating the transfected cells in hypoxia or normoxia, cells transfected with pC/EBPα or pMT were simultaneously incubated with 50 μM of $ZnCl_2$ to induce C/EBPα protein expression. Total RNA was extracted, followed by reverse transcription, and quantitative PCR analysis was performed to evaluate the expression of PAI-1 and Egr-1, HIF-1α, or C/EBP mRNA (**Fig. 7**, **Fig. 8**, **Fig. 9**).

To determine the effect of Egr-1 overexpression on PAI-1 mRNA transcription, nontransfected control cells or those cells transfected with pEgr-1 or control vector were placed in hypoxia or normoxia for 2 h before PAI-1 mRNA analysis. The expression of PAI-1 mRNA was increased 2-fold in pEgr-1-transfected hypoxic cells compared with hypoxic cells transfected with control vector ($P<0.001$) or nontransfected controls ($P<0.001$) (Fig. 7*A*). PAI-1 mRNA expression also increased in normoxic pEgr-1-transfected cells compared with normoxic control vector-transfected cells (5-fold increased, $P<0.001$) and nontransfected cells (6-fold increased, $P<0.001$) (Fig. 7*B*). In pEgr-1-transfected cells, Egr-1 mRNA expression was increased > 1000-fold compared with controls ($P<0.001$) (Fig. 7*B*). These data show that *1)* transfection of the Egr-1 containing vector increases PAI-I-1 expression under all conditions, and *2)* hypoxia

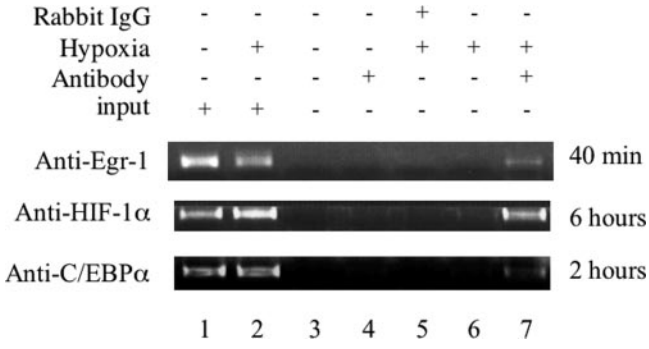

Figure 6. Chromatin immunoprecipitation of Egr-1, HIF1α, and C/EBPα interaction with PAI-1 promoter in RAW cells. Bands indicate PCR products targeting –220 to + 130 of PAI-1 promoter. RAW cells were exposed to hypoxia or normoxia for the time indicated. 5 μg of anti-Egr-1, anti-HIF-1α, or anti-C/EBPα antibodies (lane 7 for hypoxia, lane 4 for normoxia). 5 μg of rabbit IgG (lane 5 for hypoxia), no antibody (lane 6 for hypoxia, lane 3 for normoxia), and 1/100 of input (lane 2 for hypoxia, lane 1 for normoxia) were applied to the PCR reaction, respectively.

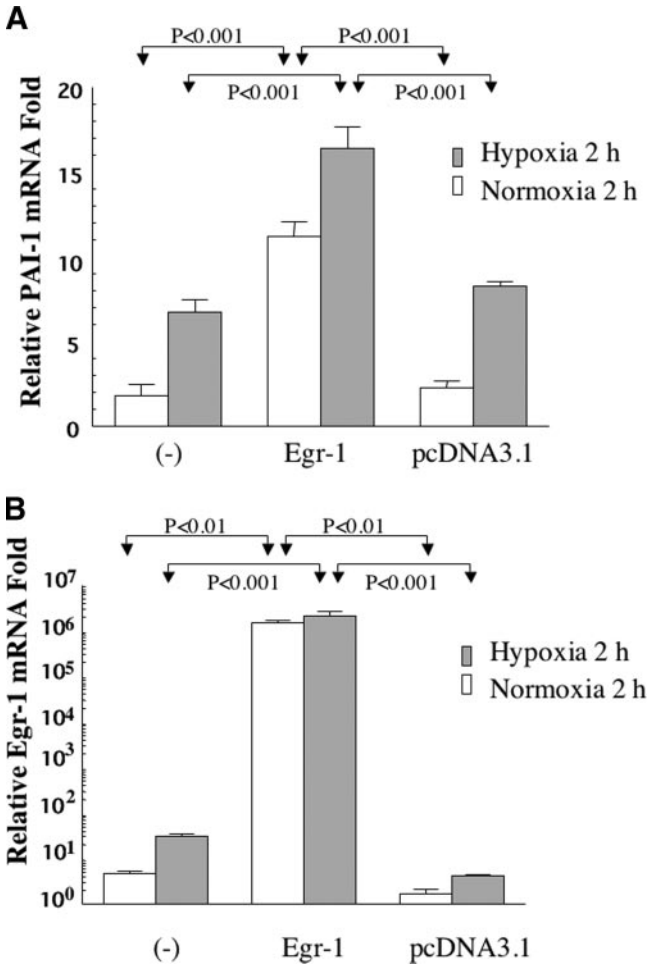

Figure 7. Effect of overexpression of Egr-1 on PAI-1 mRNA expression on RAW cells. RAW cells were transfected with Egr-1 overexpression plasmid or empty pcDNA3.1 vector. 24 h after transfection cells were exposed to hypoxia or normoxia for 40 min, then total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and Egr-1 mRNA (*B*) was measured.

augments PAI-1 expression in the Egr-1 transfectants more than the normoxic Egr-1 transfectants.

To determine the effect of HIF-1α overexpression on PAI-1 mRNA transcription, nontransfected control cells or those cells transfected with pHIF-1α or control vector were placed in hypoxia or normoxia for 6 h before PAI-1 mRNA analysis. The expression of PAI-1 mRNA was increased 48% in pHIF-1α-transfected hypoxic cells compared with hypoxic cells that were transfected only with contro1 vector ($P<0.05$) and nontransfected controls ($P<0.05$) (Fig. 8*A*). The HIF-1α mRNA expression in pHIF-1α-transfected cells was increased by >1000-fold compared with controls ($P<0.001$) (Fig. 8*B*). These data show that *1)* transfection of the HIF-1α-containing vector increases PAI-1 expression under all conditions, and *2)* hypoxia augments PAI-1 expression in the HIF-1α transfectants more than the normoxic HIF-1α transfectants. There is some increase in PAI-1 expression in the HIF-1α normoxic conditions, which may be due to the degradative pathways in the transfectants being overwhelmed.

To determine the effect of C/EBPα overexpression on PAI-1 mRNA transcription, nontransfected control cells or those cells transfected with pC/EBPα or control vector were placed in hypoxia or normoxia for 2 h (with 50 μM $ZnCl_2$ to induce expression of C/EBPα) before PAI-1 mRNA analysis. This resulted in an increase of mouse PAI-1 mRNA expression by 1.8-fold in pC/EBPα-transfected hypoxic cells compared with either hypoxic cells transfected with pMT (empty) vector ($P<0.001$) or nontransfected controls ($P<0.001$) (Fig. 9*A*). The PAI-1 mRNA expression also increased 3-fold in normoxic pC/EBPα-transfected cells compared with pMT vector-transfected cells ($P<0.001$) and 4-fold compared with nontransfected cells ($P<0.001$) (Fig. 9*A*). In pC/EBPα-transfected cells, C/EBPα mRNA expression was increased >1000-fold compared with controls ($P<0.001$) (Fig. 9*B*). These data show that *1)* transfection of the C/EBPα-containing vector increases PAI-1 expression under all conditions, and *2)* that hypoxia

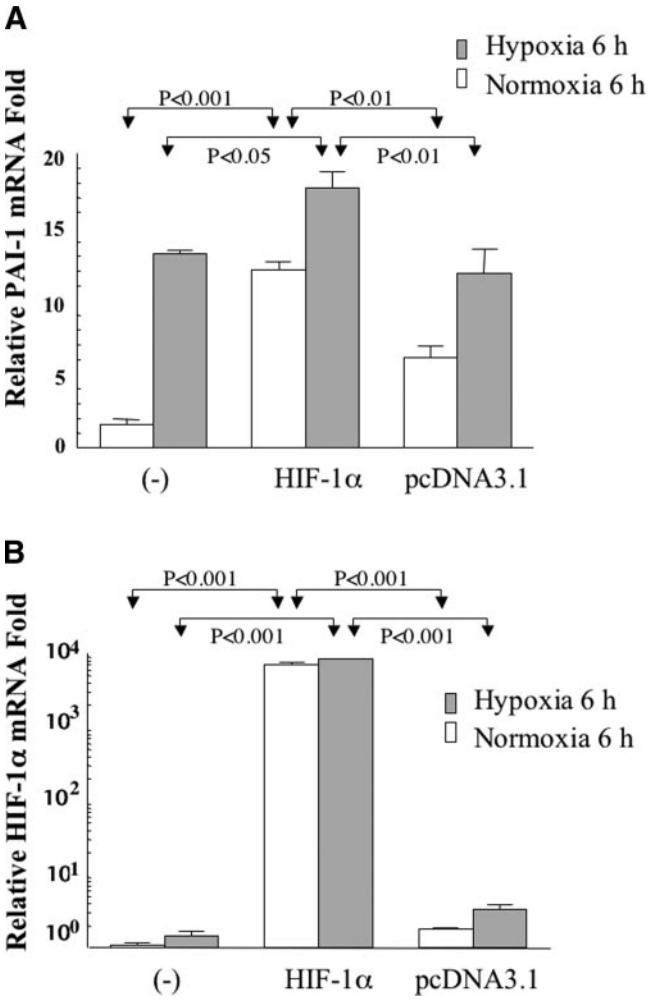

Figure 8. Effect of overexpression of HIF-1α on PAI-1 mRNA expression on RAW cells. RAW cells were transfected with HIF-1α overexpression plasmid or empty pcDNA3.1 vector. 24 h after transfection, the cells were exposed to hypoxia or normoxia for 6 h. Total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and HIF-1α mRNA (*B*) was measured.

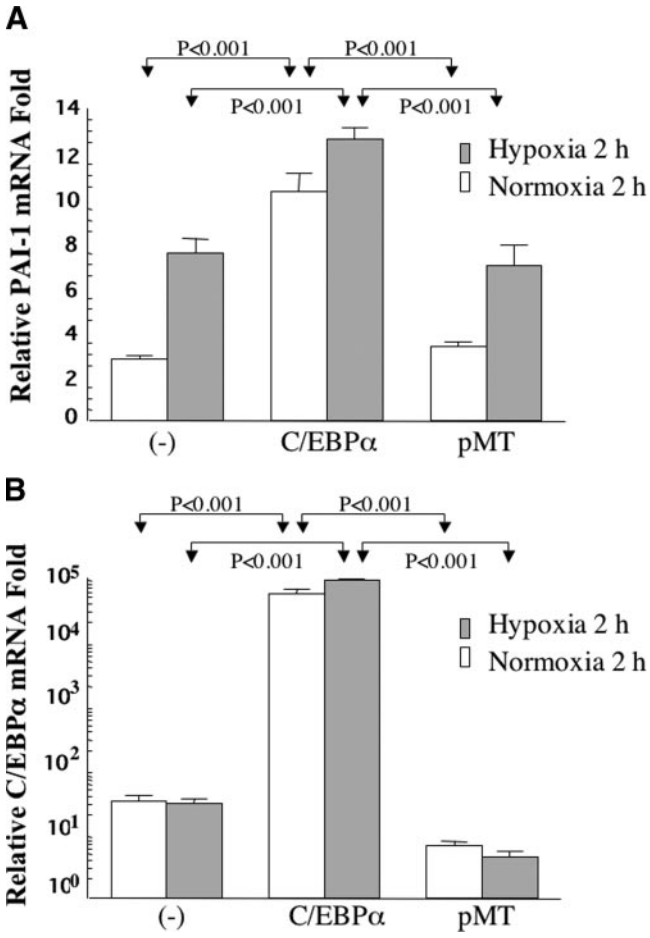

Figure 9. Effect of overexpression of C/EBPα on PAI-1 mRNA expression on RAW cells. RAW cells were transfected with C/EBPα overexpression plasmid or empty pMT vector. 24 h after transfection the cells were exposed to hypoxia or normoxia for 2 h and simultaneously incubated with 50 μM of $ZnCl_2$ to induce C/EBPα expression. Total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and C/EBPα mRNA (*B*) was measured.

augments PAI-1 expression in the C/EBPα transfectants more than the normoxic C/EBPα transfectants.

Egr-1, HIF-1α, and C/EBPα overexpression enhanced the activation of the mPAI-1 promoter in hypoxia

RAW cells were cotransfected with a luciferase reporter plasmid (pGL3PAI.Luc/287, or pGL3 basic control vector), a pCMV/β-galactosidase vector, and three overexpression constructs. The transient transfections of overexpression constructs consisted of the following plasmid combination (as indicated in (**Fig. 10**): empty control vectors (pcDNA3.1 or pMT) and/or specific overexpression constructs (pEgr-1, pHIF-1α, and/or pC/EBPα) to total transfection of 2 μg of DNA. Transfected cells were incubated in hypoxia or normoxia with 10 μM of $ZnCl_2$ for 16 h. Firefly luciferase activity of the cell lysates was then measured and normalized by control pCMV/β-galactosidase activity (Fig. 10). These data show that, in hypoxia, in cells transfected with pGL3PAI.Luc/287, luciferase activity of RAW cells also transfected with pEgr-1, pHIF-1α, or p/CEBPα was increased 68% ($P<0.01$), 170% ($P<0.001$), or 43% ($P<0.05$) compared with their respective control transfectants. pGL3PAI.Luc/287-transfected cells cotransfected with pEgr-1 and pHIF-1α had a 90% increase compared with cells transfected with pEgr-1 only ($P<0.001$) and an 18% increase compared with pHIF-1α transfectants ($P<0.05$). pGL3PAI.Luc/287-transfected cells cotransfected with pEgr-1 and pC/EBPα had a 32% increase compared with cells transfected with pEgr-1 only ($P<0.05$) and an 54% increase compared with pC/EBPα transfectants ($P<0.001$). pGL3PAI.Luc/287-transfected cells cotransfected with pHIF-1α and pC/EBPα had a 27% increase compared with cells transfected with pHIF-1α only ($P<0.001$) and an 139% increase compared with pC/EBPα transfectants ($P<0.001$). pGL3PAI.Luc/287-transfected cells, cotransfected with pEgr-1, pHIF-1α, and pC/EBPα had a 188% increase compared with cells transfected with pEgr-1 only ($P<0.001$), a 78% increase compared with pHIF-1α only ($P<0.001$), a 236% increase compared with pC/EBPα only ($P<0.001$), a 51% increase compared with pEgr-1 and pHIF-1α ($P<0.001$), an 118% increase compared with pEgr-1 and pC/EBPα ($P<0.001$), and a 41% increase compared with pHIF-1α

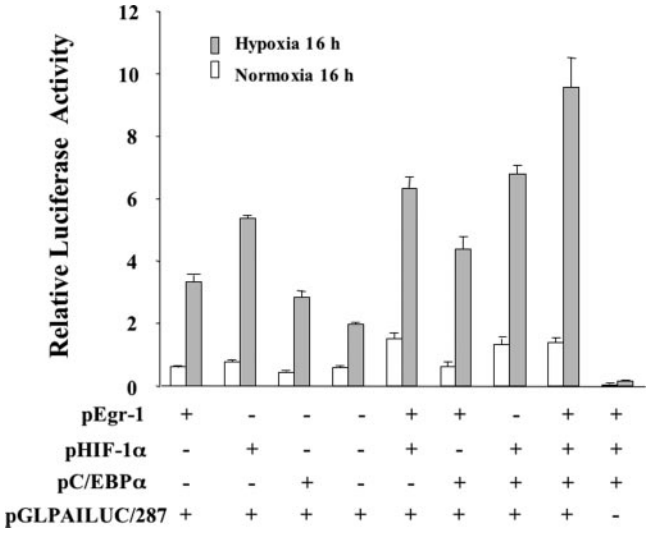

Figure 10. Egr-1, HIF-1α, and C/EBPα overexpression enhanced activation of the mPAI-1 promoter in hypoxic RAW cells cotransfected with 0.25 μg of luciferase reporter plasmid (pGL3PAI.Luc/287 or pGL3 basic control vector), 0.5 μg of pCMV/β-galactosidase, and a combination of the three overexpression constructs or controls (0.25 μg of each construct): empty control vectors (pcDNA3.1, or pMT) and/or specific overexpression constructs (pEgr-1, pHIF-1α, and/or pC/EBPα). The transfection mixture consisted of specific overexpression constructs with the balance comprised of the appropriate control vectors. These mixtures were used to equalize the total amount of DNA transfected in each well. Transfected cells were incubated in hypoxia or normoxia with 10 μM of $ZnCl_2$ for 16 h. Firefly luciferase activities of the cell lysates were then measured by the same procedure as described in Fig. 1 and normalized by control pCMV/β-galactosidase activity.

and pC/EBPα transfectants ($P<0.001$) (Fig. 10). These data showed that in hypoxia, luciferase activity was significantly increased in RAW cells cotransfected with two overexpression plasmids compared with cells transfected with only one overexpression construct. Further, luciferase activity was increased significantly in the RAW cells cotransfected with three overexpression plasmids compared with cells transfected with either two or one overexpression construct.

To verify that the Egr-1-, HIF-1α-, or C/EBPα-driven PAI-1 mRNA expression seen in RAW cells was not cell line dependent, the murine peritoneal macrophages were isolated and transfected with pEgr-1, pHIF-1α, or C/EBPα. While incubated in hypoxia or normoxia for 4 h, the cells transfected with C/EBPα or pMT empty control vector were simultaneously treated with 50 μM $ZnCl_2$ to induce expression of C/EBPα protein. The total RNA was then isolated and RT-qPCR was performed to measure the mRNA levels of PAI-1, Egr-1, HIF-1α, or C/EBPα mRNA. The results show that the expression of PAI-1 mRNA was increased by 75% in pEgr-1-transfected hypoxic cells compared with hypoxic cells transfected with control vector ($P<0.01$) or nontransfected controls ($P<0.05$) (**Fig. 11*A***). PAI-1 mRNA expression also increased in normoxic pEgr-1-transfected cells compared with normoxic control vector-transfected cells (40% increase, $P<0.05$) and nontransfected cells (1.9-fold increase, $P<0.01$) (Fig. 11*A*). In pEgr-1-transfected cells, Egr-1 mRNA expression was increased >1000-fold compared with controls ($P<0.001$) (Fig. 11*B*). PAI-1 mRNA expression was increased 3.2-fold in pHIF-1α-transfected hypoxic cells compared with hypoxic cells transfected with control vector ($P<0.001$) or nontransfected controls ($P<0.001$) (**Fig. 12*A***). The HIF-1α mRNA expression in pHIF-1α-transfected murine peritoneal macrophages was > 100-fold increased compared with controls ($P<0.001$) (Fig. 12*B*). The expression of PAI-1 mRNA was increased 1.2-fold in pC/EBPα-transfected hypoxic cells compared with the hypoxic cells transfected with control vector ($P<0.001$) or nontransfected controls ($P<0.001$) (**Fig. 13*A***). In pC/EBPα-transfected murine peritoneal macrophages, C/EBPα mRNA expression was increased >100-fold compared with controls ($P<0.001$) (Fig. 13*B*). These data confirm results from the overexpression plasmid-transfected murine peritoneal macrophages that *1)* transfection of the Egr-1, HIF-1α or C/EBPα-containing vector increases PAI-1 expression under all conditions, and *2)* hypoxia augments PAI-1 expression in the Egr-1, HIF-1α, or C/EBPα transfectants more than the normoxic transfectants.

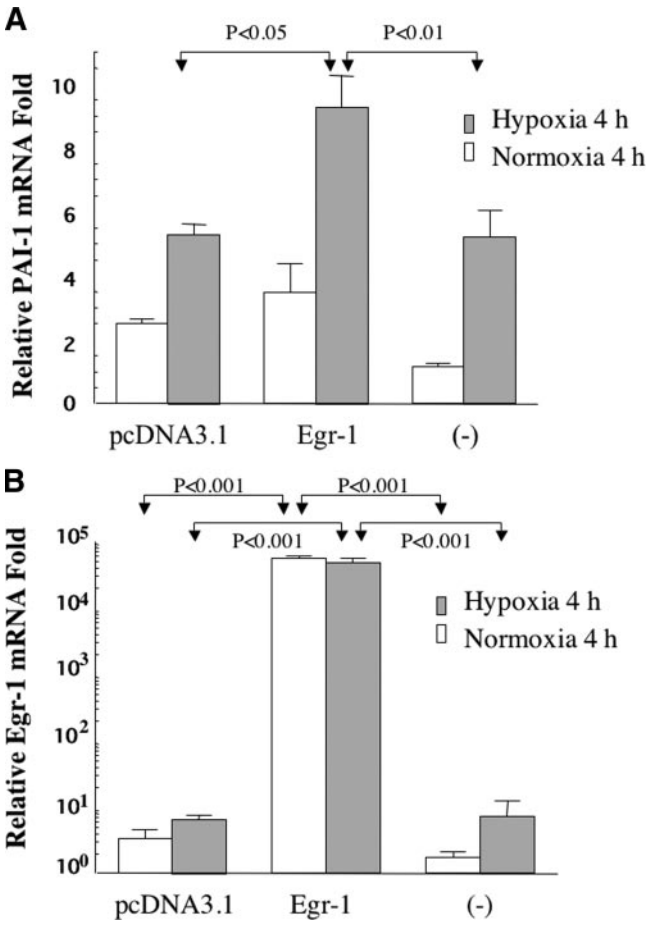

Figure 11. Effect of overexpression of Egr-1 on PAI-1 mRNA expression on peritoneal macrophages. The primary macrophages were transfected with Egr-1 overexpression plasmid or empty pcDNA3.1 vector. 24 h after transfection the cells were exposed to hypoxia or normoxia for 4 h. Total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and Egr-1 mRNA (*B*) were measured.

DISCUSSION

When cells are deprived of oxygen, genetically programmed responses either help them to adapt or, in certain instances, lead to pathological responses and tissue injury. Because of the crucial role of oxygen as life itself evolved, organisms have developed a number of highly conserved mechanisms to regulate the cellular response to low oxygen tension. In addition to energy conservation strategies, hypoxia leads cells of the vessel wall to undergo phenotypic shifts such that inflammatory and thrombotic mechanisms dominate the intravascular milieu. One of the most tightly regulated responses to periods of oxygen deprivation, inflammation, or ischemia is that governing the expression of a key regulator of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1). PAI-1 is a 50 kDa serine protease inhibitor (SERPIN) that is the major inhibitor of fibrinolysis. It binds avidly to plasminogen activators (PAs), inhibiting their lytic action on fibrinogen. PAI-1 is strongly induced by vascular cells exposed to conditions of inflammation, hypoxia, or ischemia, which results in the suppression of fibrinolysis and hence the accrual of fibrin. It is likely that the mechanism(s) underlying hypoxic induction of PAI-1 have been highly conserved over evolutionary time because fibrin accumulation provides the stromal matrix to support the ingrowth of new blood vessels into an oxygen-scarce environment.

Our group reported in 1998 that hypoxia elicits a

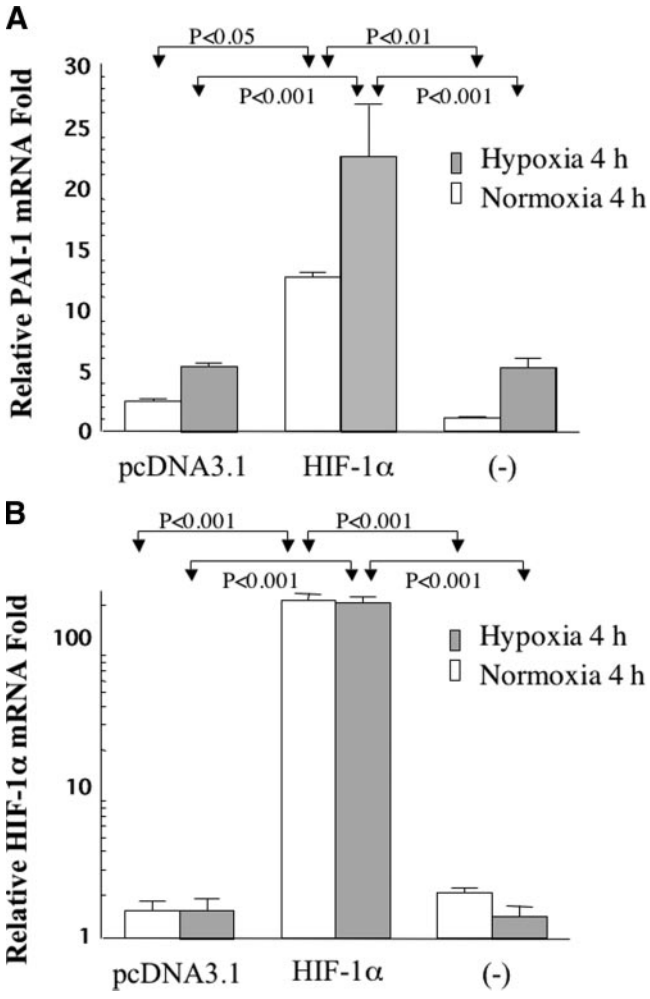

Figure 12. Effect of overexpression of HIF-1α on PAI-1 mRNA expression on peritoneal macrophages. The primary macrophages were transfected with HIF-1α overexpression plasmid or empty pcDNA3.1 vector. 24 h after transfection the cells were exposed to hypoxia or normoxia for 4 h. Total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and HIF-1α mRNA (*B*) was measured.

brisk and profound increase in PAI-1 mRNA levels and protein expression, and concomitant evidence for tissue thrombosis. Nuclear run-on analysis demonstrated that the increase in PAI-1 mRNA levels is largely driven by increases in transcription, yet the underlying factors driving the increased transcription of the PAI-1 gene under hypoxic conditions were not identified. In the current work, mechanisms underlying transcriptional activation of the PAI-1 gene by hypoxia are elucidated. These data show that the transcription factors Egr-1, HIF-1α, and C/EBPα each contribute to mouse PAI-1 gene transcription, independently and potentially cooperatively.

Early growth response gene-1 (Egr-1) is a zinc finger transcription factor recently dubbed a "master switch," which regulates the coordinated cellular response to oxygen deprivation (49). When mononuclear phagocytes are placed in a hypoxic environment, a cascade of events is triggered (19), leading to the rapid induction of Egr-1. Similarly, when a tissue (the lungs) was subject to an abrupt interruption of blood flow secondary to placement of a ligature around its arterial blood supply, there was a profound (~12-fold) increase in PAI-1 mRNA levels at ~4 h. However, when mice null for the Egr-1 gene were subjected to similar ischemic procedures, there was a significant dampening of the increment of PAI-1 expression (only a ~3-fold increase) (49). These data support our previous observation that PAI-1 expression is strongly inducible by hypoxia (5), and indicate that Egr-1 plays an important although not exclusive role in the ischemic induction of this protein. Data presented here show that the 5′-flanking DNA promoter region of the murine PAI-1 gene (from –162 to +50) [overlapping the PAI-1 start codon] is responsible in part, for the appearance of PAI-1 transcripts in mononuclear phagocytes after hypoxic exposure commences. This region encompasses a critical Egr-1 site (from –137 to –129), implicating Egr-1 as a driver of hypoxic induction of PAI-1 gene transcription. Functional analysis performed using Egr-1 cDNA overex-

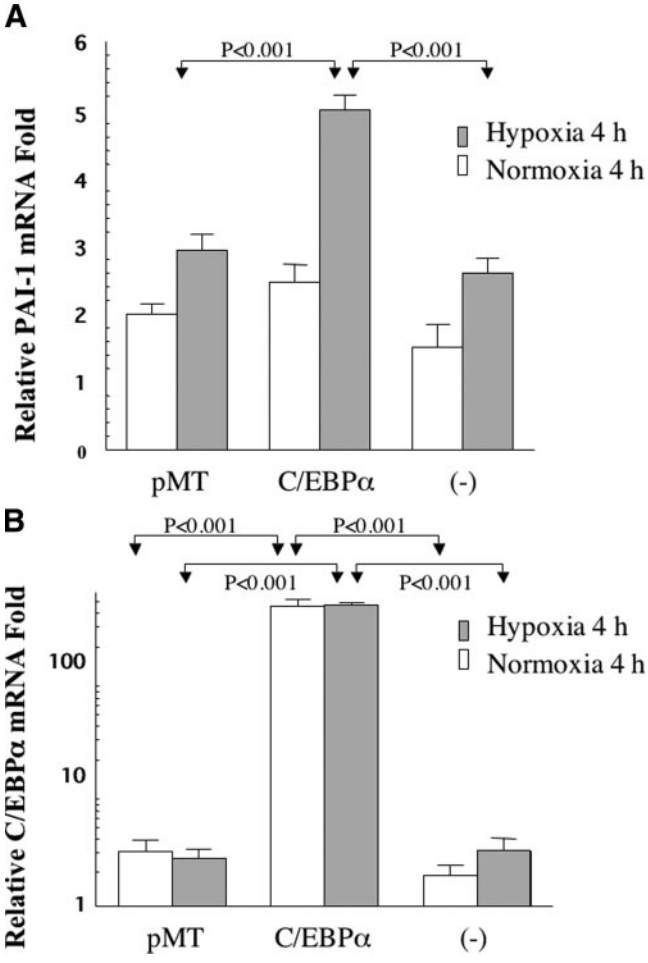

Figure 13. Effect of overexpression of C/EBPα on PAI-1 mRNA expression on peritoneal macrophages. Primary macrophages were transfected with C/EBPα overexpression plasmid or empty pMT vector. 24 h after transfection, cells were exposed to hypoxia or normoxia for 4 h and simultaneously incubated with 50 μM of $ZnCl_2$ to induce C/EBPα expression. Total RNA was extracted and the relative quantity of mPAI-1 mRNA (*A*) and C/EBPα mRNA (*B*) was measured.

pression to increase PAI-1 transcription in both RAW and murine peritoneal cells, gel mobility shift assays, chromatin immunoprecipitation assays, as well as immunoblot analysis of nuclear protein extracts for Egr-1, demonstrate an extremely brisk Egr-1 response to hypoxia, with increased Egr-1/DNA binding as early as 30 min after hypoxic exposure. Mutation of the Egr promoter site partially abolishes the hypoxic response, proving that it is actually the Egr-1 site responsible for the inductive response of the PAI-1 gene to hypoxia.

Though the Egr-1-driven response of PAI-1 to hypoxia is robust, it is by no means exclusive. Hypoxia-driven expression of PAI-1 transcripts appears to be affected by alternative mechanisms as well, including HIF-1 and C/EBP-α transcriptional mechanisms (see below). Nevertheless, even when these alternative sites are abolished (*i.e.*, use of promoter-reporter constructs retaining the Egr site but in which the promoter region encompassing HIF-1 and c/EBP-α sites as been deleted and the Egr site ligated directly to the −62 to +50 flanking region), the Egr site still retains some hypoxic response.

HIF-1 is recognized to be a key transcription factor responsible for oxygen regulation of several genes such as erythropoietin, VEGF, inducible NOS, and PAI-1 (50–53). In these and other experiments, HIF-1 DNA binding activity, a functional laboratory marker for the presence of HIF-1 protein, is detected in nuclear extracts derived from cells grown under hypoxic conditions; it is undetectable under normoxic conditions. Both HIF-1 mRNA and protein are ubiquitously expressed in all organs of human and rodents (54), both are rapidly induced by hypoxia, and both rapidly decay upon return to normoxic conditions (26, 55–57). The murine PAI-1 promoter region (–182/–167) contains two potential E-box sequences, 5′-CANNTG-3′ (58) [5′-CACGTG-3′ (–172/–167) and the reverse complement 5′-CACGTA-3′ (–182/–177)], named HRE-1 and HRE-2, respectively. E-box sequences comprise an upstream regulatory region that controls transcription of a variety of genes involved with cell development, differentiation, growth, and neoplasia. Typically, transcription factors containing a common basic helix-loop-helix (bHLH) motif (with or without a leucine zipper) bind to the E-box sequence to activate transcription. HIF-1 is an example of a bHLH-PAS protein that binds to E-box sequences, found in the promoter regions of a variety of genes, which confer hypoxia sensitivity.

Induction of PAI-1 gene expression by hypoxia was shown to depend on both HRE-1 and HRE-2 motifs in the PAI-1 promoter (59). However, no other driving motifs responsive to hypoxia were identified in that work. In the work presented here, we have shown that these HREs play an important, though not exclusive, role in hypoxic induction of PAI-1 gene transcription. Under hypoxic conditions, the HIF-1α protein is stabilized (57) and transported into the nucleus (60), leading to accumulation of the active HIF-1α/HIF-1β heterodimer. The active HIF-1 heterodimer that accumulates under conditions of oxygen scarcity leads to the induction of PAI-1 gene expression. Similarly, we showed that a transient overexpression of HIF-1α is able to induce PAI-1 gene expression in RAW and peritoneal macrophages. In our study, we have shown that HIF-1α is just one important regulator driving ischemic induction of PAI-1 protein.

The third promoter sequence that we identified as exhibiting hypoxic sensitivity was C/EBPα. This is the first demonstration of the role of this promoter element in the hypoxic regulation of gene expression. The activity and/or expression level of the three C/EBP members (α, β, and δ) is regulated by a number of inflammatory mediators, including lipopolysaccharide (LPS) and an array of cytokines (61–65). C/EBPα is known to play an important role in mediating the acute-phase response; for instance, IL-1 induces C/EBPα expression in neonatal mice's liver (41). In our study we have shown that CEB/Pα is a regulator in the ischemic induction of PAI-1 protein. After hypoxic exposure, a promoter construct that only included the C/EBP site in the upstream regulatory region of the murine PAI-1 gene increased PAI-1 transcription by ~ 3-fold. A promoter construct that retains only the intact C/EBP site, but has the Egr and other HREs mutated similarly demonstrated an ~3-fold induction by hypoxia. On the other hand, mutation of the C/EBP site diminished (by ~40%) induction of the PAI-1 gene in response to hypoxia, compared with similar hypoxic conditions in an intact WT C/EBPα sequence. Finally, direct overexpression of C/EBPα is able to induce PAI-1 gene expression in RAW and peritoneal macrophages.

Taken together, these data are the first to demonstrate that PAI-1 transcription induced by hypoxia represents a complex interplay between various promoter elements of the gene. When working in concert, the combination of all four promoter regions (1 Egr, 2 HREs, and CEB/Pα) produces the strongest inductive response to hypoxic conditions. Our data clearly show that the promoter region studied dominates the hypoxic response as its truncation nearly abrogates hypoxia-driven increases in PAI-1 transcription. Nevertheless, the minor residual hypoxic response could very well have been driven by other genetic elements located outside the region of study. There is also the possibility that full-length gene studies could identify additional regulatory regions. One can imagine a homeostatic mechanism whereby various elements in the PAI-1 promoter gene might be involved in coordination, enhancement, or fine-tuning of PAI-1 gene expression under various levels or durations of hypoxic exposure. The clinical response to ischemia, typified by PAI-1 induction as a means for promoting thrombus accrual, is redundant and highly regulated, as would be expected for an important physiological or adaptive response to oxygen deprivation. FJ

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