Plasminogen Activator Inhibitor Type 1 Enhances Neointima Formation After Oxidative Vascular Injury in Atherosclerosis-Prone Mice

Yanhong Zhu, MD; Peter M. Farrehi, MD; William P. Fay, MD

**Background**—Plasminogen activator inhibitor type 1 (PAI-1) inhibits neointima formation after vascular injury. Hyperlipidemia modulates the expression of multiple genes, however, and the effects of PAI-1 on the arterial response to injury under hyperlipidemic conditions are unknown. The purpose of this study was to examine the impact of PAI-1 on intimal hyperplasia and other vascular changes that develop after arterial injury in apolipoprotein E–deficient (apoE−/−) mice.

**Methods and Results**—Ferric chloride injury of the midportion of the common carotid arteries of apoE−/− mice (n=22) induced formation of a neointima that contained smooth muscle cells, foam cells, neutral lipid, tissue factor, and von Willebrand factor. Interactions between vascular injury and apolipoprotein E deficiency were strongly synergistic; either stimulus alone was insufficient to induce significant neointima formation. Mean intima/media ratios were significantly greater (P<0.03) in apoE−/−, PAI-1+/+ mice (5.6±1.8, n=12) than in apoE−/−, PAI-1−/− mice (1.2±0.55, n=12), as were the percentages of bromodeoxyuridine-positive cells in the intima and media (P<0.03). Transiently occlusive (<48 hours) and nonocclusive mural thrombi persisted longer in apoE−/−, PAI-1−/− mice than in apoE−/−, PAI-1+/+ mice.

**Conclusions**—In atherosclerosis-prone mice, PAI-1 promotes neointima formation after oxidative vascular injury. The apparent hyperlipidemia-dependent effect of PAI-1 may be mediated by its capacity to inhibit the clearance of platelet-fibrin thrombi that can deliver growth factors to the blood vessel wall or be incorporated into developing vascular lesions. Alternatively, hyperlipidemia may alter the pattern of gene expression in the blood vessel wall to enhance potential effects of PAI-1 on antiproliferative processes, such as transforming growth factor-β activation and apoptosis. (Circulation. 2001;103:3105-3110.)

**Key Words:** plasminogen activators ■ apolipoproteins ■ vascular biology

The arterial response to injury is characterized by cell proliferation and migration. This response plays a key role in several arterial disorders, including atherosclerosis and intimal hyperplasia after balloon angioplasty.1 The plasminogen activation system is an important determinant of vascular remodeling after injury.2 Cell-associated plasmin mediates the migration of smooth muscle and inflammatory cells within the blood vessel wall.3,4 Plasmin also degrades fibrin-platelet thrombi, which can be incorporated into atherosclerotic lesions and contribute to their growth.5 Plasminogen activator inhibitor type 1 (PAI-1), the primary inhibitor of tissue plasminogen activator and urokinase, is a key regulator of the plasminogen activation system. PAI-1 is present in plasma and platelets, and it is synthesized by vascular endothelial and smooth muscle cells.6 In humans, PAI-1 deficiency is associated with abnormal bleeding, and elevated plasma and vascular wall PAI-1 levels are associated with atherosclerosis and myocardial infarction.7–9 Vascular cell migration after injury is inhibited by PAI-1.10 This effect appears to be mediated by inhibition of plasmin formation and possibly by PAI-1 binding to vitronectin, thereby preventing its interaction with vitronectin receptors present on vascular smooth muscle cells.11

The effects of PAI-1 on arterial remodeling after injury have been studied in animals with normal lipid metabolism.10,12 Hyperlipidemia modulates the expression of multiple genes that regulate the arterial response to injury, however, such as vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and monocyte chemotactant protein 1.13 The effects of PAI-1 on the arterial response to injury under hyperlipidemic conditions are unknown. The purpose of this study was to examine the impact of PAI-1 deficiency on intimal hyperplasia and other vascular wall changes that develop after arterial injury in apolipoprotein E–deficient (apoE−/−) mice. Our results suggest that PAI-1 contributes to neointima formation under these experimental conditions and...
support the hypothesis that disordered lipid metabolism plays an important role in modulating the effect of PAI-1 on the arterial response to injury.

**Methods**

**Animals**

ApoE−/− mice were purchased from Jackson Laboratories. PAI-1−/− deficient (PAI-1−−) mice were a gift from Dr Peter Carmeliet, University of Leuven, Leuven, Belgium. All mice were backcrossed ≥8 generations into the C57BL/6J genetic background. PAI-1−/− deficient mice and apolipoprotein E−/− deficient mice were crossed to generate apoE−/−. PAI-1−/− mice. Genotyping of mice was performed by polymerase chain reaction analysis of tail DNA. All animal care and experimental procedures complied with the “Principles of Laboratory Animal Care” established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

**Carotid Injury Protocol**

Ferric chloride injury of the carotid artery was performed on 6- to 10-week-old mice as reported previously. Vascular injury and thrombosis were induced by placing filter paper saturated with 10% ferric chloride on the adventitia of the midportion of the artery for 3 minutes. After the filter paper had been removed, the incision was sutured closed. Mice subsequently were fed either high-cholesterol chow (TD 88137, Harlan Teklad) or normal chow (Rudent Diet 5001, LabDiet). Four to 8 weeks after carotid injury, the inferior vena cava was exposed, and a blood sample was collected for subsequent determination of plasma total cholesterol, HDL cholesterol, and triglycerides with colorimetric assay kits (Sigma). The arterial vasculature was perfusion-fixed, and both carotid arteries were excised and processed for histological analyses. Mice were injected with bromodeoxyuridine (BrdU) 18 hours (100 mg/kg IP) and 1 hour (25 mg/kg by tail vein injection) before they were euthanized. Selected mice were sedated 1 to 4 days after the surgical procedure, and the injured carotid artery was interrogated with a 20-MHz Doppler flow probe (provided by Dr Craig Hartley, Baylor College of Medicine, Houston, Tex) or perfusion-fixed and excised for histological analysis.

**Measurements of Carotid Artery Occlusion Time and Platelet Aggregation**

ApoE−/− mice (6 to 8 weeks old) were fed normal chow or high-cholesterol chow for 4 weeks, then subjected to ferric chloride carotid injury. Time required to form an occlusive thrombus was determined as described. In vitro platelet aggregation was studied as described.

**Histological Analyses**

Four evenly spaced cross sections were prepared from the midportion of each carotid artery and subjected to hematoxylin-eosin staining. Intima and media areas of each cross section were determined by computer-assisted planimetry (Image-Pro Plus, Media Cybernetics), and the mean intima and media cross-sectional area was calculated for each artery. Intima was defined as the area bounded by the endothelium and the internal elastic lamina. Media was defined as the area bounded by the internal and external elastic laminae. The operator was blinded to specimen genotype when performing all analyses. Oil red O staining was performed as described previously. Elastic stain (HT25-A, Sigma) was used to identify the elastic laminae. Anti-BrdU staining was performed with a BrdU staining kit (Zymed Laboratories). Tissue factor expression was detected by digoxigenin-labeled human factor VIIa staining. The chromogen for tissue factor staining was nitro blue tetrazolium chloride/X-phosphate (Digoxigenin Detection Kit, Boehringer Mannheim), and counterstaining was performed with nuclear fast red solution (Poly Scientific R&D Corp). Macrophages were identified by a rat monoclonal antibody to Mac-3 (PharMingen). Smooth muscle α-actin staining was performed with anti-human smooth muscle α-actin monoclonal antibody (clone 1A4, Dako). Fibrin was detected with a goat anti-mouse fibrinogen antibody (Accurate). von Willebrand factor (vWF) was detected with rabbit anti-human vWF antibody (Dako). All cross sections were 5 μm thick. Frozen sections were used for oil red O staining. All other sections were paraffin-embedded.

**Statistical Analyses**

Data are presented as mean±SEM. An unpaired Student’s t test was used to compare groups.

**Results**

**Characterization of the Arterial Response to Ferric Chloride Injury in ApoE−/− Mice**

Application of ferric chloride to the adventitial surface of arteries induces full-thickness injury, endothelial denudation, and platelet-rich thrombus formation. Carotid artery blood flow was monitored with a transcutaneous 20-MHz Doppler flow probe in 6 apoE−/− mice (3 female) at 24 and 48 hours after ferric chloride injury. At 24 hours, 4 of 6 arteries were patent; at 48 hours, 6 of 6 arteries were patent. Another 4 apoE−/− mice (2 female) were subjected to ferric chloride carotid artery injury. Four days later, mice were euthanized, and the injured carotid artery was excised, sectioned, and subjected to hematoxylin-eosin staining. All 4 injured arteries were patent, although residual mural thrombus was present in 2 arteries. These studies in 10 mice indicated that 10% ferric chloride carotid artery injury produced thrombi that only transiently occluded blood flow.

ApoE−/− mice were euthanized 4 weeks (n=12) or 8 weeks (n=10) after carotid injury. Of mice that were studied at 4 weeks after injury, 5 animals (3 female) received normal chow and 7 animals (4 female) received high-cholesterol chow. Of mice that were euthanized 8 weeks after injury, 5 animals (2 female) received normal chow and 5 animals (2 female) received high-cholesterol chow. Marked intimal hyperplasia was observed in all injured arteries, regardless of diet, whereas no intimal hyperplasia was observed in the contralateral (ie, noninjured) carotid arteries (Figure 1). Intima and media cross-sectional areas and intima/media ratios are shown in Table 1. Mean intima area 4 weeks after
injury was greater in mice fed high-cholesterol chow than in those fed normal chow. As controls, 5 apoE2/2 mice were subjected to ferric chloride injury, then fed a high-cholesterol chow for 8 weeks. No or only minimal neointima formation was observed (Figure 1D). These results indicated that both abnormal lipid metabolism and arterial injury were necessary to induce significant intimal hyperplasia within the midportion of the murine carotid artery.

We performed detailed histological analyses of arteries retrieved 8 weeks after injury. Immunohistochemistry confirmed the presence of smooth muscle α-actin–positive cells in neointima (Figure 2E). Anti-BrdU staining demonstrated ongoing cell proliferation within the intima and media (Figure 2B). Cholesterol clefts (Figure 1B) and numerous foam cells (Figure 1C) were observed. Oil red O staining demonstrated neutral lipid deposition within the intima and media (Figure 2A). Immunostaining confirmed the presence of macrophages in neointimal lesions (Figure 2F). Tissue factor expression, which is restricted almost entirely to the adventitia of normal arteries,19,21 was detected in the intima, media, and adventitia of injured arteries (Figure 2C and 2D). Fibrin deposition was detectable in the intima of 2 of 6 apoE2/2 mice (Figure 2H) and in 0 of 3 apoE2/2, PAI-12/2 mice. Diffuse neointimal (ie, not restricted to endothelial cells) vWF staining was observed in 5 of 5 arteries studied (Figure 2G).

Effect of High-Cholesterol Diet on the Rate of Occlusive Thrombus Formation

To explore potential interactions between diet and thrombosis, 6-week-old apoE2/2 mice were fed normal chow (n=7, 4 female) or high-cholesterol chow (n=7, 3 female) for 4 weeks, then subjected to carotid artery injury. The mean time necessary to form an occlusive thrombus was shorter in mice fed a high-cholesterol diet (12.7±2.8 minutes) than in those fed normal chow (22.3±1.7 minutes; P<0.02). Platelet-rich plasma was prepared from apoE2/2 mice after 4 weeks of a normal chow diet (n=8, 4 female) or a high-cholesterol diet (n=8, 4 female). Mean maximal percent aggregation after ADP stimulation (20 μmol/L) was 80±3% for mice fed high-cholesterol chow versus 64±6% for mice fed normal chow (P<0.05).

Effect of PAI-1 Deficiency on Remodeling After Carotid Artery Injury in ApoE2/2 Mice

We tested the hypothesis that PAI-1 enhances neointima formation after vascular injury in apoE2/2 mice. ApoE2/2, PAI-12/2 mice (n=12, 6 female) and apoE2/2, PAI-12/2 mice (n=12, 6 female) were subjected to ferric chloride carotid injury, then fed a high-cholesterol diet for 8 weeks. The gross appearance of arteries at the time of harvest differed significantly between the 2 groups (Figure 3A and 3B). Histological analyses confirmed that neointima formation was significantly greater in apoE2/2, PAI-12/2 mice than in apoE2/2, PAI-12/2 mice (Figure 3C and 3D). Mean intima and media cross-sectional areas were 10-fold and 2-fold greater, respectively, for apoE2/2, PAI-12/2 mice (intima 0.143±0.05 mm2; media 0.025±0.0035 mm2) than for apoE2/2, PAI-12/2 mice (intima 0.014±0.0049 mm2; media 0.015±0.0028 mm2). These differences were statistically significant (Figure 4). Intima/media ratios of apoE2/2, PAI-

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Injured common carotid arteries of apoE2/2 mice after 8 weeks of high-cholesterol feeding. A, Oil red O staining. Red color (arrows), which was not observed on staining of arteries from apoE2/2 mice (not shown), denotes neutral lipid deposition. B, Anti-BrdU staining. Nuclei that incorporate BrdU stain brown. C, Tissue factor staining (blue). D, Negative control for tissue factor staining, ie, cross section adjacent to that in C, was preincubated with unlabeled factor VIIa (100 nmol/L), then processed in identical fashion to that in C. E, Smooth muscle α-actin staining (red). F, Demonstration of macrophages in intima by anti–Mac-3 staining (red). G, vWF staining (red). H, Fibrin staining (red). Bar=150 μm (A and C to E) or 50 μm (B and F to H). L indicates lumen.
1/−/− mice were 5.6±1.8, compared with 1.2±0.55 for apoE−/−, PAI-1−/− mice (P<0.03). Mean intima and media cell proliferation indices, determined by anti-BrdU staining, were significantly greater for apoE−/−, PAI-1−/− mice than for apoE−/−, PAI-1−/− mice (Table 2). Four sets of adjacent cross sections obtained from 4 different arteries were immunostained with anti-smooth muscle α-actin and anti-BrdU antibodies. This analysis revealed that 96% of BrdU-positive cells (ie, 48 of 50 counted cells) were also smooth muscle α-actin–positive. To determine whether the amount of thrombus present in arteries early after ferric chloride injury differed between apoE−/−, PAI-1−/− mice and apoE−/−, PAI-1−/− mice, transcutaneous Doppler analysis was performed in 5 apoE−/−, PAI-1−/− animals. Carotid arteries were patent in 5 of 5 apoE−/−, PAI-1−/− mice 24 hours after injury. Histological analysis of multiple hematoxylin-eosin–stained cross sections from 4 apoE−/−, PAI-1−/− mice (different animals than the 5 apoE−/−, PAI-1−/− mice studied with transcutaneous Doppler ultrasound) 4 days after injury revealed no detectable thrombus. These results contrasted with those of similar experiments (described above) performed with apoE−/−, PAI-1−/− mice. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were measured in apoE−/−, PAI-1−/− mice and apoE−/−, PAI-1−/− mice. Total cholesterol and triglyceride levels were higher in mice lacking PAI-1, whereas HDL cholesterol levels did not differ between groups (Table 3). These results indicated that the enhanced neointima formation observed in mice with normal PAI-1 expression could not be explained by higher plasma lipid concentrations than in mice lacking PAI-1.

**Discussion**

We examined the arterial response to injury in atherosclerosis-prone mice. We found that topical application of ferric chloride to the murine carotid artery resulted in endothelial cell denudation, medial cell necrosis, and formation of transiently occlusive, platelet-rich thrombi. This acute response was followed by intimal and medial hyperplasia and deposition of foam cells, neutral lipid, tissue factor, vWF, and fibrin within the blood vessel wall. We examined the impact of PAI-1 on the arterial response to injury by performing morphometric analyses of apoE−/−, PAI-1−/− mice and apoE−/−, PAI-1−/− mice 8 weeks after injury. We found that PAI-1 deficiency resulted in a marked reduction in the hyperplastic response to injury. Carmeliet et al10 found greater neointima formation in PAI-1−/− mice than PAI-1−/− mice after mechanical carotid artery injury or electrical femoral artery injury. In contrast, in our experiments, PAI-1 deficiency was protective against neointima formation. Although our results and those of Carmeliet et al are potentially contradictory, the factors regulating cell migration and proliferation in the hyperlipidemic environment caused by apoE deficiency (as in our experiments) most likely differ considerably from those that control posttraumatic neointima formation in the absence of hyperlipidemia (as in the experiments of Carmeliet et al). Hyperlipidemia alters the expression of multiple genes that regulate the arterial response to injury, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1.13 The effects of mouse strain could also have contributed to the differential effects of PAI-1 in the 2 studies. The mice used in our experiments were extensively backcrossed (n=8) to the C57BL6 genetic background, whereas Carmeliet et al used mice that were 75% C57BL6/25% 129. Alternatively, the method of injury may have contributed to the differences between studies. Carmeliet et al predominantly used perivascular electric injury, whereas we used chemical injury. Electric current and ferric chloride both induce cell necrosis within the intima, media, and adventitia, and both forms of injury cause thrombosis.16 Ferric chloride induces the formation of highly reactive oxygen species, such as hydroxyl radical, that injure cells by causing lipid peroxi-

![Figure 4](image-url)  
**Figure 4.** Mean intima and media cross-sectional areas of apoE−/−, PAI-1−/− mice (solid bars, n=12) and apoE−/−, PAI-1−/− mice (open bars, n=12) 8 weeks after injury. *P<0.02 vs mean intima area of apoE−/−, PAI-1−/− mice. **P<0.04 vs mean media area of apoE−/−, PAI-1−/− mice.
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"PAI-1 paradox."22 We have shown that PAI-1 deficiency ruptured neointima, because it is lipid-rich atheroma that typically develops after arterial injury. Inhibition of cell migration is a major determinant of the pathological neointima that we observed in apoE \(^{-/-}\) mice compared with PAI-1 \(^{-/-}\) mice. These results are consistent with ours and support the hypothesis that PAI-1 contributes to neointima formation after oxidative vascular injury.

The function of PAI-1 in vascular diseases has been considered paradoxical.22 Vascular smooth muscle cell migration is a major determinant of the pathological neointima that develops after arterial injury. Inhibition of cell migration by PAI-1 might be expected to exert a "good" effect by limiting the size of arterial lesions. Inhibition of cell migration, however, could help to produce hypocellular lesions that are prone to rupture and trigger thrombosis. Such a "bad" effect of PAI-1 might be highly dependent on hypercholesterolemia, because it is lipid-rich atheroma that typically rupture. Our studies yield important data regarding the "PAI-1 paradox."22 We have shown that PAI-1 deficiency protects against lesion growth after oxidative injury in hyperlipidemic mice. Several mechanisms could account for this effect. The impact of PAI-1 on the extent and duration of mural thrombosis after injury could be involved. Platelet thrombi are a source of growth factors that can stimulate vascular cell proliferation.1 Our current and previously published data show that thrombi are cleared from injured arteries more rapidly in PAI-1 \(^{-/-}\) mice than PAI-1 \(^{+/+}\) mice.15,17 We hypothesize that enhanced stimulation of cell proliferation by thrombus-associated growth factors may have accounted for the increased intimal and medial hyperplasia observed in apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice compared with apoE \(^{-/-}\) mice fed a normal chow diet, results consistent with those of Eitzman et al.24 Hypercholesterolemia is associated with enhanced tissue factor expression, which may help to explain this observation.21 We also observed enhanced ADP-induced platelet aggregation in vitro after high-cholesterol feeding, however, suggesting a direct effect of hyperlipidemia on platelet reactivity.

A direct effect of PAI-1 on the blood vessel wall must also be considered a possible mechanism underlying our results. PAI-1 regulates processes important in cell migration, including plasmin formation and the interactions of cell-surface receptors, such as the uPA receptor and the integrin αvβ3, with vitronectin.11,25 PAI-1 deficiency, however, would be expected to enhance cell migration mediated by plasmin digestion of extracellular matrix or by interaction of vitronectin with cell-surface receptors. PAI-1 could inhibit the activation of transforming growth factor-β (TGF-β) by plasmin. The preponderance of published studies suggest that activated TGF-β inhibits vascular smooth muscle cell proliferation.26 Our results are consistent with the hypothesis that PAI-1 can promote cell proliferation by inhibiting TGF-β activation. Because hyperlipidemia enhances TGF-β expression in macrophages and vascular smooth muscle cells,27,28 the magnitude of a potential effect of PAI-1 on TGF-β expression might be expected to differ between mice with normal versus abnormal lipid metabolism. Finally, recent studies suggest that PAI-1 inhibits apoptosis in a variety of cultured cell lines, including umbilical vein endothelial cells.29 Vascular cell apoptosis is observed after arterial injury in hyperlipidemic rabbits.30 An antiapoptotic effect of PAI-1 would be consistent with the increased intimal hyperplasia that we observed in apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice compared with apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice. Additional studies are needed to explore these hypotheses.

Our study is relevant to the potential role of PAI-1 in atherosclerosis development.9 When apoE \(^{-/-}\), PAI-1 \(^{+/+}\) mice and apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice are compared, atherosclerosis within the aortic root does not differ,31 whereas atherosclerosis at the carotid artery bifurcation is reduced in mice lacking PAI-1.32 It is likely that the impact of PAI-1 on atherosclerosis development depends on the degree to which vascular injury (eg, from turbulent flow at arterial branch points) is present, because injury triggers activation of pathways that are regulated by PAI-1. Our model adds a component of injury at a readily retrievable vascular site to a widely used model of atherosclerosis (ie, apoE \(^{-/-}\) mice). Therefore, it may prove useful in studying the roles of other genetic loci that modulate atherosclerosis development after arterial injury.

### TABLE 3. Effects of Genotype and Diet on Lipid Profiles

<table>
<thead>
<tr>
<th>Genotype/Diet</th>
<th>Total Cholesterol, mg/dL</th>
<th>HDL Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
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<tr>
<td>ApoE (^{+/+}), PAI-1 (^{-/-}), NC (n=4)</td>
<td>42.4±5</td>
<td>28.8±5.3</td>
<td>59±14</td>
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<tr>
<td>ApoE (^{+/+}), PAI-1 (^{-/-}), HCC (n=5)</td>
<td>112±12.5</td>
<td>64±12</td>
<td>52±6.3</td>
</tr>
<tr>
<td>ApoE (^{-/-}), PAI-1 (^{-/-}), NC (n=5)</td>
<td>358±13</td>
<td>15.2±1.4</td>
<td>112.5±18.3</td>
</tr>
<tr>
<td>ApoE (^{-/-}), PAI-1 (^{+/+}), HCC (n=12)</td>
<td>1088±49*</td>
<td>33.1±7.8</td>
<td>169±16†</td>
</tr>
<tr>
<td>ApoE (^{-/-}), PAI-1 (^{-/-}), HCC (n=12)</td>
<td>1478±176</td>
<td>34.6±5.9</td>
<td>276±28</td>
</tr>
</tbody>
</table>

NC indicates normal chow; HCC, high-cholesterol chow. *P<0.001 vs apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice; †P<0.01 vs apoE \(^{-/-}\), PAI-1 \(^{-/-}\) mice.
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References
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