Hypoxia Induces Transcription of the Plasminogen Activator Inhibitor-1 Gene Through Genistein-Sensitive Tyrosine Kinase Pathways in Vascular Endothelial Cells

Tsuyoshi Uchiyama, Masahiko Kurabayashi, Yoshio Ohyama, Toshihiro Utsugi, Nobuhiro Akuzawa, Mahito Sato, Shouichi Tomono, Shoji Kawazu, Ryozo Nagai

Abstract—A decline in oxygen concentration perturbs endothelial function, which promotes local thrombosis. In this study, we determined whether hypoxia in the range of that observed in pathophysiological hypoxic states stimulates plasminogen activator inhibitor-1 (PAI-1) production in bovine aortic endothelial cells. PAI-1 production, measured by ELISA, was increased by 4.7-fold (P<0.05 versus normoxic control, n=4) at 12 hours after hypoxic stimulation. Northern blot analysis showed the progressive time-dependent increase in the steady-state level of PAI-1 mRNA expression by hypoxia, which reached a 7.5-fold increase (P<0.05 versus control, n=4) at 12 hours. Deferoxamine, which has been known to bind heme protein and to reproduce the hypoxic response, induced PAI-1 production at both the mRNA and protein levels. The half-life of PAI-1 mRNA, as determined by a standard decay assay, was not affected by hypoxia, suggesting that induction of PAI-1 mRNA was regulated mainly at the transcriptional level. Transient transfection assays of the human PAI-1 promoter–luciferase construct indicates that a hypoxia-responsive region lies between −414 and −107 relative to the transcription start site, where no putative hypoxia response element is found. The hypoxia-mediated increase in PAI-1 mRNA levels was attenuated by the tyrosine kinase inhibitors genistein (50 µmol/L) and herbimycin A (1 µmol/L), whereas PD98059 (50 µmol/L, MEK1 inhibitor), SB203580 (10 µmol/L, p38 mitogen-activated protein kinase inhibitor), and calphostin C (1 µmol/L, protein kinase C inhibitor) had no effect on the induction of PAI-1 expression by hypoxia and deferoxamine. Genistein but not daidzein blocked the production of hypoxia- and deferoxamine-induced PAI-1 protein. Thus, we conclude that hypoxia stimulates PAI-1 gene transcription and protein production through a signaling pathway involving genistein-sensitive tyrosine kinases in vascular endothelial cells. (Arterioscler Thromb Vasc Biol. 2000;20:1155-1161.)

Key Words: hypoxia ■ PAI-1 ■ tyrosine kinase ■ mitogen-activated protein kinase ■ endothelial cells

There is growing evidence indicating that vascular endothelium plays an obligatory role in regulating fibrinolytic activity, such as vascular tone and platelet activity.1 Endothelial dysfunction leads to the derangement of vascular endothelial anticoagulant properties and contributes to the pathophysiology of several cardiovascular disorders, including intravascular thrombosis, intimal growth, and plaque rupture.2

Endothelial cells have been shown to produce many vasoactive substances that intervene in the fibrinolytic and coagulation processes. Of the many components regulating the balance between procoagulant and anticoagulant properties of the endothelium, pathways controlling plasminogen activator activity are particularly important. This activity is controlled primarily by plasminogen activator inhibitor-1 (PAI-1), which is synthesized by endothelial cells,3 smooth muscle cells,4,5 platelets,6 monocytes,7 and hepatocytes.8 Increased levels of PAI-1 activity resulting in decreased fibrinolytic capacity have been reported in patients with coronary artery disease and metabolic syndrome of insulin resistance.9 In addition, increased PAI-1 levels have also been demonstrated in atherosclerotic lesions within the vessel wall.10 Therefore, both systemically and locally increased PAI-1 concentrations could have a pathogenic role in the development of atherosclerotic disease.

PAI-1 synthesis has been shown to be regulated by a number of relevant factors, including endotoxin, inflammatory cytokines,11 lipoprotein,12 angiotensin II,13 transforming growth factor-β,14 and phorbol ester15 in endothelial cells, and by low oxygen levels (hypoxia) in trophoblasts.16 Hypoxia is often associated with thrombosis, which is a major cause of morbidity and mortality and can appear in many clinical contexts. For example, pulmonary emboli seem to be a common complication in acute respiratory failure.17 In
addition, stasis of blood, a condition that, if severe enough, causes a decline in oxygen tension, predisposes to the development of thrombosis. Despite the well-appreciated association between hypoxia and thrombosis, the precise molecular mechanism of prothrombotic diathesis under hypoxic conditions remains unknown. We hypothesize that hypoxia induces PAI-1 expression, which contributes to the predisposition to thrombosis.

In this study, we examined whether hypoxia in the range of that observed in pathophysiological hypoxic states induces PAI-1 expression in endothelial cells. Furthermore, we investigated the molecular mechanisms underlying the increased production of PAI-1 in endothelial cells in response to hypoxia. Our results showed that hypoxia stimulates transcription from the PAI-1 promoter gene via genistein-sensitive tyrosine kinase–dependent pathways. Promoter analysis by transient transfection assays delineated the hypoxia response region between −414 and −107, which contains no canonical hypoxia response element (HRE). These findings emphasize the role of tyrosine kinases in transducing the hypoxic signals to nuclei and provide new insights into the pathogenesis of thrombosis originating from vascular disorders in which oxygen tensions are decreased.

**Methods**

**Materials**

The hypoxic chamber and Anaeropack (disposable agent) were purchased from Mitsubishi Gas Chemical. PD98059, SB203580, calphostin C, genistein, herbimycin A, and tyrphostin 23 (RG-50810) were purchased from Calbiochem. RPMI-1640 medium, FCS, streptomycin, and penicillin were purchased from Gibco-BRL. A random primer kit was purchased from Boehringer Mannheim. Deferoxamine was purchased from Sigma. [α-32P]dCTP (3000 Ci/mmol) was obtained from Amersham.

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were isolated, as previously described, by mechanical scraping of the intima of the descending bovine aorta. The cells were plated at a density of 10^5 cells on 10-cm plastic Petri dishes. The cells were cultured at 37°C in RPMI-1640 medium supplemented with 10% FCS, 50 U/mL penicillin, and 50 mg/mL streptomycin.

**Hypoxia**

The hypoxic condition was achieved by use of anaerobic jars equipped with Anaeropack as previously described (disposable aerating agent, Mitsubishi Gas Chemical). In this study, we examined whether hypoxia in the range of that observed in pathophysiological hypoxic states induces PAI-1 expression in endothelial cells. Furthermore, we investigated the molecular mechanisms underlying the increased production of PAI-1 in endothelial cells in response to hypoxia. Our results showed that hypoxia stimulates transcription from the PAI-1 promoter gene via genistein-sensitive tyrosine kinase–dependent pathways. Promoter analysis by transient transfection assays delineated the hypoxia response region between −414 and −107, which contains no canonical hypoxia response element (HRE). These findings emphasize the role of tyrosine kinases in transducing the hypoxic signals to nuclei and provide new insights into the pathogenesis of thrombosis originating from vascular disorders in which oxygen tensions are decreased.

**RNA Extraction and Northern Blot Analysis**

Total cellular RNA was isolated from BAECs with IsoQuick (Nippon Gene) as described previously. Equal amounts of RNA were resolved by electrophoresis on 1.2% agarose gel containing 25 mmol/L MOPS buffer (pH 7.0), 1 mmol/L EDTA, and 2.2 mol/L formaldehyde and were transferred onto Hybond N membrane (Amersham) as described previously.22 Equal amounts of RNA were resolved by electrophoresis on 1.2% agarose gel containing 25 mmol/L MOPS buffer (pH 7.0), 1 mmol/L EDTA, and 2.2 mol/L formaldehyde and were transferred onto Hybond N membrane (Amersham) as described previously.

**PAI-1 Production**

PAI-1 production was evaluated with a commercially available ELISA kit (American Diagnostica Inc). Briefly, cells were grown on 6-cm dishes to 80% confluence in RPMI-1640 supplemented with 10% FCS, and medium was switched to serum-free RPMI-1640 before the cells were exposed to hypoxia. Culture supernatants were removed after 12 hours, and PAI-1 production, normalized to cellular protein concentrations, was evaluated.

**DNA Transfection and Luciferase Assay**

DNA transfections into cultured BAECs were performed by using Tfx-50 (Promega) according to the manufacturer’s procedure. Cells were transfected with 1 μg of reporter plasmid or transfected with 1 μg of reporter plasmid and 2 μg of expression vector. After transfection, cultures were maintained with RPMI-1640 containing 10% FCS. Luciferase activity was measured with a Berthold Lumat LB9501 luminometer and was normalized to cellular protein concentrations. Each transfection was repeated at least 3 times in duplicate.

**Data Analysis**

Data are presented as the mean ± SEM for ≥3 separate experiments. Statistical analysis was performed with a paired Student’s t test, with significant differences determined as p < 0.05.

**Results**

**Hypoxia Uregulated PAI-1 Production by BAECs**

To investigate whether hypoxia at physiological levels directly affects homeostasis in endothelial culture, we used the Anaeropack, which is a disposable oxygen–absorbing and CO2 (≥5%)–generating agent. Under our experimental conditions, oxygen tension within the culture medium dropped to 53.7 ± 9.1 mm Hg after 4 hours and remained unchanged thereafter in the presence of Anaeropack; oxygen tension was 52.2 ± 7.3 mm Hg after 6 hours and 53.6 ± 8.4 mm Hg after 12 hours of incubation. There were no detectable variations in cell morphology at the microscopic level between cells exposed to ambient air and cells maintained in the hypoxic chamber (data not shown). Under these conditions, BAECs

**Expression Plasmid for Hypoxia-inducible Factor (HIF)-1α-like factor (HIF)**

The expression plasmid for hypoxia-inducible factor (HIF)-1α-like factor (HIF) was described.

**Promoter-Luciferase Vector and Expression Plasmid**

For generation of PAI-1 promoter luciferase reporter genes, the following forward primers and reverse primer were used in a PCR reaction using a plasmid containing an ∼3.4-kb DNA insert (courtesy of Dr Douglas E. Vaughan, Vanderbilt University Medical Center, Nashville, Tenn) as a template. Sequences for PCR forward primers with a KpnI site (italicized) were nt-414, 5′-CCCGTACCTGGTTCGCAAAGAAAGCA-3′ and nt-107, 5′-CCGTTAATGGTGTCGACAAGGATCGCAGAG-3′; the reverse primer (nt +82) with a XhoI site (italicized) was 5′-GGGCTCGAG-CITGAGAATATCCGGTGG-3′. PCR products were gel-purified, digested, and subcloned into the KpnI/XhoI sites of the promoterless luciferase reporter gene vector pGL3-Basic (Promega). The expression plasmid for hypoxia-inducible factor (HIF)-1α-like factor (HIF) was described.

**PAI-1 Production**

PAI-1 production was evaluated with a commercially available ELISA kit (American Diagnostica Inc). Briefly, cells were grown on 6-cm dishes to 80% confluence in RPMI-1640 supplemented with 10% FCS, and medium was switched to serum-free RPMI-1640 before the cells were exposed to hypoxia. Culture supernatants were removed after 12 hours, and PAI-1 production, normalized to cellular protein concentrations, was evaluated.

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**Data Analysis**

Data are presented as the mean ± SEM for ≥3 separate experiments. Statistical analysis was performed with a paired Student’s t test, with significant differences determined as p < 0.05.
were cultured for 12 hours and tested for PAI-1 production with ELISA. As shown in Figure 1A, exposure to hypoxia significantly (P<0.01, n=4) increased PAI-1 production in a time-dependent manner. To determine whether the observed increase represents an upregulation in PAI-1 mRNA expression, Northern blot analysis was performed. BAECs were harvested for RNA analysis at the indicated times after hypoxic stimulation. As shown in Figure 1, B and C, progressive time-dependent increases in PAI-1 mRNA levels were observed in cells cultured in hypoxic conditions, whereas β-actin mRNA levels were only modestly changed. Induction of PAI-1 mRNA levels was evident by 4 hours and reached a maximum by 12 hours. In contrast, tissue-type plasminogen activator (tPA) and urokinase-like PA (uPA) mRNA levels were not noticeably induced in response to hypoxia (data not shown).

To assess the possible involvement of heme proteins in the regulation of PAI-1 expression by hypoxia, the effects of deferoxamine on the PAI-1 mRNA levels were examined because this reagent is known to prevent binding of molecular oxygen to heme proteins and thus reproduce the hypoxic response.27 Exposure of BAECs to deferoxamine led to a smaller but consistent increase in both PAI-1 protein and mRNA levels (Figure 2, A and B).

Figure 1. Hypoxia induces PAI-1 production by BAECs. A, BAECs were cultured in 6-cm dishes for ~5 days, then exposed to hypoxia. After 12 hours, the supernatants were collected and analyzed for PAI-1 protein with ELISA. Relative PAI-1 protein levels are presented as percentage of PAI-1 protein levels of normoxic control cells. B, Northern blots. Total RNA (12 µg) was extracted from BAECs at indicated times after stimulation with hypoxia and analyzed by Northern blotting for PAI-1 mRNA and β-actin. C, Densitometric analysis of PAI-1 mRNA levels. PAI-1 mRNA levels at indicated times were normalized by β-actin mRNA levels. Values of control cells were set at 1.0. Each column and bar represents mean±SEM for 3 separate experiments in duplicate. *P<0.01 vs normoxic cells (n=4).

Hypoxia Increased PAI-1 mRNA Expression at the Transcriptional Levels

To determine whether hypoxia increases steady-state levels of PAI-1 mRNA at the transcriptional level or posttranscriptional level involving, for example, decreased degradation rate, measurement of PAI-1 mRNA half-life was performed in the presence or absence of actinomycin D (5 µg/mL). The results show that there is no significant difference in the half-life of PAI-1 mRNA between the absence and the presence of hypoxia or deferoxamine (Figure 3, A and B). Thus, hypoxia does not appear to affect the stability of PAI-1 mRNA, suggesting that the observed increase in PAI-1 mRNA levels by hypoxia was due to an increase in transcription from the PAI-1 promoter. It is also interesting to note that the stability of PAI-1 mRNA is considerably higher than that of eNOS mRNA, because eNOS mRNA levels were decreased by 80% of control at 10 hours after treatment with actinomycin D.

Figure 2. Deferoxamine induces PAI-1 production by BAECs. A, BAECs were cultured in 6-cm dishes for ~5 days, then treated with deferoxamine (DFX, 130 µmol/L). After 12 hours, the supernatants were collected and analyzed for PAI-1 protein with ELISA. Relative PAI-1 protein levels are presented as percentage of PAI-1 protein levels of normoxic control cells. B, Northern blots. Total RNA (12 µg) was extracted from BAECs at indicated times after stimulation with deferoxamine and analyzed by Northern blotting for PAI-1 and β-actin mRNAs. C, Densitometric analysis of PAI-1 mRNA levels. PAI-1 mRNA levels at indicated times were normalized by β-actin mRNA levels. Values of control cells were set at 1.0. Each column and bar represents mean±SEM for 3 separate experiments in duplicate. *P<0.01 vs control untreated cells (n=4).

Figure 3. Effects of hypoxia on mRNA stability in BAECs. A, Top, Northern blots. Total RNAs isolated from BAECs cultured in normoxic conditions and hypoxic conditions after incubation with actinomycin D (5 mg/mL) for 2 hours, 4 hours, and 10 hours were separated on agarose-formaldehyde gels, blotted, and hybridized with probes for PAI-1, eNOS, and β-actin. B, Bottom, Densitometric analysis of PAI-1 mRNA levels. PAI-1 mRNA levels at indicated times were normalized by β-actin mRNA levels. Values of control cells were set at 1.0. Each graph and bar represents mean±SEM for 3 separate experiments in duplicate. *P<0.01 vs control untreated cells (n=4).
Hypoxia Increased PAI-1 Promoter Activity

We then performed transient transfection assays to test whether hypoxia stimulates transcription from the PAI-1 promoter. The human PAI-1 promoter, which contains 414 bp of the 5'-flanking region and 80 bp of the 5'-untranslated region, was linked to the luciferase gene, and the resultant construct, -414PAI-1Luc, was transiently transfected into BAECs. Exposure of the cells to hypoxia for 12 hours increased luciferase activity modestly but reproducibly (2.0±0.1-fold, P<0.05 compared with normoxia, n=8) (Figure 4A). To verify the specificity of an increase in luciferase activity, β-actin promoter luciferase construct was transfected into BAECs, and luciferase activity was measured in the same way. No significant change was observed in luciferase activity from the β-actin promoter region, thus suggesting that the induction of -414PAI-1Luc activity by hypoxia is promoter-specific. Transcriptional activation of PAI-1 promoter by deferoxamine was also examined by transient transfection assays. Incubation of transfected cells with deferoxamine (130 μmol/L) increased luciferase activity derived from the -414PAI-1Luc construct, whereas β-actin promoter activity was not affected by deferoxamine (Figure 4B). Deletion to -107 significantly attenuated the induction of promoter activity in response to hypoxia, thus indicating that the hypoxia-mediated increase in PAI-1 promoter activity is mediated at least partly through the sequence between -414 and -107. Because no canonical HRE (5'-RCGTG-3') was found within this region, these data suggest that hypoxia induces PAI-1 promoter activity through HRE-independent cis-regulatory elements.

Hypoxia-Induced PAI-1 mRNA Expression Was Mediated Through Tyrosine Kinase–Dependent Pathway

To determine the signal transduction pathways that play a major role in inducing PAI-1 mRNA expression, we examined the effects of a variety of protein kinase inhibitors on the steady-state levels of PAI-1 mRNA. We used the inhibitors at the concentrations that have been proved to be effective in blocking the phosphorylation of the substrates.29 As shown in Figure 5, pretreatment of BAECs with the protein kinase C inhibitor calphostin C (1 μmol/L), or the mitogen-activated protein kinase (MAP)/extracellular signal-regulated kinase (ERK) (MEK1)-specific inhibitor PD98059 (50 μmol/L), had no effects on either basal or hypoxia-stimulated increases in PAI-1 mRNA levels. In contrast, pretreatment of BAECs with SB203580 (10 μmol/L), a specific inhibitor for p38 MAP kinase, led to a significant decrease in basal PAI-1 mRNA levels, whereas it had little effect on the multiples of induction of hypoxia-mediated PAI-1 mRNA levels. Conversely, the nonselective tyrosine kinase inhibitor genistein (50 μmol/L),30 but not daidzein (negative control of genistein), reduced both basal and hypoxia-stimulated increases in PAI-1 mRNA expression. Likewise, herbinycin A (1 μmol/L), which irreversibly inhibits the nonreceptor tyrosine kinases, including Src31 and Bcr-Abl,32 reduced the basal and hypoxia-induced PAI-1 mRNA levels. Tyrophostin 23 (100 μmol/L), which potently inhibits epithelial growth factor (EGF) receptor tyrosine kinases,33 reduced basal levels of PAI-1 mRNA but had no effect on the induction of PAI-1 mRNA expression by hypoxia. These results indicate that genistein and herbinycin A are effective in reducing both basal and hypoxia-stimulated expression of PAI-1 mRNA. As shown in Figure 6, studies with deferoxamine showed the same results as those observed in hypoxic stimulation: SB203580, genistein, and herbinycin A led to a significant drop in basal PAI-1 mRNA in response to hypoxia. As shown in Figure 6, studies with deferoxamine showed the same results as those observed in hypoxic stimulation: SB203580, genistein, and herbinycin A but not SB203580 prevented hypoxia-mediated increases in PAI-1 mRNA expression.

Genistein Inhibited Hypoxia-Mediated PAI-1 Protein Production

The effects of genistein on hypoxia-induced PAI-1 protein were examined by ELISA (Figure 7). The results show that
increased production of PAI-1 in response to hypoxia is blunted in the presence of genistein, whereas daidzein had little inhibitory effect on this response. Calphostin C, PD98059, and SB203580 appeared to attenuate the hypoxic response but did not play a major role in preventing the induction of PAI-1 protein by hypoxia.

**HLF Induced PAI-1 Promoter**

We next examined whether PAI-1 promoter is regulated by HLF, a transcription factor that has been shown to be involved in gene expression in response to hypoxia. As shown in Figure 8, cotransfection of expression vector for HLF (pBOS-HLF) along with the PAI-1 promoter–luciferase constructs showed that HLF increased the luciferase activity derived from -414PAI-1Luc but not from -107PAI-1Luc. These findings were consistent with the results in Figure 4, in which -414PAI-1Luc but not -107PAI-1Luc was responsive to hypoxia. The promoter of β-actin gene was not affected by pBOS-HLF, further indicating that the response of PAI-1 promoter to pBOS-HLF was promoter-specific.

**Discussion**

Although there has been an increasing appreciation for the role of oxygen tension in regulating endothelial function, little is known about the molecular mechanisms responsible for the alteration in gene expression by hypoxia in endothelial cells. In this study, we investigated the effects of hypoxia on PAI-1 gene expression in BAECs in a hypoxic chamber in which oxygen tension was decreased to a level observed in pathophysiological conditions. One of the major conclusions drawn from this study was that the physiological range of low oxygen tension is sufficient to induce PAI-1 mRNA expression at the transcriptional level in endothelial cells.

Previous studies on the effects of hypoxia on PAI-1 expression have yielded conflicting results. Wojta et al. showed that exposure of bovine lung endothelial cells increases PAI-1 activity, but they claimed that this response is due to the impaired release of plasminogen activator with a consequent increase in the levels of available PAI-1. Studies by Bach et al. showed a decrease in PAI-1 activity in anoxic cultures of human umbilical vein endothelial cells and a concomitant increase in tPA activity. Our data are consistent with a recent report in which PAI-1 mRNA was increased in response to hypoxia in trophoblastic cells. To the best of our knowledge, the present work is the first demonstration that reduced oxygen tension led to an increase in PAI-1 mRNA expression in endothelial cells. In contrast to PAI-1 induction, tPA and uPA mRNA levels were not noticeably induced in response to hypoxia, which suggests that hypoxia increases procoagulant activity without increasing anticoagulant activity.

We demonstrated that genistein-sensitive tyrosine kinase is involved in hypoxia-induced PAI-1 expression. Although genistein is a broad-spectrum tyrosine kinase inhibitor, the effect of genistein on PAI-1 expression was rather selective because another tyrosine kinase inhibitor, tyrphostin 23, had no effect. The importance of tyrosine kinases in signal transduction by hypoxia has been well documented. In the nitrogen-fixing gene in the bacterium *Rhizobium meliloti*, hypoxia induces oxygen dissociation from the heme group, which activates tyrosine kinase and results in the phosphor-
ylation of transcription factors. 37 In higher organisms, Mukhopadhyay et al38 reported that hypoxia activates pp60src, which, in turn, phosphorylates downstream transcription factors responsible for the hypoxic induction of vascular EGF (VEGF). Because genistein is an inhibitor of pp60src,39 these mechanisms appear to conform to our findings. Further studies will be necessary to test the role of pp60src in inducing PAI-1 gene expression in response to hypoxia.

In this study, a MEK1 inhibitor, PD98059, had no effects on hypoxia-mediated induction of PAI-1 mRNA levels. This finding was somewhat surprising, because previous studies have demonstrated that activation of the ERK/MAP kinase cascade is implicated in the hypoxic response. Mukhopadhyay et al38 documented that a plasmid-overexpressing dominant negative form of Raf1 mutant or the ERK inhibitor 6-thioguanine clearly inhibit the hypoxia-mediated increase in VEGF mRNA levels. Muller et al40 reported the induction of c-fos gene transcription through a MAP kinase–dependent pathway in HeLa cells. Although a variety of stimuli, including phorbol ester, endothelin-1,41 transforming growth factor-β,14 and high glucose,25 induce PAI-1 mRNA expression, little evidence exists for the role of ERK/MAP kinase pathways in the inducible expression of the PAI-1 gene. Consistent with these results, we found that overexpression of a constitutive active mutant of MEK1 had only minimal effects on PAI-1 promoter activity (T. Uchiyama, M. Kurabayashi, and R. Nagai, unpublished results). These findings may account for the inability of MEK1 inhibitor to block the inducible expression of the PAI-1 gene in response to hypoxia.

Deletion analysis of PAI-1 promoter indicated that the region spanning between –414 and –107 contains element(s) mediating the hypoxic response. In agreement with these results, forced expression of HLF markedly induces luciferase activity of –414PAI-1Luc but not that of –107PAI-1Luc. A search for the putative transcription factor–binding sites within this region revealed that it contains elements mediating the hypoxic response. In conclusion, the present study showed that PAI-1 production was significantly induced in response to a physiological range of hypoxic conditions at the mRNA and protein levels through a signaling pathway involving genistein-sensitive protein tyrosine kinases. Although an induction of PAI-1 mRNA was regulated primarily at the transcriptional levels, the regulatory region for PAI-1 promoter activity by hypoxia does not contain consensus HRE. Identification of a signaling cascade mediating the transcriptional response to hypoxia will facilitate our understanding of the molecular mechanisms responsible for endothelial dysfunction caused by hypoxia.

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References


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