Plasminogen Activator Inhibitor-1 Inhibits Angiogenic Signaling by Uncoupling Vascular Endothelial Growth Factor Receptor-2-α₃β₃ Integrin Cross Talk

Jianbo Wu, Tammy L. Strawn, Mao Luo, Liqun Wang, Rong Li, Meiping Ren, Jiyi Xia, Zhuo Zhang, Weizhong Ma, Tingting Luo, Daniel A. Lawrence, William P. Fay

Objective—Plasminogen activator inhibitor-1 (PAI-1) regulates angiogenesis via effects on extracellular matrix proteolysis and cell adhesion. However, no previous study has implicated PAI-1 in controlling vascular endothelial growth factor (VEGF) signaling. We tested the hypothesis that PAI-1 downregulates VEGF receptor-2 (VEGFR-2) activation by inhibiting a vitronectin-dependent cooperative binding interaction between VEGFR-2 and α₃β₃.

Approach and Results—We studied effects of PAI-1 on VEGF signaling in human umbilical vein endothelial cells. PAI-1 inhibited VEGF-induced phosphorylation of VEGFR-2 in human umbilical vein endothelial cells grown on vitronectin, but not on fibronectin or collagen. PAI-1 inhibited the binding of VEGFR-2 to β₃ integrin, VEGFR-2 endocytosis, and intracellular signaling pathways downstream of VEGFR-2. The anti-VEGF effect of PAI-1 was mediated by 2 distinct pathways, one requiring binding to vitronectin and another requiring binding to very low-density lipoprotein receptor. PAI-1 inhibited VEGF-induced angiogenesis in vitro and in vivo, and pharmacological inhibition of PAI-1 promoted collateral arteriole development and recovery of hindlimb perfusion after femoral artery interruption.

Conclusions—PAI-1 inhibits activation of VEGFR-2 by VEGF by disrupting a vitronectin-dependent proangiogenic binding interaction involving α₃β₃ and VEGFR-2. These results broaden our understanding of the roles of PAI-1, vitronectin, and endocytic receptors in regulating VEGF-2 activation and suggest novel therapeutic strategies for regulating VEGF signaling. (Arterioscler Thromb Vasc Biol. 2015;35:111-120. DOI: 10.1161/ATVBAHA.114.304554.)

Key Words: plasminogen activator inhibitor-1 ■ vascular endothelial growth factor A ■ very low-density lipoprotein receptor ■ vitronectin

Angiogenesis is an intensely regulated process that is integral to the evolution of many human diseases, including ischemic cardiovascular disease and cancer metastasis. Several studies have implicated plasminogen activator inhibitor-1 (PAI-1), the primary endogenous inhibitor of tissue-type plasminogen activator, and urinary-type plasminogen activator (uPA), as playing an important role in regulating angiogenesis. PAI-1 exerts both proangiogenic and antiangiogenic properties, with the concentration of PAI-1 being an important determinant of which effect is observed. The proangiogenic effect of PAI-1 seems to be mediated by stabilizing the extracellular matrix (ECM) through inhibition of proteolysis and by stimulation of fibronectin-dependent cell migration. The antiangiogenic effect of PAI-1 seems to be mediated by binding of PAI-1 to vitronectin, an ECM protein that promotes cell adhesion and migration by binding to cell surface receptors, including α₃β₃ integrin, and to the uPA receptor (uPAR). The binding site for PAI-1 on vitronectin overlaps with vitronectin’s binding sites for α₃β₃ and uPAR. Therefore, binding of PAI-1 to vitronectin blocks cell adhesion and migration, and hence, inhibits angiogenesis.

Although PAI-1 regulates angiogenesis through downstream effects on ECM proteolysis and cell migration, no studies have implicated PAI-1 in controlling upstream signaling pathways that initiate angiogenesis. Critical among these is the activation of endothelial cells, mediated by binding of a vascular endothelial growth factor (VEGF) to its main angiogenic receptor, VEGF receptor-2 (VEGFR-2). A cooperative binding interaction between VEGFR-2 and α₃β₃ integrin plays a key role in regulating VEGF signaling in endothelial cells. This receptor cross talk depends on binding of α₃β₃ to vitronectin. Given that PAI-1 regulates binding of α₃β₃ to vitronectin, we hypothesized that PAI-1 regulates VEGF-mediated endothelial cell activation by disrupting VEGFR-2–α₃β₃ cross talk. In this study, we performed a series of biochemical, cell culture, and in vivo experiments to test our hypothesis. Our
results identify a previously unrecognized role of PAI-1 in regulating VEGF signaling, which is vitronectin-dependent and mediated by 2 distinct PAI-1 ligands.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results

PAI-1 Blocks VEGFR-2-β3 Integrin Complex Formation
Vitronectin is a major ligand for αβ3, and it significantly enhances VEGF-mediated activation of endothelial cells via VEGFR-2.10-11 Given that PAI-1 binds to vitronectin and blocks its binding to αβ3,6 we hypothesized that PAI-1 inhibits the vitronectin-dependent binding interaction between αβ3 and VEGFR-2. To test this hypothesis, we cultured human umbilical vein endothelial cells (HUVECs) in vitronectin-coated wells and prepared cell extracts before and after treating the cells with VEGF in the absence or in the presence of PAI-1-wild-type (WT). VEGFR-2-αβ3 complexes in extracts were captured with an immobilized anti-VEGFR-2 antibody and detected by Western blotting with an anti-β3 integrin antibody. Coimmunoprecipitation of β3 integrin and VEGFR-2 was significantly enhanced by VEGF and inhibited by PAI-1-WT (Figure 1A), suggesting that VEGF induces VEGFR-2-αβ3 complex formation and PAI-1 inhibits this process.

PAI-1 Inhibits VEGFR-2 Phosphorylation, Internalization, and Signaling
To determine whether PAI-1 inhibits VEGF-induced VEGFR-2 activation, we cultured HUVECs in wells coated with collagen, fibronectin, or vitronectin in the presence of PAI-1-WT or vehicle control. After 4 hours, VEGF or vehicle control was added for 10 minutes, after which cell lysates were captured with an immobilized anti-VEGFR-2 antibody and detected by Western blotting with an activated protein kinase (1E). PAI-1-WT also inhibited VEGF-induced phosphorylation of focal adhesion kinase and p44/42 mitogen-activated protein kinases (1E). PAI-1-WT also inhibited VEGF-induced coimmunoprecipitation of VEGFR-2 and β3 integrin, and phosphorylation of both VEGFR-2 and p44/p42 in human microvascular endothelial cells (Figure III in the online-only Data Supplement). We did not observe any significant detachment of HUVECs from wells in response to PAI-1 treatment (data not shown). As a whole, these results suggested that PAI-1 inhibits VEGF-induced VEGFR-2 activation by a vitronectin-dependent mechanism.

Inhibition of VEGF Signaling by PAI-1 Depends on Vitronectin and Low-Density Lipoprotein Receptor Family Member Binding
To determine which of functional interactions of PAI-1 mediate its effects on VEGFR-2 activation, we studied the capacity of recombinant PAI-1 proteins harboring specific loss-of-function mutations to inhibit VEGFR-2 phosphorylation. We performed these experiments with HUVECs whose endogenous PAI-1 expression was silenced with PAI-1 siRNA. This approach decreased PAI-1 expression by ≈80% (Figure 2A). Under these conditions, PAI-1-WT efficiently inhibited VEGF-R2 phosphorylation, as did PAI-1-R, a mutant with preserved vitronectin binding, but lacking anti-protease activity (Figure 2B). These results suggested that the anti-protease activity of PAI-1 was not absolutely required to inhibit VEGF-R2 activation and supported the hypothesis that PAI-1 inhibits VEGF signaling by a mechanism involving the PAI-1-vitronectin binding domain. Unexpectedly, PAI-1-AK, which retains full anti-protease activity, but does not
Figure 1. Plasminogen activator inhibitor-1 (PAI-1) inhibits vascular endothelial growth factor receptor-2 (VEGFR-2) signaling. A, PAI-1 inhibits β3 integrin–VEGFR-2 complex formation. Human umbilical vein endothelial cells (HUVECs) were cultured on vitronectin (VN) in the presence of vehicle control, VEGF (50 ng/mL), or VEGF and PAI-1-wild-type (WT) (10 μg/mL), as indicated (+ indicates added and − indicates omitted, replaced by vehicle control). Cell lysates were prepared and incubated with a resin-bound anti-VEGFR-2 antibody. Cap- tured proteins were analyzed by Western blotting with anti-β3 integrin antibodies. Representative images of 3 independent experiments are shown. *P<0.05 vs negative control. B, Inhibition of VEGF-induced VEGFR-2 phosphorylation by PAI-1 is VN-dependent. HUVECs were cultured on VN, collagen, or fibronectin in the presence of vehicle control, VEGF (50 ng/mL), VEGF, and PAI-1-WT (10 μg/mL), or PAI-1-WT, as indicated. *P<0.05 vs negative control. Internalized VEGFR-2 was detected by Western blotting. C, PAI-1 inhibits VEGFR-2 endocytosis. HUVEC surface proteins were biotinylated, after which cells were cultured in the presence of vehicle control, VEGF, or VEGF and PAI-1-WT, as shown. Internalized VEGFR-2 was detected by Western blotting. D, PAI-1 inhibits translocation of VEGFR-2 to perinuclear endosomes. HUVECs were cultured on VN in the presence of vehicle control, VEGF (50 ng/mL), VEGF, and PAI-1-WT (10 μg/mL), or PAI-1-WT, as indicated. VEGFR-2 and early endosome antigen 1 (EEA1) were detected by immunofluorescence confocal microscopy. 4′,6-Diamidino-2-phenylindole (DAPI) (nuclear) and merged VEGFR-2/EEA1/DAPI images are also shown. Images are representative of 3 independent experiments. *P<0.05 vs negative control group (first bar).

bind to vitronectin, also inhibited VEGF-induced VEGFR-2 phosphorylation (Figure 2B). This result suggested that the PAI-1–vitronectin binding interaction was sufficient, but not required, for PAI-1 to inhibit VEGFR-2 signaling. It also suggested that although PAI-1-mediated downregulation of VEGF signaling was vitronectin-dependent, direct binding of PAI-1 to vitronectin was not absolutely required to exert this effect. In addition to a vitronectin binding site, PAI-1 contains a cryptic binding site for low-density lipoprotein (LDL) receptor–related protein 1 and related endocytic receptors of the LDL receptor family, which becomes exposed after PAI-1 binds to uPA.15 We hypothesized that binding of PAI-1 to an endocytic receptor inhibits VEGF-2 activation by VEGF because endocytic receptor binding capacity is retained in PAI-1-AK.16 Therefore, we studied the capacity of PAI-1, a mutant with markedly reduced binding capacity for LDL receptor family members,15 to inhibit VEGF-induced VEGFR-2 phosphorylation. PAI-E inhibited VEGF-induced VEGFR-2 phosphorylation (Figure 2B). However, because the inhibitory effect of PAI-1-E could be mediated by its retained capacity to bind to vitronectin, we also constructed a compound mutant, PAI-1-AKE, which contained all of the mutations in PAI-1-AK and PAI-1-E. PAI-1-AKE retains full antiprotease activity, but does not bind to vitronectin or LDL receptor family members with high-affinity. Unlike PAI-1-AK and PAI-1-E, PAI-1-AKE did not significantly inhibit VEGF-induced VEGFR-2 phosphorylation (Figure 2B). Consistent with this result, the receptor-associated protein (RAP), which...
blocks binding of PAI-1 to LDL receptor–related protein 1 and other LDL receptor family members, completely inhibited the capacity of PAI-1-AK to downregulate VEGFR-2 phosphorylation (Figure 2C). As a whole, these findings suggested that PAI-1 binding to either vitronectin or an endocytic receptor was sufficient to inhibit VEGFR-2 signaling. We also studied the capacity of PAI-AK to inhibit VEGFR-2 phosphorylation in the presence of a synthetic peptide, D2A-Ala, which blocks binding of uPAR to αvβ3. D2A-Ala potently inhibited the capacity of PAI-1-AK to disrupt VEGFR-2 activation, whereas a control (scrambled) peptide had no significant effect (Figure IV in the online-only Data Supplement), suggesting that PAI-1 binding to an endocytic receptor family member inhibits VEGFR-2 signaling by a pathway involving a uPAR–integrin binding interaction.

**PAI-1 Inhibits VEGF-Induced Endothelial Cell Adhesion, Migration, and Cord Formation in a Vitronectin-Dependent Manner**

We examined the effects of PAI-1 on the physiological responses of endothelial cells to VEGF stimulation. In the absence of added PAI-1, VEGF significantly stimulated adhesion and migration of HUVECs grown on vitronectin, collagen, or fibronectin (Figure 3A and 3B, white bars), with the stimulatory effect being maximal in cells grown on vitronectin. In the absence of added VEGF, PAI-1-WT and PAI-1-R each significantly inhibited, but did not completely block, adhesion and migration of HUVECs cultured in vitronectin-coated wells, whereas PAI-1-AK had no significant effect, consistent with its defect in vitronectin binding. However, PAI-1-AK completely inhibited the upregulation of adhesion and migration induced by VEGF in HUVECs cultured on vitronectin, as did PAI-1-WT and PAI-1-R. PAI-1-WT, PAI-1-R, and PAI-1-AK had no significant effect on adhesion or migration of HUVECs cultured on fibronectin or collagen, either in the presence or in the absence of added VEGF.

VEGF stimulates cultured endothelial cells to form cord-like structures that mimic forming blood vessels. To examine the effect of PAI-1 on this process, HUVECs were cultured on Matrigel formed in the presence or in the absence of supplemental vitronectin. PAI-1-WT inhibited the augmentation of tubule formation by VEGF in vitronectin-supplemented

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**Figure 2.** Inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) phosphorylation by plasminogen activator inhibitor-1 (PAI-1) depends of vitronectin (VN) and endocytic receptor binding. A, Human umbilical vein endothelial cells (HUVECs) were transfected with a human PAI-1 siRNA expression vector or a control (Con-siRNA) plasmid. Western blot analysis of PAI-1 and β-actin expression in transfected HUVECs is shown. B, PAI-1-silenced HUVECs were incubated with recombinant PAI-1 (wild-type [WT], AKE, E, R, or AK mutant) or vehicle control (−) and stimulated with VEGF (+, 50 ng/mL) or vehicle control (−), as shown. Cell lysates were prepared and phosphorylated (p) and total VEGFR-2 were assessed by Western blotting. C, Preincubation of HUVECs with a receptor-associated protein (RAP, 0.2 μM) blocks the capacity of PAI-1-AK to inhibit VEGF-induced VEGFR-2 phosphorylation. All graphs correspond to the blots above them and represent densitometric analyses of 3 independent experiments. *P<0.05 vs negative control (first bar).
Matrigel, but not in Matrigel-lacking supplemental vitronectin (Figure 3C and 3D).

To explore the functional significance of interactions of PAI-1 with vitronectin and endocytic receptor(s) in regulating VEGF signaling further, we studied the effects of recombinant PAI-1 mutants on VEGF-induced migration of vitronectin-adherent HUVECs in the presence and in the absence of RAP. In the absence of RAP, PAI-1-WT, PAI-1-R, and PAI-1-AK each inhibited VEGF-induced migration (Figure VA in the online-only Data Supplement, bars 6–8). In the presence of RAP, PAI-1-WT and PAI-1-R each retained the capacity to inhibit VEGF-induced migration (bars 9–10), whereas the inhibitory effect of PAI-1-AK was lost (bar 11). Furthermore, in a separate set of experiments performed in the absence of RAP, PAI-1-AKE did not inhibit VEGF-induced HUVEC migration (Figure VB in the online-only Data Supplement, bar 4), whereas PAI-1-E, which is defective in endocytic receptor binding, but retains normal vitronectin binding affinity, inhibited VEGF-induced HUVEC migration (bar 5). Together, these results supported the functional significance of PAI-1 binding to either vitronectin or an LDL receptor family member in regulating VEGF signaling. They also suggested that the endocytic-receptor–dependent role of PAI-1 in VEGF signaling although not requiring binding of PAI-1 to vitronectin, still exhibited vitronectin-dependence because the effect of PAI-1-WT, which binds to LDL receptor–related protein 1 and other LDL receptor family members, was lost in the absence of vitronectin.

**Binding of PAI-1 to Very Low-Density Lipoprotein Receptor Inhibits VEGF Signaling**

Because the very LDL receptor (VLDLR) is thought to be the primary LDL receptor family member present on HUVECs, we examined whether the ability of PAI-1-AK to inhibit VEGF-induced cell migration could be reversed by an antibody that specifically blocks ligand binding to VLDLR. These studies demonstrated that the VLDLR antibody completely inhibited the anti-VEGF effect of PAI-1-AK (Figure VC in the online-only Data Supplement), suggesting that
VEGF-Induction of Angiogenesis
Ex Vivo and In Vivo is Vitronectin-Dependent and Inhibited by PAI-1

To examine the role of vitronectin in VEGF-induced angiogenesis under physiological conditions, we cultured segments of aorta from WT and Vn−/− mice ex vivo in Matrigel in the presence or in the absence of VEGF. The capacity of VEGF to stimulate microvessel sprouting was significantly greater in WT mice than in Vn−/− mice (Figure 4A and 4B). Immunostaining confirmed the presence of vitronectin in aorta of WT mice (Figure VIA in the online-only Data Supplement). PAI-1-WT significantly inhibited VEGF-induced microvessel sprouting from WT aorta; however, PAI-1-AKE had no significant effect (Figure 4C). To study the significance of our findings in vivo, we injected VEGF-impregnated Matrigel into subcutaneous tissue of WT and Vn−/− mice. After 14 days, solidified gels were retrieved, and blood vessel invasion into them was measured. VEGF significantly increased endothelial cell invasion into Matrigels in WT mice, but not in Vn−/− mice (Figure 4D and 4E). Immunostaining confirmed that host-derived vitronectin diffused into Matrigel implants (Figure VIB in the online-only Data Supplement). To determine whether PAI-1 inhibits VEGF-induced angiogenesis in vivo, we injected Matrigel supplemented with PAI-1-WT (10 μg/mL) into WT and Vn−/− mice. PAI-1-WT completely inhibited VEGF-induced angiogenesis in WT mice, but had no significant effect in Vn−/− mice (Figure 4D and 4E). These results suggested that PAI-1 and vitronectin play key roles in regulating VEGF-induced angiogenesis in vivo, and that PAI-1 could be used to inhibit VEGF-induced angiogenesis pharmacologically. However, downregulation of fibrinolysis by active PAI-1 could promote thrombosis. Given that PAI-1-R does not inhibit plasminogen activators, we tested its capacity to inhibit VEGF-induced angiogenesis in vivo in WT mice. PAI-1-R significantly inhibited VEGF-induced angiogenesis (Figure 4F and 4G).
Pharmacological Inhibition of PAI-1 Promotes Reperfusion of Ischemic Hindlimb Tissue

To examine the significance of our findings in a pathological model of PAI-1 overexpression relevant to human cardiovascular disease, we fed mice high-fat chow for 14 weeks, which produced obesity, hyperglycemia, and a significant increase in plasma PAI-1 concentration (Figure VII in the online-only Data Supplement). Hindlimb ischemia was induced in high-fat chow-fed mice by ligation and excision of the femoral artery, after which mice received PAI-039, a specific PAI-1 inhibitor, or vehicle control. After 14 days of treatment, plasma PAI-1 activity was significantly lower in PAI-039–treated mice (0.61±0.09 ng/mL) than in controls (2.66±0.35 ng/mL; *P<0.05; n=6 per group). Laser Doppler imaging revealed that the recovery of perfusion of ischemic hindlimb tissue after femoral artery interruption was significantly increased in PAI-039–treated mice compared with vehicle controls (Figure 5A and 5B). Consistent with these results, arteriole density in ischemic gastrocnemius muscle 14 days after induction of ischemia was significantly increased in PAI-039–treated mice versus controls (Fig. 5C and 5D). Capillary density in ischemic gastrocnemius muscle did not differ significantly between experimental groups (P>0.5). HPF indicates high-power field.

Discussion

We describe a previously unrecognized role of PAI-1 in regulating VEGF-induced activation of its main angiogenic receptor, VEGFR-2. Specifically, PAI-1 inhibits VEGFR-2 phosphorylation and translocation to perinuclear endosomes, as well as intracellular signaling downstream of VEGFR-2. The antiprotease function of PAI-1 is not absolutely required for it to inhibit VEGFR-2 activation because PAI-1-R, a mutant devoid of antiprotease activity, inhibited VEGF signaling. This observation is consistent with the high-affinity binding interaction between PAI-1 and vitronectin, which inhibits binding of vitronectin to its cell surface receptors. Our data suggest that the antiangiogenic property of PAI-1-R is mediated by its capacity to competitively inhibit binding of αVβ3 integrin to vitronectin. However, an intriguing aspect of our study is the observation that inhibition of VEGF-2 activation and angiogenic signaling by PAI-1, while requiring vitronectin, is not mediated solely by binding to vitronectin, as evidenced by the preserved antiangiogenic effect.
of PAI-1-AK, which has no measurable vitronectin binding affinity. Experiments examining how PAI-1 can inhibit VEGF signaling in a vitronectin-dependent manner without binding vitronectin led us to uncover another previously unrecognized regulator of VEGFR-2 activation, which seems to be the VLDLR. This conclusion is supported by 3 lines of evidence. First, the antiangiogenic activity of PAI-1-AK is inhibited by RAP, an antagonist of LDL receptor family members; second, the antiangiogenic activity of PAI-1-AK is lost on introduction of an additional glutamate mutation (PAI-1-AKE) that ablates the capacity of PAI-1to bind to LDL receptor family members; and third, the ability of PAI-1-AK to inhibit VEGF-induced HUVEC migration is blocked by an antibody directed against the ligand binding repeats of VLDLR. These results suggest that VLDLR, which is expressed by endothelial cells, is the LDL receptor family member that mediates the antiangiogenic effect of PAI-1 and is consistent with a study showing that VLDLR signaling exerts an antiangiogenic effect on retinal endothelial cells.

On the basis of our data and published studies, we hypothesize that αvβ3 integrin and its ligand, vitronectin, underpin the capacity of PAI-1 to inhibit VEGFR-2 activation, whether by binding to vitronectin or VLDLR, as illustrated in Figure 6. Previous studies showed that αvβ3 and vitronectin potently enhance VEGFR-2 activation by VEGF, demonstrating a key role of αvβ3–VEGFR-2 cross talk in VEGF signaling and the vitronectin-dependent nature of this interaction (Figure 6A). By binding to the somatomedin B domain of vitronectin, PAI-1 competitively inhibits binding of αvβ3 to the vitronectin’s immediate adjacent integrin binding site. Our experiments involving recombinant PAI-1 mutants suggest that blockade of the αvβ3–vitronectin binding interaction by binding of PAI-1 to vitronectin directly uncouples αvβ3–VEGFR-2 cross talk and downregulates VEGF signaling (Figure 6B). Alternatively, binding of PAI-1 to VLDLR (most likely in the form of PAI-1–uPA complex) has the potential to uncouple αvβ3 from VN, thereby uncoupling the proangiogenic binding interaction between αvβ3 and VEGFR-2, which downregulates VEGF signaling (Figure 6C). Our data suggest that binding of PAI-1 to VLDLR is required for PAI-1 to dissociate

![Figure 6. Proposed model by which plasminogen activator inhibitor-1 (PAI-1) inhibits vascular endothelial growth factor (VEGF) signaling.](http://arch.ahajournals.org/content/ajahd/1/1/1560)
αβ3 from vitronectin functionally via the pathway that does not require direct PAI-1 binding to vitronectin, because the antiangiogenic effect of PAI-1-AK (which does not bind to vitronectin, but binds to the uPA–uPAR complex) was lost on introduction of an additional mutation into PAI-1-AK that ablated LDL receptor family member binding. The VLDLR-dependent antiangiogenic effect of PAI-1 could be mediated by endocytic-receptor–mediated internalization of αβ3 (along with PAI-1, uPA, and uPAR, as has been described)23,26 or by conformational changes in αβ3 that are not accompanied by its cellular internalization.25 This issue was not resolved by our experiments. However, we showed that peptide-mediated inhibition of uPAR–integrin binding potently inhibited the anti-VEGF effect of PAI-1-AK, suggesting that PAI-1 disrupts VEGF signaling by a pathway involving uPAR–integrin complex formation. Consistent with our results, Alexander et al.27 showed that uPAR is an important determinant of VEGF signaling in endothelial cells. Additional studies are necessary to define the molecular events underlying the VLDLR-dependent downregulation of VEGFR-2 signaling by PAI-1 more precisely.

Our experiments involving microvessel sprouting from aortic rings ex vivo and microvessel invasion into subcutaneous ECM implants in vivo suggest that PAI-1 is a physiologically relevant regulator of VEGFR-2 activation. On the basis of our data, we hypothesize that enhanced PAI-1 expression, which occurs under pathological conditions, such as diabetes mellitus,28 inhibits VEGFR-2 activation in vivo. Consistent with this hypothesis, we showed that PAI-039, a highly specific pharmacological inhibitor of PAI-1 with no discernable effect on angiogenesis in PAI-1-deficient mice,29,30 promoted the recovery of tissue perfusion and development of collateral arterioles after induction of hindlimb ischemia in mice with diet-induced PAI-1 overexpression. Previous studies have shown that collateral arteriole development and recovery of tissue perfusion after femoral artery occlusion are dependent on VEGFR-2 activation by VEGF.31,32 Therefore, our findings with PAI-039 and another murine study involving pharmacological PAI-1 inhibition33 support the in vivo relevance of the inhibition of VEGFR-2 activation by PAI-1. We did not observe an increase in capillary density in ischemic gastrocnemius muscle in response to PAI-039 treatment. However, this does not rule out an effect of PAI-1 on capillary remodeling. Furthermore, the dominant determinant of tissue reperfusion after femoral artery ligation is collateral arteriole development,34 and we observed a positive nant of tissue reperfusion after femoral artery ligation is col-

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We did not observe an increase in capillary density in ischemic
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However, this does not rule out an effect of PAI-1 on
capillary remodeling. Furthermore, the dominant determi-
nant of tissue reperfusion after femoral artery ligation is
collateral arteriole development,34 and we observed a positive
effect of PAI-039 on this angiogenic response. A limitation of
our hindlimb ischemia model experiment is that we can-
not definitively conclude that pharmacological inhibition of
PAI-1 promoted tissue reperfusion solely by blocking the
negative effect of PAI-1 on VEGFR-2 activation, because
pharmacological inhibition of PAI-1 could potentially
modulate the angiogenic response to ischemia by VEGFR-
2–independent pathways. Nevertheless, our hindlimb isch-
emia model data support the significance of our proposed
molecular mechanisms in a clinically relevant, in vivo con-
text, thereby complementing our cell culture, ex vivo aortic
ring, and in vivo Matrigel plug data, which demonstrated
that PAI-1 inhibits VEGFR-2 activation. Additional in vivo
studies will be necessary to dissect further and characterize
better the significance of our newly reported regulatory path-
way on VEGF-dependent angiogenic signaling in normal
vascular development and other disease models.

In summary, we have shown that PAI-1 inhibits VEGFR-2
activation by VEGF in a vitronectin-dependent manner. Our
data suggest that PAI-1 mediates its effect by inhibit-
ing binding of αβ3 to its ECM ligand, vitronectin, thereby
disrupting the proangiogenic binding interaction between
αβ3 and VEGFR-2. The antiangiogenic effect of PAI-1
seems to be mediated, although not exclusively, by direct
binding to vitronectin, which competitively blocks binding
of vitronectin to αβ3. We also have shown that binding of
PAI-1 to an endocytic receptor of the LDL receptor family
inhibits VEGFR-2 activation, and that this effect is inhib-
ited by an antibody that blocks ligand binding to VLDLR.
Our results suggest that VLDLR plays an important role
in VEGF signaling, one which is sensitive to inhibition by
PAI-1 and mediated by functional disengagement of αβ3
from vitronectin. We have shown that the anti-VEGF effect
of PAI-1 is evident under physiological conditions in vitro
and in vivo. Overall, our results broaden our understanding
of the roles of PAI-1, vitronectin, and the LDL receptor
family in regulating angiogenic signaling. They also sup-
port proceeding to additional preclinical studies involving
recombinant PAI-1 mutants and pharmacological PAI-1
inhibitors as therapeutic strategies to inhibit or promote
angiogenesis in vivo.

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Disclosures
None.

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The plasminogen activation system regulates a diverse array of mammalian physiological and pathological processes. We report a previously unrecognized role of plasminogen activator inhibitor-1, a central member of the plasminogen activation system, in regulating the activation of vascular endothelial growth factor receptor-2. We show that plasminogen activator inhibitor-1 inhibits vascular endothelial growth factor receptor-2 activation by blocking the proangiogenic binding interaction between vascular endothelial growth factor receptor-2 and α<sub>V</sub>β<sub>3</sub> integrin, and that the antiangiogenic effect of plasminogen activator inhibitor-1 involves 2 pathways, one entailing binding to vitronectin and another requiring binding to a very-low-density lipoprotein receptor. Our findings significantly expand our understanding of the roles of plasminogen activator inhibitor-1, vitronectin, and the low-density lipoprotein receptor family in regulating angiogenic signaling and suggest novel strategies for pharmacologically controlling angiogenesis.
Integrin Cross Talk

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Endothelial Growth Factor Receptor-2- αVβ3 Integrin Cross Talk
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Supplement Material

Supplementary Figures

Supplementary Figure I. The inhibition of VEGFR-2 activation by PAI-1 is dose-dependent. (A) HUVECs were seeded in wells coated with VN. After achieving 80% confluency, cells were incubated with or without PAI-1-WT as indicated for 4 hr, followed by addition of VEGF (50 ng/mL) for 5 min. Cell lysates were prepared and analyzed for phosphorylated (pVEGFR-2) and non-phosphorylated VEGFR-2. (B) Densitometric analysis of 3 independent experiments. *P<0.05 vs. control; **P<0.05 vs. HUVECs incubated with VEGF in the absence of any PAI-1-WT.

![Graph A](image)

![Graph B](image)
**Supplementary Figure II.** (A). PAI-1-WT does not bind to immobilized VEGF. (B). PAI-1-WT does not inhibit VEGF binding to VEGFR-2/Fc. Anti-VEGF monoclonal antibody 2C3 (ATCC, which blocks binding of VEGF to VEGFR-2), was used as a positive control.
Supplementary Figure III. PAI-1 inhibits VEGFR-2 signaling in human microvascular endothelial cells (HMVECs). (A) PAI-1 inhibits VEGF-induced β3 integrin-VEGFR-2 complex formation and VEGFR-2 phosphorylation. HMVECs were grown in vitronectin (VN)-coated wells and incubated with vehicle control (-), VEGF (50 ng/mL), or VEGF and PAI-1-WT (10 µg/mL), as indicated. To assess β3 integrin-VEGFR-2 complex formation, cell lysates were prepared and incubated with resin-bound anti-VEGFR-2 antibody. Captured proteins were analyzed by Western blotting using anti-β3 integrin anti-VEGFR-2 antibodies. To assess VEGFR-2 phosphorylation, cell lysates were subjected to Western blotting using anti-phosphorylated-VEGFR-2 (pVEGFR-2) antibody. (B) PAI-1-WT inhibits VEGF-induced phosphorylation of p44/p42. HMVECs cell lysates, prepared as described in panel A, were subjected to Western blotting using antibodies specific for phosphorylated-p44/p42 (Phos-p44/p42) and total p44/p42. For both panel A and panel B, representative images and band densitometry analysis of 3 independent experiments are shown. *P<0.05 vs. negative control (HUVECs not treated with either VEGF or PAI-1-WT; i.e. 1st lane and 1st bar of graphs).
Supplementary Figure IV. Inhibition of VEGFR-2 phosphorylation by PAI-1-AK is blocked by a peptide (D2A-Ala) which inhibits uPAR-integrin binding interactions. HUVECs were grown in vitronectin-coated wells and treated with control peptide or D2A-Ala (100 pM) for 30 min, as shown. Thereafter, HUVECS were treated with vehicle control (-), VEGF (50 ng/mL), VEGF and PAI-1-AK (10 µg/mL), or PAI-1-AK, as shown, after which cell lysates were prepared and analyzed by Western blotting using antibodies specific for phosphorylated VEGFR-2 (pVEGFR-2) and total VEGFR-2. A representative blot is shown as well as band densitometry analysis of 3 independent experiments. Band intensities were normalized to control conditions, which were HUVECs grown in presence of control peptide, but not treated with either VEGF of PAI-1-AK (i.e. 1st bar). *P<0.05 vs. control conditions.
Supplementary Figure V. PAI-1 inhibits VEGF-induced HUVEC migration by binding to very-low-density-lipoprotein receptor (VLDLR). (A) The inhibitory effect of PAI-1-AK (but not of PAI-1-WT or PAI-1-R) on VEGF-induced HUVEC migration is blocked by RAP. HUVECs were incubated for 30 min on VN-coated porous membranes in presence (+) of RAP (0.2 µM) or vehicle control (-), after which recombinant PAI-1 (WT, R, or AK forms, each at 10 µg/mL) or vehicle control (-) was added, followed 30 min later by stimulation with VEGF (50 ng/mL, +) or vehicle control (-), as shown. After 24 hr cell migration through pores was measured. (B) PAI-1-AKE (which is defective in VN and endocytic receptor binding) does not inhibit VEGF-induced HUVEC migration. (C) Ligand-blocking antibody to VLDLR inhibits the anti-VEGF effect of PAI-1-AK. HUVECs were treated sequentially with 1) anti-VLDLR IgG, control (non-immune) IgG, or vehicle control; 2) PAI-1-AK or vehicle control; and 3) VEGF or vehicle control, as shown. Data shown are mean of triplicate experiments and expressed as % of control (1st bar of each graph). *P<0.05 vs. control. Groups other than those denoted by an asterisk did not differ significantly (P>0.05) vs. control.
Supplementary Figure VI. Vitronectin (VN) immuno-staining of murine tissues. (A) VN is present in mouse aorta. Thoracic aortic rings from wild-type (WT) mice and Vn<sup>−/−</sup> mice were incubated in serum-free medium for 24 hr, homogenized in RIPA buffer, and centrifuged. Supernatants were subjected to SDS-PAGE (reducing conditions) and Western blotting using anti-VN antibody. A sample of wild-type murine plasma was analyzed as a positive control. To assess for equal loading of wild-type and Vn<sup>−/−</sup> aorta samples, blots were stripped and re-probed with an antibody against β-actin (expected to be present in aorta, but not in plasma). (B) VN is present in Matrigel plugs retrieved from WT mice, but is not detectable in Matrigel plugs retrieved from Vn<sup>−/−</sup> mice. Plugs were retrieved 14 days after insertion. Cross-sections were prepared and subjected to immuno-histochemical staining with anti-VN antibody. Nuclei were stained with methyl green. Brick red color represents positive VN immuno-staining. Data shown are representative of 3 plugs retrieved from each experimental group. Scale bar = 5 µm.
**Supplementary Figure VII.** High-fat diet induces obesity, hyperglycemia, and increased plasma PAI-1 in mice. Mice were fed high-fat chow (HFC) or normal chow (NC) for 14 weeks, after which (A) body weight, (B) plasma glucose, (C) plasma PAI-1 antigen, and (D) plasma PAI-1 activity were measured (n=6/group); *P<0.05 vs. NC group.
Materials and Methods

Recombinant PAI-1 mutants
Recombinant human PAI-1 was expressed and purified as described.¹ These were: 1) PAI-1-14-1b (PAI-1 N150H, K154T, Q319L, M354I), which inhibits u-PA and t-PA and binds VN with wild-type (WT) activities. PAI-1-14-1b is resistant to conversion to the inactive (i.e. latent) form (half-life >140 hrs). Throughout this study PAI-1-14-1b is referred to as “PAI-1-WT.” 2) PAI-1-R (T333R, A335R), a reactive center loop mutant that binds VN normally, but has no detectable anti-proteolytic activity and cannot assume a latent conformation.² 3) PAI-1-AK (PAI-1 N150H, K154T, Q319L, M354I, R101A, Q123K), an active, stable mutant with no detectable VN binding.³ 4) PAI-1-E (PAI-1 N150H, K154T, Q319L, M354I, R76E), an active stable mutant with markedly reduced binding affinity for LRP1 and other LDL receptor family members, but normal VN binding affinity.⁴ 5) PAI-1-AKE (PAI-1 N150H, K154T, Q319L, M354I, R101A, Q123K, R76E), an active, stable mutant with markedly reduced binding to LRP1 and other LDL receptor family members and no detectable binding to VN.

Reagents
Collagen type I was from Sigma. Growth-factor-reduced BD Matrigel Matrix (BD Biosciences) and anti-α,β3 integrin antibody were from Chemicon International. Human VN was from Promega. Human fibronectin and antibodies to focal adhesion kinase (FAK), β3 integrin, platelet/endothelial cell adhesion molecule-1 (PECAM-1), and PAI-1 were from Santa Cruz Biotechnology. Mouse anti-human early endosome antigen 1 (EEA1) antibody was from Abcam. Antibodies to phosphorylated and non-phosphorylated p44/42, phosphorylated VEGFR-2 (Tyr 1175), and total VEGFR-2 were from Cell Signaling Technology. Antibody against very low density lipoprotein receptor (VLDLR), which specifically blocks ligand binding to this receptor (clone 1H10), was from Molecular Innovations.⁵ Rat anti-mouse VN monoclonal antibody was from R&D Systems. Anti-smooth muscle α-actin antibody was from Sigma. Anti-β actin polyclonal antibody was from Cell Signaling Technology. Recombinant VEGF-A was from R&D Systems. The latent form of human PAI-1 and receptor-associated protein (RAP) were from Molecular Innovations. Anti-VEGF monoclonal antibody 2C3 was from ATCC. Peptide D2Ala (IQEQAAGRPKDDR), derived from domain 2 of uPAR, and which blocks uPAR binding to αvβ3,⁶ as well as a control (scrambled) peptide (DEIGQDKERPGRE) were from NeoBioLab. PAI-039, a pharmacological inhibitor of PAI-1, was from Pfizer.

Animals
C57BL/6J mice were from Jackson Labs. C57BL/6J-congenic VN-deficient (Vn⁻) mice were a gift from Dr. David Ginsburg, University of Michigan.⁷ All animal care and experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Cell culture
Human umbilical vein endothelial cells (HUVECs, Cascade Biologics) and human dermal microvascular endothelial cells (Cascade Biologics) were grown in Medium 200 (Cascade Biologics) containing low-serum growth supplement. Cells used were passaged 3-7 times.

Immunoassays
To screen for potential binding of PAI-1 to VEGF, microtiter plate wells were coated with VEGF. After washing and blocking, PAI-1-WT (0.01-10 µg/mL) was added and incubated at room temperature for 1hr, after which biotinylated anti-PAI-1 antibody and streptavidin-HRP conjugate were sequentially added. After washing wells, HRP substrate was added and OD450 was measured. To screen for potential inhibition of binding of VEGF to VEGFR-2 by PAI-1, 96-well
plates were coated with recombinant VEGFR-2/Fc (R&D Systems). After washing and blocking wells, VEGF (25 ng/mL) was added in the presence or absence of PAI-1-WT (0.01-10µg/mL) and incubated for 1h. Bound VEGF was detected by sequential addition of biotinylated anti-human VEGF antibody, streptavidin-HRP conjugate, and HRP substrate and measurement of OD450.

**Western blotting**
HUVECs cell lysates were prepared as described. Supernatants (25µg total protein) were subjected to SDS-PAGE (using either 7.5% homogeneous or 4-20% gradient acrylamide gels) and Western transfer. After blocking, membranes were incubated with rabbit or mouse IgG raised against human PAI-1, FAK, p44/42, or phosphorylated and non-phosphorylated forms of VEGFR-2. Secondary antibody was horseradish-peroxidase (HRP)-conjugated goat IgG against rabbit or mouse IgG (Santa Cruz Biotechnology). Blots were developed with ECL substrate (Pierce).

**VEGFR-2 internalization**
Internalization of VEGFR-2 was studied as described. HUVECs surface membrane proteins were biotinylated using Cell Surface Isolation Kit (Thermo Scientific). Cells were incubated with PAI-1 (10 µg/mL) or vehicle control for 1 hr, then stimulated with VEGF (50 ng/mL) or vehicle control for 10min. Cell surface biotin was removed with glutathione, after which cell lysates were prepared and incubated with NeutrAvidin Agarose (Thermo Scientific). Captured proteins were subjected to SDS-PAGE and Western blotting with anti-VEGFR-2 antibody. Internalization of VEGFR-2 was also studied by immunofluorescence microscopy, as described, with minor modification. Cell surface VEGFR-2 was labeled by incubating HUVECs grown on VN-coated coverslips with rabbit anti-human-VEGFR-2 antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit-IgG (Invitrogen). Cells were incubated with PAI-1-WT (10 µg/mL) or vehicle control for 4 hr, followed by VEGF (50 ng/mL) or vehicle control for 30 min. Cells were washed with ice-cold phosphate-buffered saline (PBS, pH 2.5) to remove remaining cell-surface labeled proteins, fixed with 4% paraformaldehyde, and permeabilized by treatment with 2% BSA,5% goat serum, and 0.1% Triton X-100 for 30 min. Cells were incubated with mouse anti-human EEA1 antibody followed by Alexa Fluor 647-conjugated goat anti-mouse IgG (Invitrogen). Nuclei were stained with DAPI, after which immunofluorescent staining was observed with an LSM 510 2-photon confocal microscope (Zeiss).

**Silencing of PAI-1 gene expression**
HUVECs were transfected with siRNA duplexes (5nM) directed against human PAI-1 (target sequence TTCACGAGTCTTTTCGACCAA) or negative control siRNA (Qiagen) using HiPerFect reagent (Qiagen).

**Co-immunoprecipitation**
HUVECs (2x10^5) were incubated 4 hr in wells pre-coated with VN (10 µg/mL). PAI-1 (10 µg/mL) was added. One hr later cells were stimulated with VEGF (50 ng/mL) for 10min, then lysed in RIPA buffer (Cell Signaling Technology). Protein co-immunoprecipitation was performed with
Co-IP kit (Pierce). Anti-VEGFR-2 antibody was immobilized on coupling resin and incubated with lysates that were pre-cleared with control agarose resin. After extensively washing anti-VEGFR-2-bearing resin, bound proteins were eluted, resolved by SDS-PAGE, and analyzed by Western blotting. VEGFR-2 and β3 integrin subunit were detected with appropriate antibodies. Secondary antibody was HRP-conjugated goat IgG raised against rabbit IgG (Santa Cruz Biotechnology).

Cell adhesion, migration, and tubule formation assays
HUVECs adhesion was studied with InnoCyte™ ECM Cell Adhesion Assay kit (Calbiochem) using 96-well plates coated with VN, fibronectin, or collagen. Cells (1 x 10⁴) were added to wells, incubated 1 hr at 37°C, treated with recombinant PAI-1-WT (10 µg/mL) or vehicle control for 30 min, followed by addition of VEGF (50 ng/mL). HUVEC migration was studied using Transwell migration chambers (Life Technologies) with bottom membranes containing 8 µm pores. Membranes were coated with VN, fibronectin, or collagen, after which cells (2x10⁴) were added to the upper chamber and treated with PAI-1-WT (10 µg/mL) or vehicle control for 30 min, followed by VEGF (50 ng/mL). After 24 hr at 37°C membranes were excised and stained. Cells that migrated to the lower-chamber were counted. Formation of tubules by HUVECs was studied as described, using 24-well plates coated with growth-factor-reduced Matrigel. For some experiments VN (10 µg/mL) was added to Matrigel. A suspension of cells in culture medium was added into each coated well in the presence or absence of PAI-1 (10 µg/mL) 30 min before addition of VEGF (50 ng/mL). Cells were incubated 24 hr at 37°C, washed, fixed, and viewed through an IX70 inverted microscope (Olympus). Total tube length was measured in 5 fields by computer-assisted image analysis using ImagePro Plus software.

Ex vivo tissue culture
Tissue culture was performed as described previously with minor modifications. Briefly, thoracic aortic rings isolated from WT and Vn−/− mice were embedded between two layers of growth-factor-reduced Matrigel (250 µL/layer) in a 24-well plate in the presence of Medium 200 (Cascade Biologics; 300 µL), Medium 200 containing VEGF (50 ng/mL), Medium 200 containing VEGF and recombinant PAI-1 (10 µg/mL), or Medium 200 containing only PAI-1. Medium was changed every 3 days. Aortic rings were photographed 14 days later. The number of sprouting microvessels was quantified by computer-assisted images analysis using ImagePro Plus software.

In vivo angiogenesis assay
Matrigel implant experiments were performed as described, with minor modifications. Growth-factor-reduced Matrigel WT (300 µL) was injected into subcutaneous tissue of WT- and Vn−/− mice. Matrigel contained heparin (60 U/mL) and one of the following 1) VEGF (250 ng/mL), 2) VEGF and PAI-1 (10 µg/mL), 3) PAI-1, or 4) vehicle control, yielding 8 experimental groups (n=6/group). After 14 days, Matrigel implants were excised, fixed, and sectioned. PECAM-1-positive infiltrating microvessels were quantified in 5 microscopic fields in each of 3 cross-sections of each implant using ImagePro Plus software.

Mouse model of diet-induced PAI-1 over-expression
Obesity, hyperglycemia, and increased PAI-1 expression were induced in male C57BL/6J mice by feeding them a high-fat chow (HFC) diet (D12451; Research Diet, New Brunswick, NJ) for 14 weeks, as described previously. Age-matched male mice fed a normal chow (NC) diet served as controls. Blood glucose levels were measured from tail vein blood samples using an automatic glucometer (Accu-Check; Roche Diagnostics, Mannheim, Germany).

Mouse hindlimb ischemia model
Unilateral hindlimb ischemia was induced in mice by ligation and excision of a segment of the left femoral artery, as previously described. After recovering from surgery, mice were treated with PAI-039 (4 mg/kg/day; dissolved in vehicle consisting of sterile water containing 0.5% methylcellulose and 2% Tween 80), or vehicle control, administered for 14 days after surgery by twice daily oral gavage, with the first dose given 1 hour after surgery. PAI-039- and vehicle-treated mice continued to be fed HFC after surgery. Perfusion of the ischemic and non-ischemic hindlimb was measured in each mouse by laser-Doppler imaging (LDI) immediately before surgery, 1 hour after surgery, and at several subsequent time points using a moorLDI2-HIR high resolution laser Doppler imager (Moor Instruments). Mice were euthanized 14 days after surgery. Ischemic gastrocnemius muscle was excised, embedded in paraffin, and cross-sections were prepared for immunohistochemical analysis. Arterioles within gastrocnemius muscle were immunostained with anti-smooth muscle α-actin antibody and arteriole density was measured, as described previously. Capillaries within gastrocnemius muscle were immunostained with anti-platelet endothelial cell adhesion molecule (PECAM-1) antibody, and capillary density was measured as described previously.

**Measurement of plasma PAI-1**

Blood was collected into citrate anticoagulant and plasma was prepared by centrifugation. PAI-1 antigen was measured using a mouse PAI-1 total antigen assay ELISA kit (Molecular Innovations). PAI-1 activity was measured using an active mouse PAI-1 functional assay ELISA kit (Molecular Innovations).

**Data analysis**

Image analyses were performed in blinded fashion. Data are presented as mean ± standard error of the mean. Experimental groups were compared by the two-tailed Student’s t-test or one-way analysis of variance (ANOVA).

**References**


