

Enhanced Thrombosis in Atherosclerosis-Prone Mice Is Associated With Increased Arterial Expression of Plasminogen Activator Inhibitor-1

Katrin Schäfer, Katja Müller, Anneke Hecke, Emmanuelle Mounier, Julia Goebel, David J. Loskutoff, Stavros Konstantinides

Objectives—This study was undertaken to investigate the origin and pathophysiological importance of plasminogen activator inhibitor (PAI-1) in atherosclerosis.

Methods and Results—We used the ferric chloride model to induce carotid artery injury in apolipoprotein E knockout (apoE<sup>−/−</sup>) and wild-type (WT) mice. ApoE<sup>−/−</sup> mice fed high-fat diet for 4 months developed severe hypercholesterolemia and had significantly elevated plasma PAI-1 levels (2.3±0.3 versus 0.6±0.1 ng/mL in WT mice; <i>P</i>&lt;0.05). These mice exhibited a prothrombotic phenotype with shortened times to thrombotic arterial occlusion (8.6 versus 11.5 minutes; <i>P</i>&lt;0.001) and reduced recanalization rates (12% versus 51%; <i>P</i>&lt;0.0001) compared with WT mice. In situ hybridization, reverse transcriptase–polymerase chain reaction, and immunohistochemistry showed a significantly upregulated PAI-1 expression in P-selectin–positive (activated) endothelial cells lining normal-appearing arterial segments and within the advanced atherosclerotic lesions of apoE<sup>−/−</sup> mice. No significant upregulation of PAI-1 expression was found in the other organs studied, and only trace amounts of PAI-1 mRNA were detected in murine platelets. Importantly, deletion of the PAI-1 gene reversed the thrombotic tendency and reduced neointimal growth after injury in apoE<sup>−/−</sup> mice despite the persistence of excessive hypercholesterolemia.

Conclusions—These results suggest that increased vascular expression of PAI-1 may contribute to the elevated circulating levels of the inhibitor and be responsible, at least in part, for the prothrombotic phenotype in apoE<sup>−/−</sup> mice. (Arterioscler Thromb Vasc Biol. 2003;23:2097-2103.)

Key Words: atherosclerosis ■ thrombosis ■ plasminogen activator inhibitor ■ arteries ■ mouse models
suggest a direct link between PAI-1 and the risk of thrombotic complications in atherosclerosis.

Methods

Animals and Genotyping

C57BL/6J WT mice and apoE/{sup}−/− or PAI-1/{sup}−/− mice were purchased from Jackson Laboratories (Bar Harbor, Maine). ApoE/{sup}−/− mice had been backcrossed to the C57BL/6J genetic background for at least 10 generations and PAI-1/{sup}−/− mice for ≥7 generations. ApoE/{sup}−/− mice lacking the PAI-1 gene (double-knockout [DKO] mice) were generated by intercrossing apoE/{sup}−/− mice with PAI-1/{sup}−/− mice. The genotype of the offspring at each crossing stage was confirmed using polymerase chain reaction (PCR) analysis of mouse tail DNA using primer sequences and protocols provided by Jackson Laboratories. At 6 to 8 weeks of age, mice were either switched to a HFD containing 21% wt/wt fat and 0.15% wt/wt cholesterol (No. 88137; Harlan Winkelmann) or maintained on normal chow. All animal care and experimental procedures were approved by the Animal Research Committee of the University of Goettingen.

Measurement of Cholesterol, Triglyceride, and PAI-1 Levels

In deeply anesthetized noninjured mice, blood was collected by cardiac puncture in 3.8% sodium citrate (1:9 final dilution) and plasma was prepared by centrifugation at 3000 rpm for 5 minutes. To obtain murine platelets, anticoagulated blood was centrifuged at 900 rpm for 15 minutes at room temperature and the platelet-rich plasma (PRP) layer was carefully removed. To pellet platelets, 1 μmol/L prostaglandin E, and 1 U/mL apyrase were added and the mixture was centrifuged at 1600 rpm. The supernatant (platelet-poor plasma [PPP]) was removed and the platelet pellet was used for RNA extraction.

Plasma cholesterol and triglyceride levels were determined enzymatically (Sigma). Total PAI-1 levels in plasma, PRP, and PPP were measured using an enzyme-linked immunosassay specific for murine PAI-1 (MPAIKT-TOT; Molecular Innovations). The sensitivity of measurement was assessed using an enzyme-linked immunoassay specific for murine PAI-1 (MPAIKT-TOT; Molecular Innovations). The sensitivity of measurement was assessed using an enzyme-linked immunoassay specific for murine PAI-1 (MPAIKT-TOT; Molecular Innovations). The sensitivity of measurement was assessed using an enzyme-linked immunoassay specific for murine PAI-1 (MPAIKT-TOT; Molecular Innovations). The sensitivity of measurement was assessed using an enzyme-linked immunoassay specific for murine PAI-1 (MPAIKT-TOT; Molecular Innovations).

Carotid Injury in Mice

Mice were anesthetized with methoxyflurane (Metofane, Janssen) and subjected to carotid artery injury using 10% ferric chloride. In apoE/{sup}−/− mice, a normal-appearing segment of the common carotid artery (identified under the dissecting microscope; magnification ×10) was subjected to injury with FeCl3. In contrast to a previous study, we avoided direct injury of spontaneously occurring atherosclerotic lesions at the carotid bifurcation. Carotid blood flow was monitored before and for 25 minutes after injury. Tissue harvest and processing were performed as previously described.

Immunohistochemical Studies

Immunohistochemistry was performed on paraffin-embedded carotid artery sections. Activated endothelial cells were identified using a rabbit anti-mouse von Willebrand factor (vWF) antibody (Dako; dilution, 1:100), a rabbit anti-mouse P-selectin antibody (PharMingen; dilution, 1:40), and a rabbit anti-mouse vascular cellular adhesion molecule-1 (VCAM-1) antibody (Santa Cruz; dilution, 1:20). Macrophages were detected using a rat anti-mouse Mac-3 antibody (PharMingen; dilution, 1:200). PAI-1 was localized using a polyclonal rabbit anti-mouse PAI-1 antibody (dilution, 1:200). The presence of smooth muscle cells was assessed using a monoclonal anti-mouse α-actin antibody (peroxidase-labeled; Dako), followed by incubation with aminoethyl-carbazole reagent (Zytochem). For quantitative morphometric analysis, carotid vessels were harvested 3 weeks after injury and paraffin sections were stained with Verhoeff’s elastic stain. The neointima and media area, the intima/media ratio, and the degree of luminal stenosis were determined (ImagePro Plus, Media Cybernetics). Five sections equally spaced throughout the injured arterial segment (at 200-μm intervals) were evaluated, and the results were averaged for each animal. Mean±SEM values were calculated from 9 to 11 mice in each group.

Analysis of PAI-1 Gene Expression

Total messenger RNA from mouse tissues and isolated murine platelets was extracted using RNAwiz (Ambion) and chloroform, and reverse transcriptase (RT)-PCR analysis was performed for quantitative determination of PAI-1 and β-actin mRNA expression. For the preparation of digoxigenin-labeled PAI-1 riboprobes, the pGEM-3Z vector (Promega) carrying the 1085-bp EcoRI/ HindIII fragment of the mouse PAI-1 cDNA was linearized and used as a template for the in vitro transcription of digoxigenin-labeled antisense or sense riboprobes using SP6 or T7 RNA polymerases, respectively, in the presence of digoxigenin-UTP (Roche). For in situ hybridization, sections were dewaxed by treatment with xylene, postfixed in 4% paraformaldehyde for 20 minutes, and treated with 200 mmol/L HCl for 10 minutes to denature proteins. After incubation in 0.5% acetic anhydride in 100 mmol/L Tris buffer, sections were treated with 5 μg/mL proteinase K for 20 minutes at 37°C. Hybridization was carried out overnight at 65°C, followed by stringency washes for 10 minutes in 2×SSC (0.3 mol/L NaCl and 0.03 mol/L sodium citrate), 50% deionized formamide in 1×SSC (0.15 mol/L NaCl and 0.015 mol/L sodium citrate; 3×20 minutes), and 1×SSC (2×15 minutes). For the detection of RNA, sections were incubated for 30 minutes with 10% FCS in blocking buffer (Roche) followed by incubation with alkaline phosphatase–conjugated anti-digoxigenin antibody (1:500 in blocking buffer; Roche) for 2 hours. Bromo-chloro-indoxyl phosphate and nitro blue tetrazolium were used as color reagents, and sections were counterstained with Nuclear Fast Red. Parallel sections were hybridized using PAI-1 sense riboprobe as control for nonspecific hybridization.

Statistical Analysis

For comparison of the times to thrombotic occlusion after injury, the Mann-Whitney test was used. Mouse vessels that did not occlude completely were regarded as having times to thrombosis corresponding to the end of the flow-monitoring period (25 minutes). Differences between mean values were tested by Student’s t test or by ANOVA followed by the Bonferroni t test for pairs of means when more than 2 groups were analyzed. Qualitative variables were tested by Fisher’s exact test.

Results

Thrombotic Response to Arterial Injury in WT and ApoE/{sup}−/− Mice

The metabolic parameters of mice at the time of vascular injury are displayed in the Table. As expected, excessively high cholesterol levels were found in apoE/{sup}−/− mice placed on HFD for 4 months (apoE/{sup}−/−+HFD).

Figure 1 summarizes the thrombotic response of 22- to 24-week-old WT and apoE/{sup}−/− mice to carotid injury with FeCl3. After induction of injury, thrombotic occlusion occurred in 82% of the WT (n=43) mice fed normal chow. The median time to thrombotic occlusion was 11.5 minutes
(Figure 1A). At the end of the 25-minute flow-monitoring period after injury, 51% of the WT mouse vessels were patent (Figure 1B). Mildly hypercholesterolemic WT mice fed HFD for 4 months (WT+HFD; n=23) had only slightly shortened times to thrombotic occlusion (panel A) but formed more stable arterial thrombi, as indicated by the lower carotid patency rates in this group (panel B). In contrast to the findings in WT mice, apoE−/− mice placed on HFD for 4 months exhibited a markedly enhanced thrombotic response when a normal-appearing segment of the common carotid artery was subjected to FeCl3 injury. The median time to thrombotic occlusion was 8.6 minutes (P<0.001 versus WT on normal chow; P<0.05 versus WT on HFD; Figure 1A), and only 12% of apoE−/− mouse vessels were patent 25 minutes after injury (Figure 1B).

To investigate the possibility that lack of apoE per se might be the underlying cause for enhanced arterial thrombosis,18 we also induced injury in 6- to 8-week-old apoE−/− mice (n=13) fed normal chow. These mice exhibited significant but not excessive hypercholesterolemia (267±67 mg/dL; P<0.001 versus WT) and hypertriglyceridemia (104±28 mg/dL; P<0.001 versus WT). Neither the median time to thrombosis (9.6 minutes; P=NS versus WT) nor the patency rate at 25 minutes (39%; P=NS versus WT) supported the presence of a pronounced prothrombotic phenotype in young apoE−/− mice.

**Circulating PAI-1 Levels**

Mean PAI-1 concentrations were moderately increased in plasma from 22- to 24-week-old WT mice fed HFD for 4 months compared with their counterparts fed normal chow (1.1±0.2 versus 0.6±0.1 ng/mL; P=NS; Figure 2A). On the other hand, age-matched apoE−/− mice placed on HFD for the same time period (ie, those mice with a marked thrombotic reaction to vascular injury; Figure 1) exhibited an almost 4-fold increase in plasma PAI-1 levels (2.3±0.3 ng/mL; P<0.05 versus WT on normal chow).

In additional experiments, we examined whether the elevated levels of PAI-1 in apoE−/− mice were attributable to hyperlipidemia or to the presence of advanced atherosclerotic lesions. Young (6- to 8-week-old), nonatherosclerotic apoE−/− mice fed normal chow had 1.4±0.3 ng/mL PAI-1 in plasma (P<0.05 versus WT mice on normal chow; P=NS versus WT mice on HFD; Figure 2A). Feeding young apoE−/− mice with HFD for a short period (10 days) resulted in severe acute hyperlipidemia, increasing their cholesterol levels from 267±67 to 428±60 mg/dL (P<0.001). However, the change...
in plasma concentrations of PAI-1 was minimal (from 1.4±0.3 to 1.6±0.1 ng/mL; P=NS), and the PAI-1 levels remained lower than those of 22- to 24-week-old apoE/−/− mice. As expected, 6- to 8-week-old apoE/−/− mice exhibited signs of endothelial cell activation (P-selectin and VCAM-1 expression) but had not yet developed extensive atherosclerotic lesions (not shown). This was in contrast to their 22- to 24-week-old counterparts, which exhibited severe advanced atherosclerosis (Figure 3).

Expression of PAI-1 in the Arteries, Platelets, and Other Tissues

To determine the origin of the elevated plasma PAI-1 levels in the apoE/−/− mice, RNA was extracted from various mouse tissues and analyzed for PAI-1 mRNA expression using quantitative RT-PCR (Figure 2B). In most of the tissues examined, PAI-1 mRNA expression was similar when WT mice fed normal chow, WT mice fed HFD, young nonatherosclerotic apoE/−/− mice fed normal chow, and severely hypercholesterolemic, atherosclerotic apoE/−/− mice fed HFD were compared. However, when PAI-1 expression in the vasculature (aorta and major branches, including the carotid arteries) was analyzed (Figure 2B), mRNA levels were found to be significantly elevated in apoE/−/− mice placed on HFD compared with the other mouse groups (mean levels, 72±10% of β-actin expression; P<0.05 versus WT, WT+HFD, or young apoE/−/− mice). On the other hand, RT-PCR excluded the possibility that murine platelets were a major source of circulating PAI-1. Only trace amounts of PAI-1 mRNA (1% to 2% of β-actin expression) were detected in platelets from WT mice on normal chow, and there was no upregulation of PAI-1 expression in platelets from WT mice on HFD, young apoE/−/− mice, or apoE/−/− mice on HFD (not shown). In agreement with these findings, PAI-1 ELISA revealed no differences between the levels of the inhibitor in PRP and those in PPP regardless of the genotype (WT versus apoE/−/−) or the type of diet (normal chow versus HFD).

In situ hybridization and immunohistochemistry studies were performed to localize PAI-1 mRNA and protein expres-
sion in the arterial wall. Cross-sections of the abdominal aorta and carotid arteries were examined, and representative results are shown in Figure 3. In contrast to WT animals (panel A), apoE\textsuperscript{−/−} mice fed HFD (panel B) exhibited a strong PAI-1 expression signal in the intima of uninjured, normal-appearing segments of the aorta or common carotid arteries. This latter finding was confirmed by immunohistochemistry (not shown). Staining of serial sections with antibodies specific for vWF (not shown), P-selectin (panel C), and VCAM-1 (panel D) revealed a positive signal in the intimal monolayer of apoE\textsuperscript{−/−} mouse arteries, thus identifying the PAI-1–expressing cells as activated endothelium in the early stages of atherosclerosis.\textsuperscript{21} In comparison, WT mouse arteries were positive for vWF but not for P-selectin or VCAM-1 antigen (not shown), suggesting normal, nonactivated endothelium.

In addition to the above findings, analysis of advanced atherosclerotic lesions (for example, at the carotid bifurcation) of 22- to 24-week-old apoE\textsuperscript{−/−} mice placed on HFD for 4 months revealed a strong, multifocal PAI-1 gene (panel E) and protein (panel F) expression signal. Immunohistochemical analysis of serial sections identified the PAI-1–producing cells in these lesions as \(\alpha\)-actin-positive vascular smooth muscle cells (panel G) and, particularly, Mac-3–positive tissue macrophages (panel H).

**Lack of PAI-1 Reverses the Prothrombotic Tendency of ApoE\textsuperscript{−/−} Mice**

ApoE\textsuperscript{−/−} PAI-1\textsuperscript{−/−} DKO mice were placed on HFD for 4 months and underwent carotid artery injury at 22 to 24 weeks of age. As shown in the Table and in agreement with a previous report,\textsuperscript{13} plasma cholesterol and triglyceride levels were similar in DKO and apoE\textsuperscript{−/−} mice on HFD. However, Figure 1A demonstrates that the median time to arterial thrombosis after injury with FeCl\textsubscript{3} was significantly prolonged in the DKO mice compared with their apoE\textsuperscript{−/−} counterparts. In fact, the median time to thrombosis in DKO mice fed HFD did not differ significantly from that of age-matched WT animals fed normal chow. The characteristic flow profiles displayed in Figure 4 demonstrate that thrombi forming in DKO mice (panels B and C) were unstable and embolized more frequently than those of apoE\textsuperscript{−/−} mice (panel A). As a result, a high proportion of vessels were patent in DKO mice 25 minutes after injury (Figure 1B).

**Effect of PAI-1 on Neointimal Growth After Injury**

The effects of PAI-1 on vascular remodeling are summarized in Figure 5. Morphometric analysis performed 3 weeks after injury revealed that young apoE\textsuperscript{−/−} and, particularly, 22- to 24-week-old apoE\textsuperscript{−/−} mice fed HFD for 4 months (and throughout the 3-week period after injury) exhibited enhanced neointimal growth (panel A) and luminal stenosis (panel B) compared with WT mice fed normal chow. On the other hand, PAI-1 deficiency was associated with a significant reduction in both the neointima area and the severity of luminal stenosis in apoE\textsuperscript{−/−} mice fed HFD.

**Discussion**

In the present study, we applied an established model of arterial injury to induce platelet-rich thrombi in the carotid artery of atherosclerosis-prone apoE\textsuperscript{−/−} mice. Our experiments show that 22- to 24-week-old apoE\textsuperscript{−/−} mice maintained on HFD for 4 months had significantly shorter times to thrombotic occlusion and more stable arterial thrombi in response to injury compared with their WT counterparts fed normal chow (Figure 1). The prothrombotic phenotype of apoE\textsuperscript{−/−} animals, which is in agreement with previous reports,\textsuperscript{17,21} was associated with an approximately 4-fold increase in plasma PAI-1 levels (Figure 2A).

Because PAI-1 gene expression has been demonstrated in a variety of murine tissues, we investigated the origin of the elevated circulating PAI-1 in the apoE\textsuperscript{−/−} animals. PAI-1 expression was significantly upregulated in the arterial vessels (but not in the other organs examined) of 22- to 24-week-old apoE\textsuperscript{−/−} mice fed HFD (Figure 2B). Additional
analysis (Figure 3) revealed that PAI-1 was expressed in vascular smooth muscle cells and, particularly, macrophages of advanced atherosclerotic lesions. These results are in agreement with observations in human atherosclerotic plaques. Importantly, however, a positive signal for PAI-1 mRNA and protein also was found in endothelial cells lining apparently normal arterial segments without intimal thickening. The PAI-1–producing endothelial cells stained positive for both P-selectin and the cell adhesion molecule VCAM-1, indicating endothelial activation and inflammatory activity as a marker of beginning atherosclerosis.19 Our findings thus suggest that upregulated expression of PAI-1 both in the activated endothelium and within the advanced atherosclerotic lesions may have contributed to the increased circulating levels of the inhibitor in 22- to 24-week-old apoE−/− mice. On the other hand, young (6- to 8-week-old) apoE−/− mice that exhibited signs of endothelial cell activation but had not yet developed advanced atherosclerosis had moderately elevated circulating PAI-1 levels compared with WT mice (1.4±0.3 versus 0.6±0.1 ng/mL). These levels were lower than those of their 22- to 24-week-old counterparts fed HFD for 4 months (2.3±0.3 ng/mL). Moreover, when the 6- to 8-week-old apoE−/− mice were placed on HFD for a short (10-day) period, they developed severe acute hypercholesterolemia, which was, however, not accompanied by significant increases in plasma PAI-1 concentrations (1.6±0.1 ng/mL). This is possibly related to the fact that the duration of (diet-induced) hyperlipidemia was too short to induce severe advanced atherosclerosis in these animals. Admittedly, the contribution of spontaneous or diet-induced hyperlipidemia to the elevation of systemic PAI-1 levels cannot be completely dissected from that of atherosclerosis in vivo, because the 2 conditions coexist in apoE−/− mice (and, frequently, in humans). Of note, in previous studies, the effect of acute hyperlipidemia on the thrombotic response of young apoE−/− mice to arterial injury was either pronounced21 or moderate,16 possibly depending on the injury model used.

Although PAI-1 has been shown to stabilize arterial thrombi in normolipidemic mice,2 the inhibitor might be expected to have minor pathophysiological relevance in the presence of a potent thrombogenic stimulus such as severe hypercholesterolemia and of the other prothrombotic factors encountered during the atherosclerotic process.9 In the present study, the importance of the elevated PAI-1 was confirmed by the results obtained in apoE−/− animals, which also lacked the PAI-1 gene. Although these latter mice also had very high plasma cholesterol levels, their thrombotic reaction to injury was significantly weaker than that of their apoE−/− counterparts and the time to thrombotic occlusion was, in fact, indistinguishable from that of normolipidemic WT mice (Figures 1 and 4). These results extend the observations of other authors who also reported an attenuated thrombotic response in double-knockout mice.13,21 Additional studies are needed to dissect the mechanisms by which PAI-1 may enhance arterial thrombosis in the apoE−/− mouse.

The effects of high cholesterol levels on thrombosis seem to involve primarily the modulation (enhancement) of platelet function.16,21–24 Importantly, it was recently reported that, in the mouse, the concentration of PAI-1 in platelets is very low (in contrast to its levels in human platelets) and that PAI-1 derived from murine platelets may not contribute to the thrombotic response to injury.25 Using RT-PCR, we detected only trace amounts of PAI-1 mRNA in isolated murine platelets and also found that HFD did not alter PAI-1 mRNA or antigen levels in platelets. These data add additional support to the pathophysiological importance of the vascular expression of PAI-1 in the apoE−/− mouse model of hypercholesterolemia and atherosclerosis.

We also examined the effects of PAI-1 on vascular remodeling after carotid artery injury in apoE−/− mice. In agreement with a previous report,21 histological and quantitative morphometric analysis of injured arterial segments (Figure 5) revealed that lack of PAI-1 was associated with a significant reduction in both the neointima area and the severity of luminal stenosis in apoE−/− mice fed HFD. These results suggest that, at least in the FeCl3 model of arterial injury (and in other models that are characterized by a prominent thrombotic reaction and subsequent fibrinolysis), the antifibrinolytic factor PAI-1 may promote neointima formation in the vessel wall.26 Although carotid artery injury using ferric chloride results in lesions that exhibit several histological characteristics of human atherosclerotic

\[ \text{Figure 5. Effects of PAI-1 deficiency on the neointima area (a) and the degree of luminal stenosis (b) in apoE}^{−/−} \text{mice 3 weeks after arterial injury with ferric chloride; quantitative morphometric analysis. Mean±SEM was calculated from 9 to 11 mice in each group.} \]

\[ \text{**}P<0.001 \text{for young apoE}^{−/−} \text{mice and apoE}^{−/−}+\text{HFD compared with WT mice; and} \]

\[ \text{^A}P<0.05 \text{for the difference between DKO+HFD and apoE}^{−/−}+\text{HFD.} \]

\[ \text{Figure 5.} \]

\[ \text{Arterioscler Thromb Vasc Biol. November 2003} \]
plaques, the effects of PAI-1 on neointima formation and atherosclerosis involve complex mechanisms that still are incompletely understood. In conclusion, the findings presented above suggest that upregulated expression of PAI-1 in the vasculature may contribute to the prothrombotic phenotype and promote lesion growth in atherosclerosis-prone apoE−/− mice. Our results thus propose an important direct link between PAI-1 and the risk of arterial thrombosis in atherosclerosis.

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References

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